National Comprehensive Cancer Network®

NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®)

Pancreatic Adenocarcinoma

Version 1.2016

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NCCN Guidelines Version 1.2016 Panel Members
Pancreatic Adenocarcinoma

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‡ Hematology/Hematology oncology
Þ Internal medicine
~ Interventional radiology
≠ Pathology
¥ Patient advocacy
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Clinical Trials: NCCN believes that the best management for any cancer patient is in a clinical trial. Participation in clinical trials is especially encouraged.

To find clinical trials online at NCCN Member Institutions, click here: nccn.org/clinical_trials/physician.html.

NCCN Categories of Evidence and Consensus: All recommendations are category 2A unless otherwise specified.

See NCCN Categories of Evidence and Consensus.

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NCCN Guidelines Version 1.2016 Updates Pancreatic Adenocarcinoma

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Updates in Version 1.2016 of the NCCN Guidelines for Pancreatic Adenocarcinoma from Version 2.2015 include:

PANC-1

- Under workup, "Obtain family history" has been added with the following footnote: "If pancreatic cancer is diagnosed, consider referral for genetic counseling for patients who are young or who have a family history of cancer."
- "Chest imaging" has been changed to "Chest CT (preferred) or x-ray."

PANC-3

- Footnote "i" has been revised: "*For patients with tumors that are clearly resectable and who do not have high-risk features, neoadjuvant therapy is only recommended in a clinical trial. For patients with high-risk features* (ie, very highly elevated CA 19-9, large primary tumors, large regional lymph nodes, excessive weight loss, extreme pain)*, neoadjuvant chemotherapy may be considered,* which requires biopsy confirmation of adenocarcinoma (see PANC-4). *Acceptable neoadjuvant regimens include FOLFIRINOX or gemcitabine + albumin-bound paclitaxel. Subsequent chemoradiation is sometimes included. Most NCCN Member Institutions prefer neoadjuvant therapy at a high-volume center."* ~~In selected patients who appear technically resectable but have poor prognostic features (ie, very highly elevated CA 19-9, large primary tumors, large regional lymph nodes, excessive weight loss, or extreme pain) consider neoadjuvant therapy (clinical trial preferred), which requires biopsy confirmation of adenocarcinoma (see PANC-4). For patients with biliary obstruction, durable biliary decompression is required.~~

PANC-4

- "Baseline CA 19-9" has been added to the initial workup, and "Post-treatment CA 19-9" has been added to the workup following neoadjuvant therapy.
- After neoadjuvant therapy, the first bullet has been revised and split into two bullets: "*Pancreatic protocol CT or MRI (*abdomen *and pelvis)*; and, Chest ~~imaging~~ *CT (preferred) or x-ray."*
- If unresectable at surgery, the options for patients with jaundice have been revised: "~~Self-expanding metal stent or~~ *Consider surgical* biliary bypass ± gastrojejunostomy..."

PANC-5

- Former algorithm for "Borderline Resectable Disease, Planned Resection" has been removed.
- New algorithm for "Borderline Resectable, No Metastases, Cancer Not Confirmed" has been added.

PANC-6

- The second adjuvant therapy option has been revised: "Systemic gemcitabine or 5-FU/leucovorin or continuous infusion 5-FU before ~~or~~ *and* after chemoradiation..."
- The frequency of surveillance after two years has been changed from "annually" to "every 6–12 mo."
- The following has been added to footnote "o": "The adjuvant therapy options are dependent on the response to neoadjuvant therapy and other clinical considerations."

PANC-8

- The second-line therapy options have been separated into recommendations for those "previously treated with gemcitabine-based therapy" or "previously treated with fluoropyrimidine-based therapy." (Also on PANC-9)
- Footnote "v" has been added: "FOLFIRINOX should be limited to those with ECOG 0-1. Gemcitabine + albumin-bound paclitaxel is reasonable for patients with KPS ≥70." (Also on PANC-9)

PANC-9

- The following second-line therapy option has been added for patients with metastatic disease previously treated with gemcitabine-based therapy: "5-FU + leucovorin + liposomal irinotecan (category 1)." (Also on PANC-G, 1 of 3)

Continued on next page

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UPDATES

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NCCN Guidelines Version 1.2016 Updates Pancreatic Adenocarcinoma

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Discussion

Updates in Version 1.2016 of the NCCN Guidelines for Pancreatic Adenocarcinoma from Version 2.2015 include:

PANC-A

- The following reference has been added: Al-Hawary MM, Francis IR, Chari ST, et al. Pancreatic ductal adenocarcinoma radiology reporting template: consensus statement of the Society of Abdominal Radiology and the American Pancreatic Association. Radiology 2014 Jan; 270(1):248-260. (Also on PANC-B)

PANC-A (2 of 8)

- The following has been added to #8: "Intraoperative ultrasound can be used as a diagnostic adjunct during staging laparoscopy."
- #10 has been added: "For locally advanced/metastatic disease, the panel recommends serial CT (routine single portal venous phase or dedicated pancreatic protocol if surgery is still contemplated) or MRI of known sites of disease to determine therapeutic benefit. It is recognized that patients can demonstrate progressive disease clinically without objective radiologic evidence of disease progression."

PANC-A (5 of 8)

- The Pancreatic Cancer Radiology Reporting Template has been included and adapted from:
  - Al-Hawary MM, Francis IR, Chari ST, et al. Pancreatic ductal adenocarcinoma radiology reporting template: consensus statement of the Society of Abdominal Radiology and the American Pancreatic Association. Radiology 2014 Jan; 270(1):248-260.

PANC-C

- Under distal pancreatectomy, the following bullet has been removed: "Utilization of radical resection is associated with an increase in blood loss, transfusion requirements, operating time, length of stay, and whether morbidity/mortality remains acceptable."

PANC-D (2 of 4)

- Under histologic sectioning, the last sub-bullet has been revised: "*Consider* frozen section analysis of the pancreatic neck and bile duct ~~is recommended~~."

PANC-E

- The third bullet has been revised: "Severe tumor-associated abdominal pain *that is unresponsive to optimal, around-the-clock narcotic administration, or if patient experiences undesirable narcotic associated side effects (See NCCN Guidelines for Adult Cancer Pain)."*
- Footnote "c" has been added: "A randomized trial examing the effects of prophylactic low-molecular-weight heparin showed a decrease in VTE but no effect on survival. (Pelzer U, Opitz B, Deutschinoff G, et al. Efficacy of prophylactic low–molecular weight heparin for ambulatory patients with advanced pancreatic cancer: Outcomes from the CONKO-004 trial. J Clin Oncol 2015;33:2028–2034.)"

PANC-F (2 of 6)

- The following adjuvant therapy option has been removed: "Upfront fluoropyrimidine- (CI 5-FU or capecitabine) or gemcitabine-based chemoradiation followed by maintenance 5-FU or gemcitabine."
- Footnote "b" has been added: "Adjuvant options listed apply only to patients who did not receive prior neoadjuvant therapy. For those who received prior neoadjuvant therapy, the adjuvant therapy options are dependent on the response to neoadjuvant therapy and other clinical considerations."

PANC-G (1 of 3)

- After gemcitabine + cisplatin, the text in parenthesis has been revised: "*Can be considered as an alternative to FOLFIRINOX* ~~especially for~~ *in* patients with possible hereditary cancers *involving DNA repair mutations.*

PANC-G (2 of 3)

- The following bullet has been added: "Recommended adjuvant therapy options apply to patients who did not receive prior neoadjuvant therapy. For those who received prior neoadjuvant therapy, the adjuvant therapy options are dependent on the response to neoadjuvant therapy and other clinical considerations."

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Clinical Trials: NCCN believes that the best management of any cancer patient is in a clinical trial. Participation in clinical trials is especially encouraged.

INTRODUCTION

Decisions about diagnostic management and resectability should involve multidisciplinary consultation at a high-volume center with use of appropriate imaging studies.

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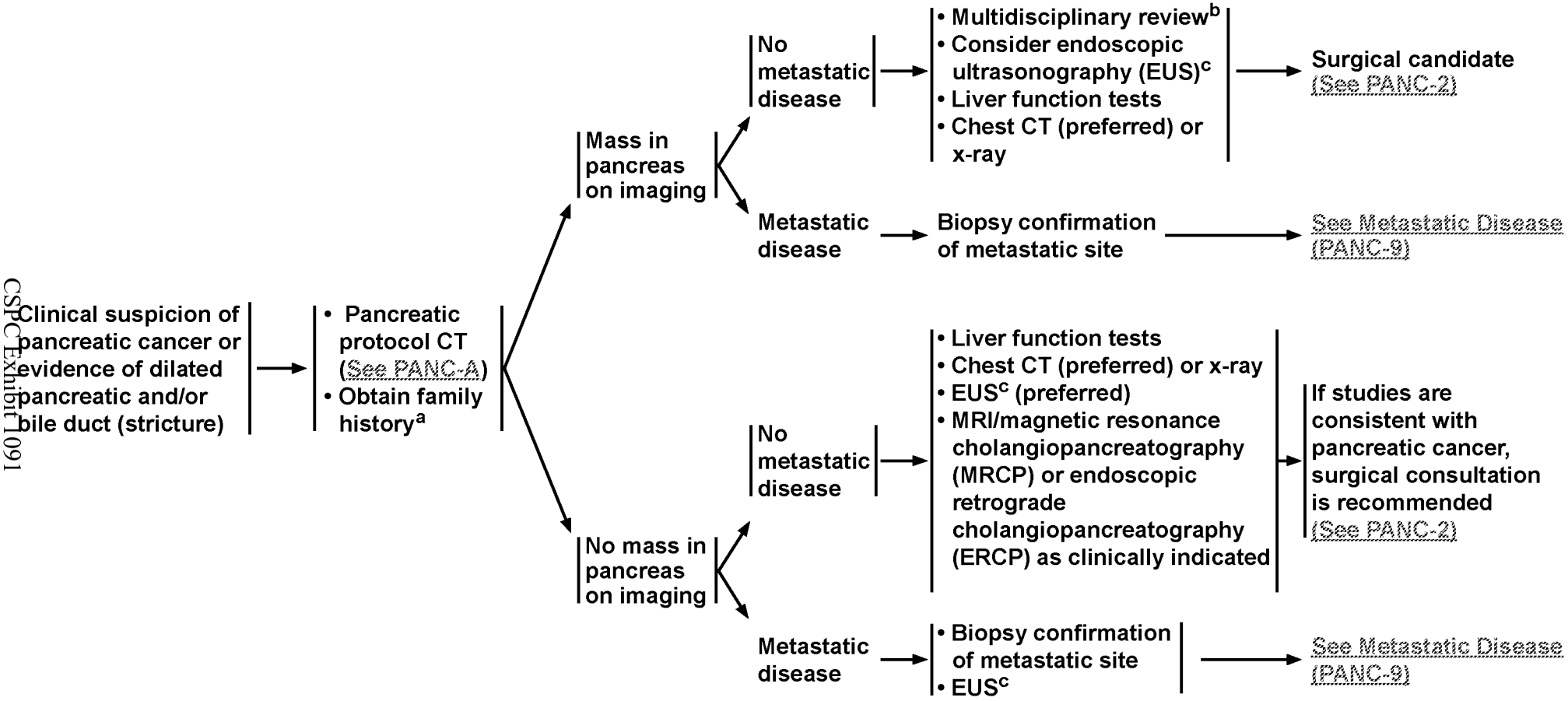
Pancreatic Adenocarcinoma



[a]If pancreatic cancer is diagnosed, consider referral for genetic counseling for patients who are young or who have a family history of cancer.
[b]Multidisciplinary review should ideally involve expertise from diagnostic imaging, interventional endoscopy, medical oncology, radiation oncology, surgery, and pathology.
[c]EUS-FNA if clinically indicated.

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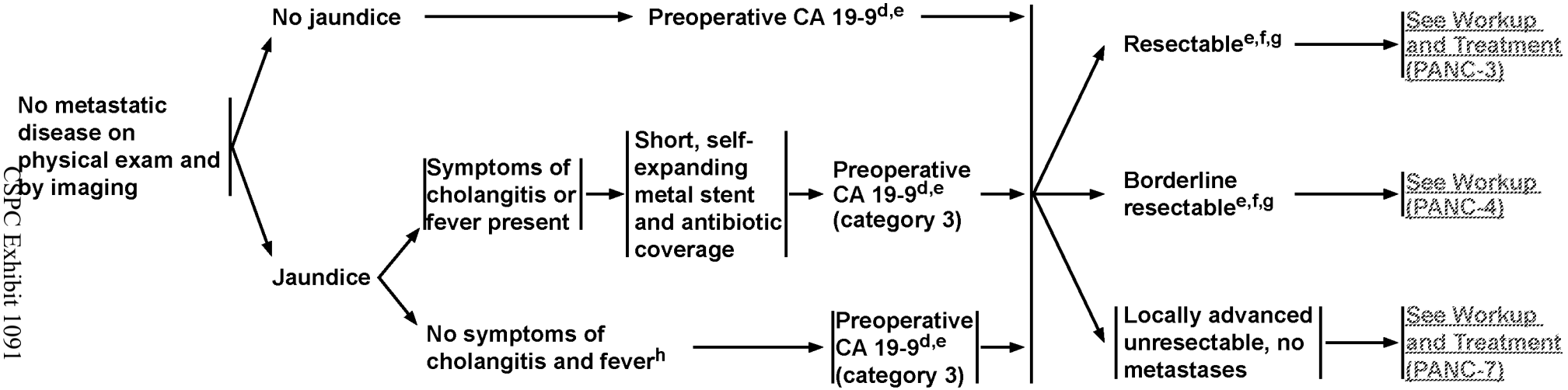
CLINICAL PRESENTATION

WORKUP



[d]Elevated CA 19-9 does not necessarily indicate cancer or advanced disease. CA 19-9 may be elevated as a result of biliary infection (cholangitis), inflammation, or obstruction, benign or malignant. In addition, CA 19-9 may be undetectable in Lewis antigen-negative individuals. (See Discussion)
[e]See Principles of Diagnosis, Imaging, and Staging (PANC-A).
[f]See Criteria Defining Resectability Status (PANC-B).
[g]See Principles of Surgical Technique (PANC-C) and Pathologic Analysis: Specimen Orientation, Histologic Sections, and Reporting (PANC-D).
[h]Self-expanding metal stent as clinically indicated in patients with select comorbidities or when surgery may be delayed. (See Discussion)

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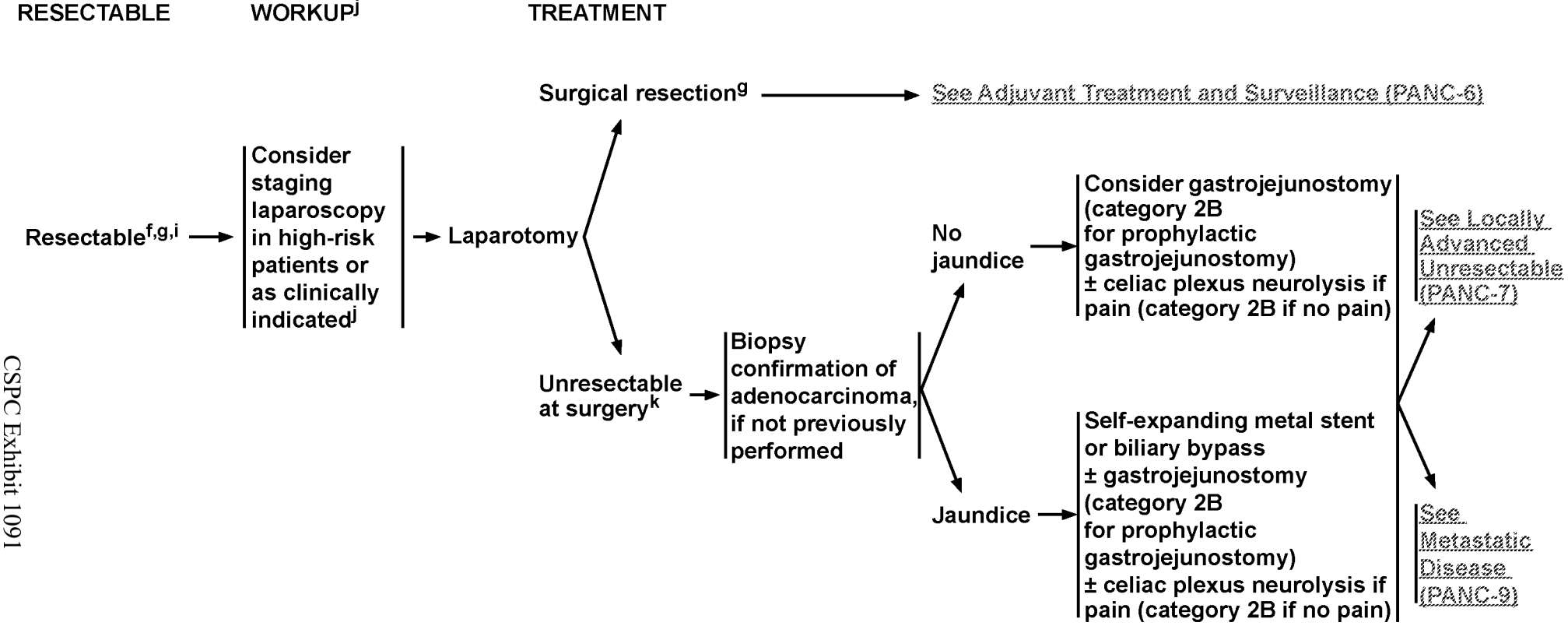

[f] See Criteria Defining Resectability Status (PANC-B).
[g] See Principles of Surgical Technique (PANC-C) and Pathologic Analysis: Specimen Orientation, Histologic Sections, and Reporting (PANC-D).
[i] For patients with tumors that are clearly resectable and who do not have high-risk features, neoadjuvant therapy is only recommended in a clinical trial. For patients with high-risk features (ie, very highly elevated CA 19-9, large primary tumors, large regional lymph nodes, excessive weight loss, extreme pain), neoadjuvant chemotherapy may be considered, which requires biopsy confirmation of adenocarcinoma (see PANC-4). Acceptable neoadjuvant regimens include FOLFIRINOX or gemcitabine + albumin-bound paclitaxel. Subsequent chemoradiation is sometimes included. Most NCCN Member Institutions prefer neoadjuvant therapy at a high-volume center.
[j] See Principles of Diagnosis, Imaging, and Staging #8 (PANC-A).
[k] See Principles of Palliation and Supportive Care (PANC-E).

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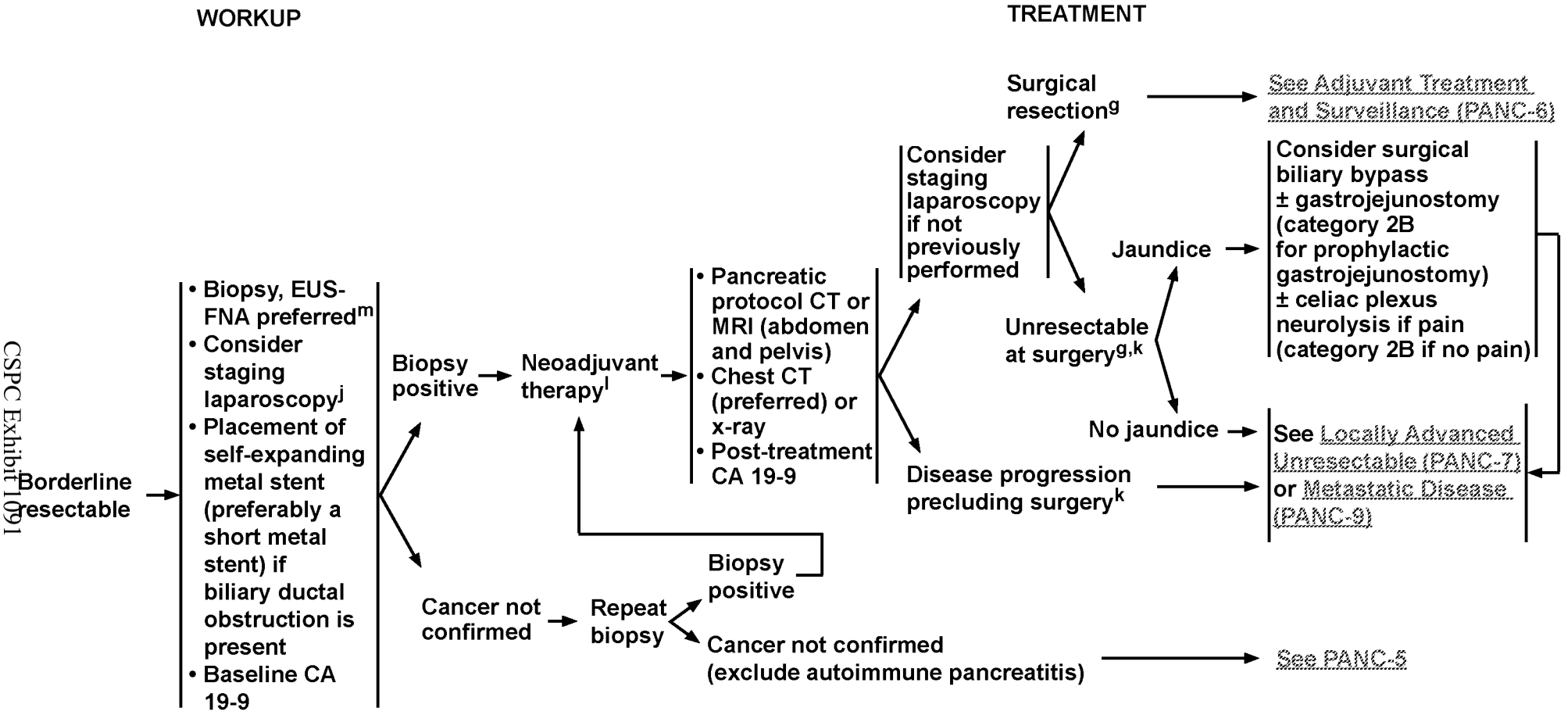
BORDERLINE RESECTABLE[e,f] NO METASTASES



[e] See Principles of Diagnosis, Imaging, and Staging (PANC-A).
[f] See Criteria Defining Resectability Status (PANC-B).
[g] See Principles of Surgical Technique (PANC-C) and Pathologic Analysis, Specimen Orientation, Histologic Sections, and Reporting (PANC-D).
[j] See Principles of Diagnosis, Imaging and Staging #8 (PANC-A).
[k] See Principles of Palliation and Supportive Care (PANC-E).
[l] There is limited evidence to recommend specific neoadjuvant regimens off-study, and practices vary with regard to the use of chemotherapy and chemoradiation. Acceptable regimens include FOLFIRINOX or gemcitabine + albumin-bound paclitaxel (see PANC-G). Subsequent chemoradiation is sometimes included (see PANC-F). Most NCCN Member Institutions prefer neoadjuvant therapy at a high-volume center. Performing surgery with a high likelihood of a positive margin is not recommended.
[m] See Principles of Diagnosis, Imaging and Staging #1 and #7 (PANC-A).

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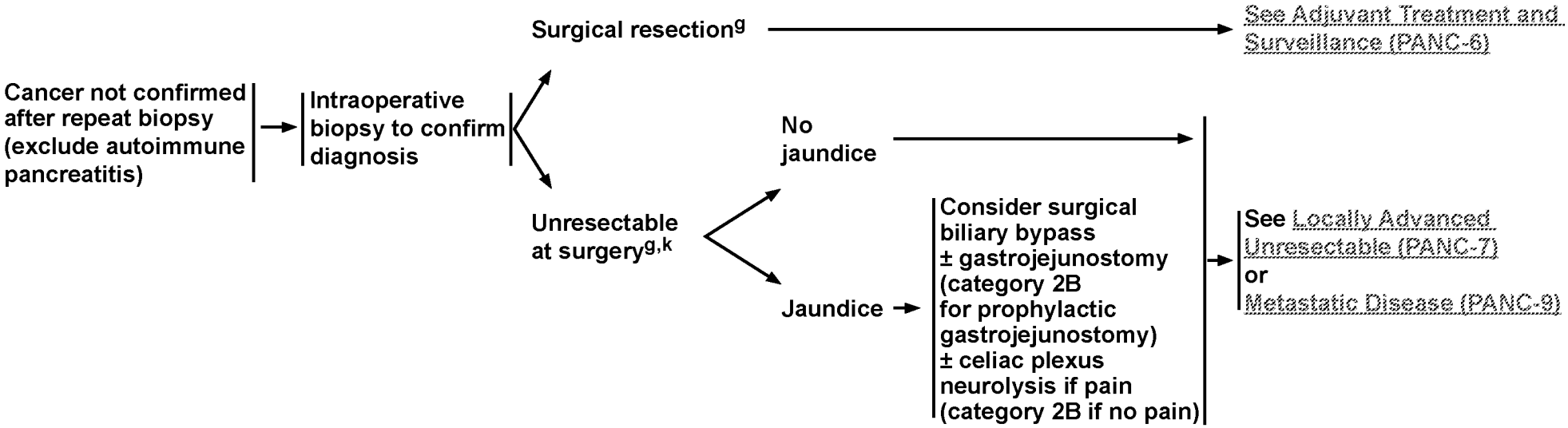
BORDERLINE RESECTABLE[f] NO METASTASES, CANCER NOT CONFIRMED

TREATMENT



[f] See Criteria Defining Resectability Status (PANC-B).
[g] See Principles of Surgical Technique (PANC-C) and Pathologic Analysis: Specimen Orientation, Histologic Sections, and Reporting (PANC-D).
[k] See Principles of Palliation and Supportive Care (PANC-E).

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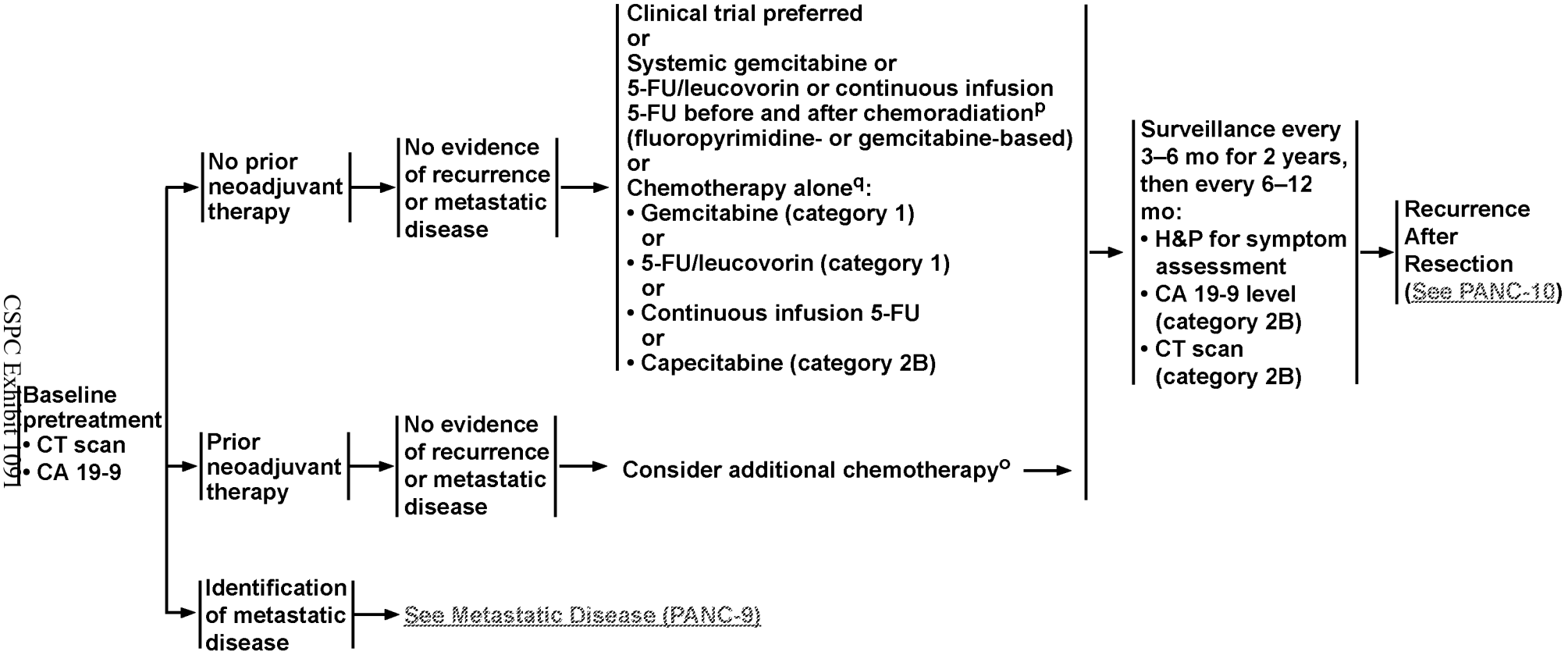
POSTOPERATIVE ADJUVANT TREATMENT[n,o]

SURVEILLANCE



[n]Adjuvant treatment should be administered to patients who have not had neoadjuvant chemotherapy and who have adequately recovered from surgery; treatment should be initiated within 12 weeks. If systemic chemotherapy precedes chemoradiation, restaging with imaging should be done after each treatment modality.

[o]Patients who have received neoadjuvant chemoradiation or chemotherapy may be candidates for additional chemotherapy following surgery and multidisciplinary review. The adjuvant therapy options are dependent on the response to neoadjuvant therapy and other clinical considerations.

[p]See Principles of Radiation Therapy (PANC-F).

[q]See Principles of Chemotherapy (PANC-G).

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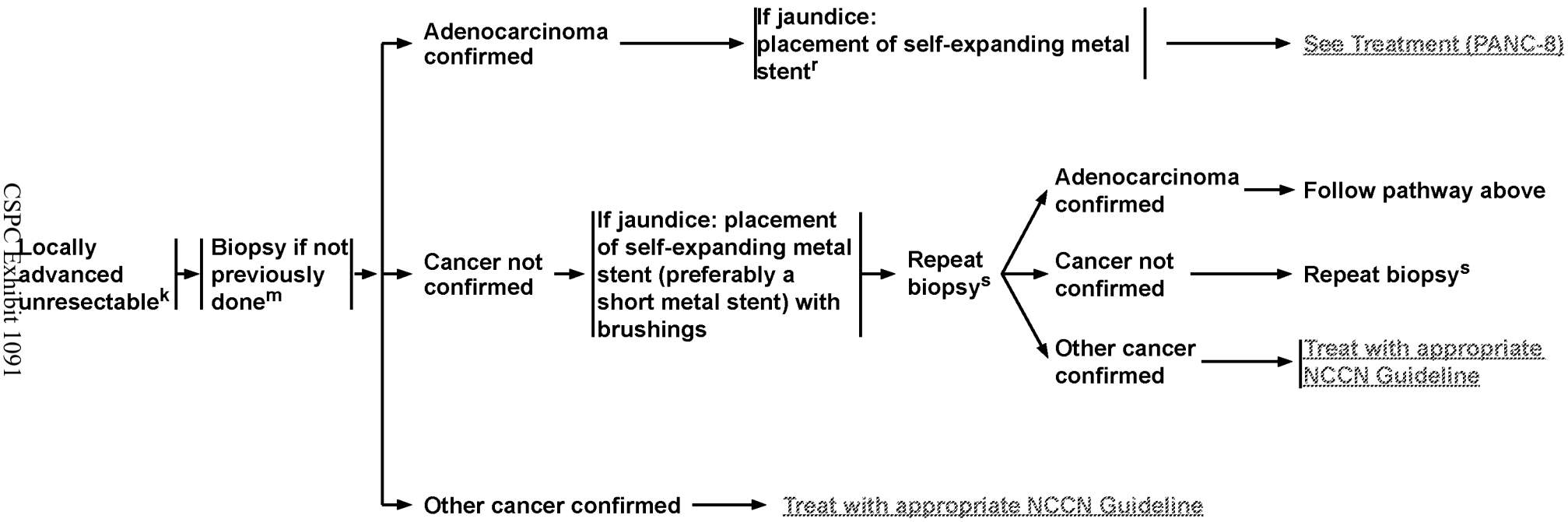
LOCALLY ADVANCED UNRESECTABLE — **WORKUP**



[k] See Principles of Palliation and Supportive Care (PANC-E).
[m] See Principles of Diagnosis, Imaging and Staging #1 and #7 (PANC-A).
[r] Unless biliary bypass performed at time of laparoscopy or laparotomy.
[s] EUS-FNA ± core biopsy at a center with multidisciplinary expertise is preferred.

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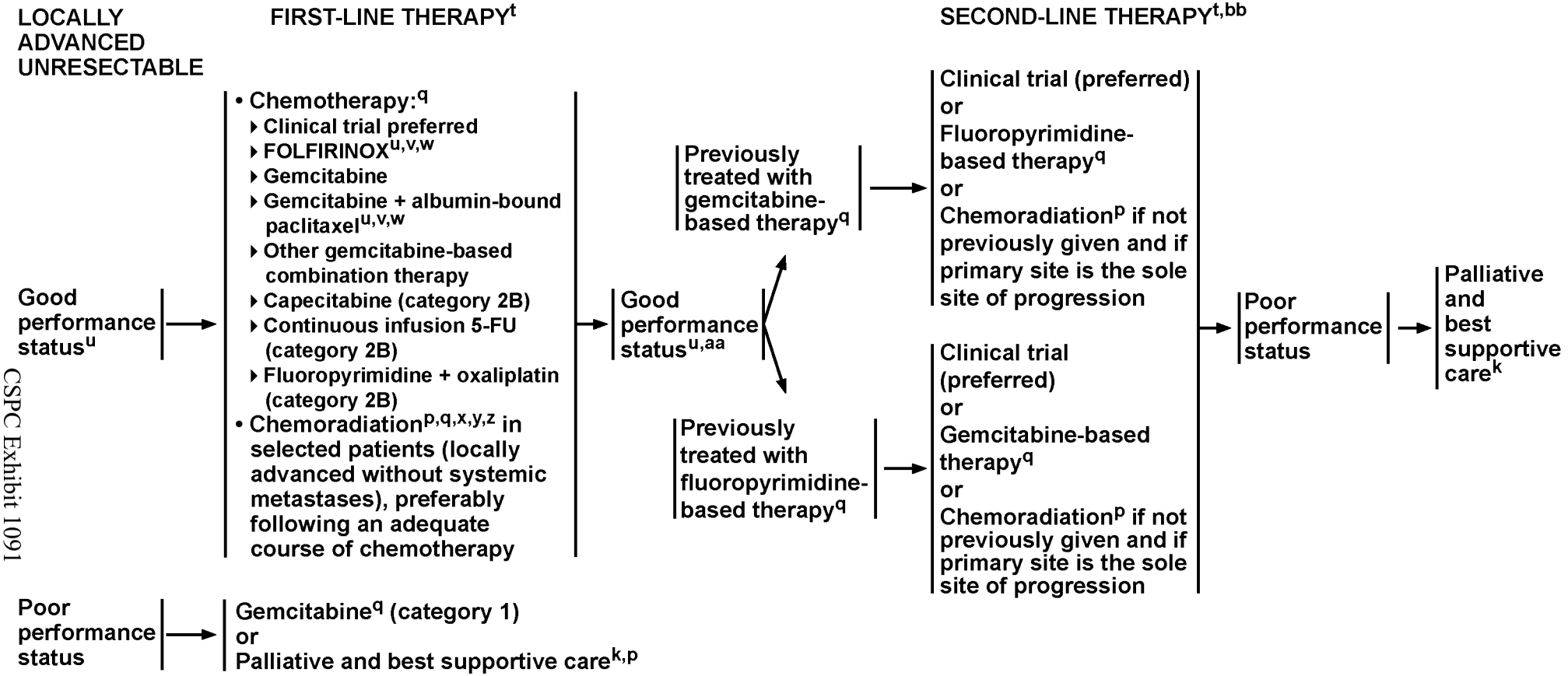

[k] See Principles of Palliation and Supportive Care (PANC-E).
[p] See Principles of Radiation Therapy (PANC-F).
[q] See Principles of Chemotherapy (PANC-G).
[t] See Principles of Diagnosis, Imaging and Staging #10 (PANC-A).
[u] Defined as ECOG 0-1 with good pain management, patent biliary stent, and adequate nutritional intake.
[v] FOLFIRINOX should be limited to those with ECOG 0-1. Gemcitabine + albumin-bound paclitaxel is reasonable for patients with KPS ≥70.
[w] The recommendations for FOLFIRINOX and gemcitabine + albumin-bound paclitaxel in patients with locally advanced disease are based on extrapolations from randomized trials in patients with metastatic disease.
[x] Chemoradiation should be reserved for patients who do not develop metastatic disease while receiving systemic chemotherapy.
[y] Based on preliminary data from the LAP-07 trial, there is no clear survival benefit with the addition of conventional chemoradiation following gemcitabine monotherapy. Chemoradiation may improve local control and delay the need for resumption therapy. (Huguet F, Hammel P, Vernerey D, et al. Impact of chemoradiotherapy on local control and time without treatment in patients with locally advanced pancreatic cancer included in the international phase III LAP 07 study. J Clin Oncol 2014; 32:5s. Abstract 4001.)
[z] Laparoscopy as indicated to evaluate distant disease.
[aa] Patients with a significant response to therapy may be considered for surgical resection.
[bb] Best reserved for patients who maintain a good performance status.

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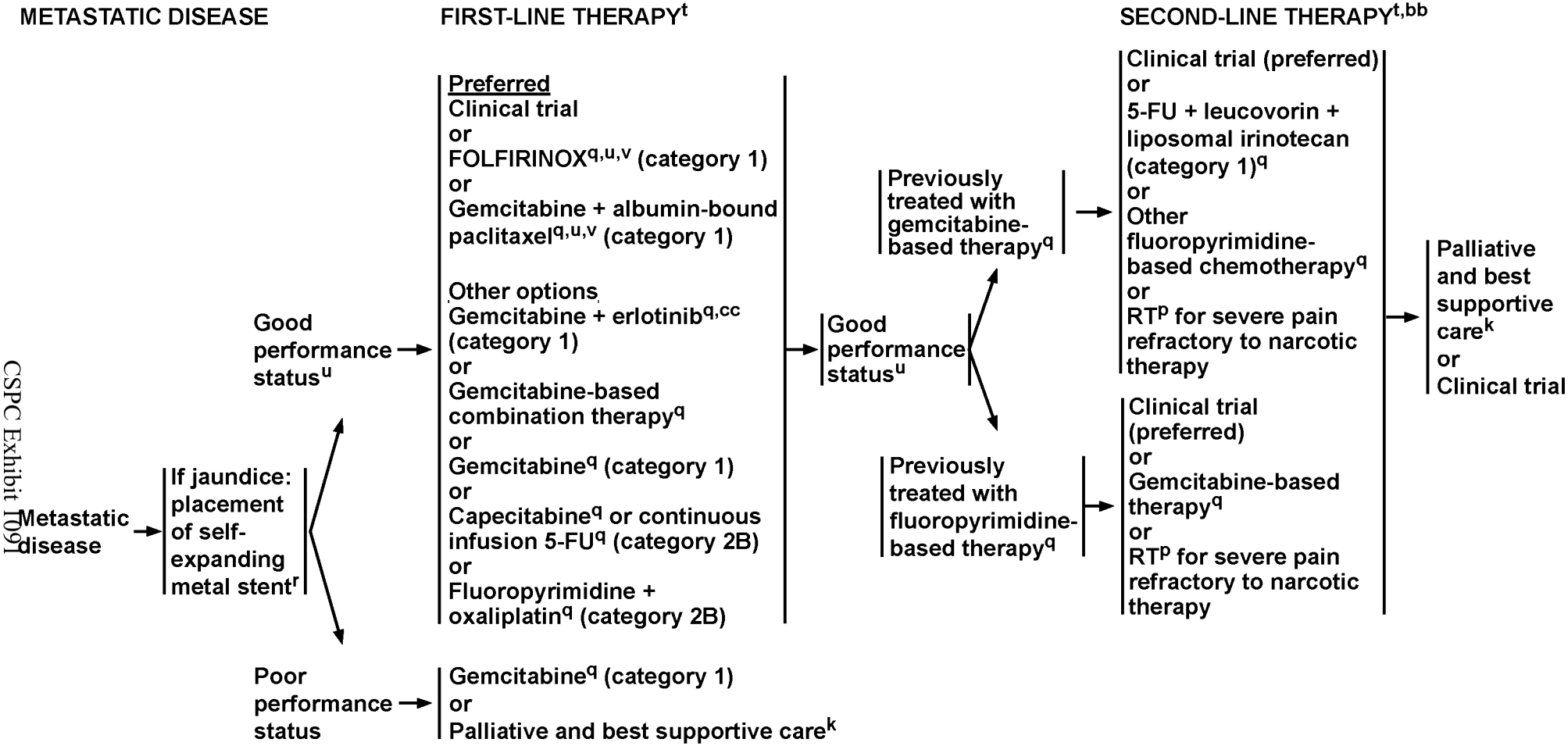

[k] See Principles of Palliation and Supportive Care (PANC-E).
[p] See Principles of Radiation Therapy (PANC-F).
[q] See Principles of Chemotherapy (PANC-G).
[r] Unless biliary bypass performed at time of laparoscopy or laparotomy.
[t] See Principles of Diagnosis, Imaging and Staging #10 (PANC-A).
[u] Defined as ECOG 0-1 with good pain management, patent biliary stent, and adequate nutritional intake.
[v] FOLFIRINOX should be limited to those with ECOG 0-1. Gemcitabine + albumin-bound paclitaxel is reasonable for patients with KPS ≥70.
[bb] Best reserved for patients who maintain a good performance status.
[cc] Although this combination significantly improved survival, the actual benefit was small, suggesting that only a small subset of patients benefit.

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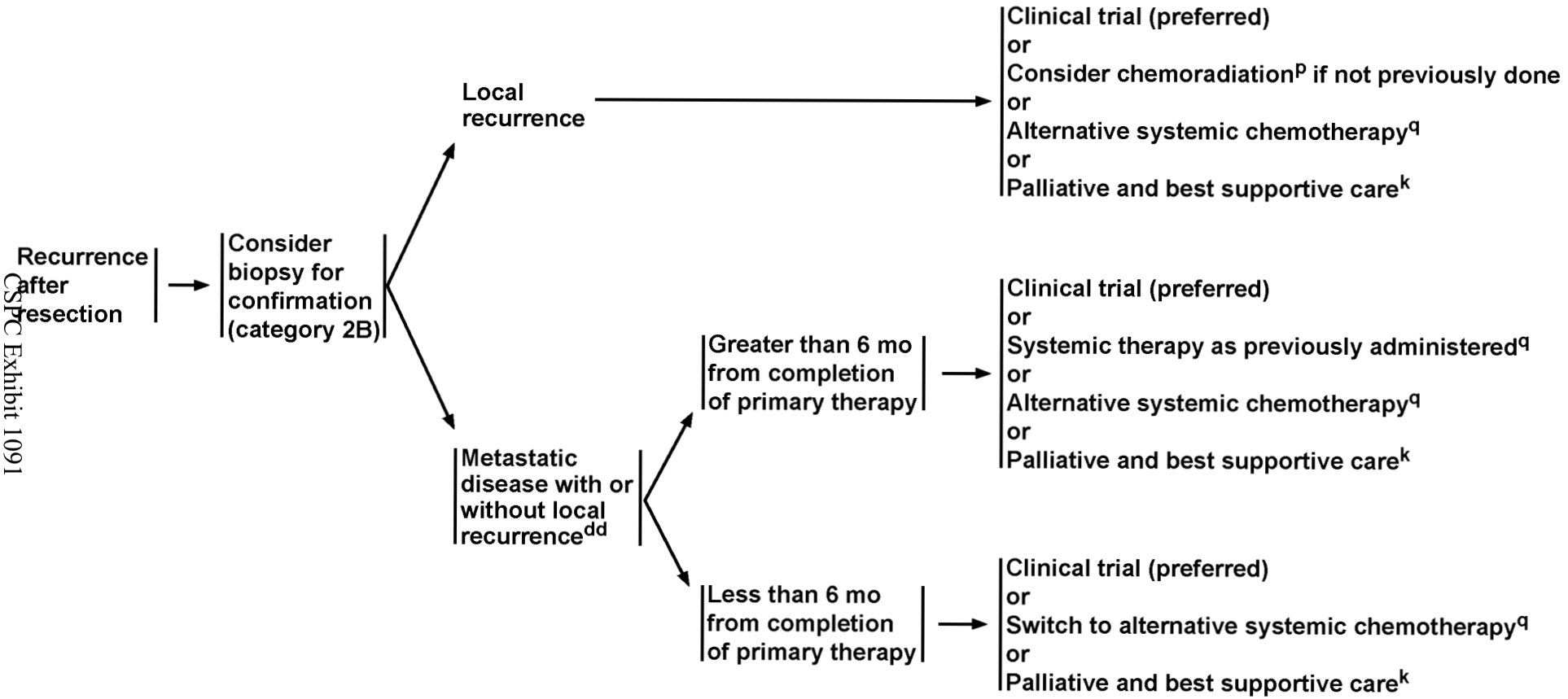
RECURRENCE AFTER RESECTION

SECOND-LINE THERAPY[bb]



[k] See Principles of Palliation and Supportive Care (PANC-E).
[p] See Principles of Radiation Therapy (PANC-F).
[q] See Principles of Chemotherapy (PANC-G).
[bb] Best reserved for patients who maintain a good performance status.
[dd] For more information about the treatment of isolated pulmonary metastases, see Discussion.

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING

#1 Decisions about diagnostic management and resectability should involve multidisciplinary consultation at a high-volume center with reference to appropriate high-quality imaging studies to evaluate the extent of disease. Resections should be done at institutions that perform a large number (at least 15–20) of pancreatic resections annually.

#2 High-quality dedicated imaging of the pancreas should be performed at presentation (even if standard CT imaging is already available) and following neoadjuvant treatment to provide adequate staging and assessment of resectability status.

#3 Imaging should include dedicated pancreatic CT (preferred) or MRI.

- **Maximum computed tomography (MDCT) angiography, performed by acquiring thin, preferably sub-millimeter, axial sections using a dual-phase pancreatic protocol, with images obtained in the pancreatic and portal venous phase of contrast enhancement, is the preferred imaging tool for dedicated pancreatic imaging.[1] Scan coverage can be extended to cover the chest and pelvis for complete staging as per institutional preferences. Multiplanar reconstruction is preferred as it allows precise visualization of the relationship of the primary tumor to the mesenteric vasculature as well as detection of subcentimeter metastatic deposits.** **See MDCT Pancreas Adenocarcinoma Protocol, PANC-A (3 of 8).**
- **MRI is most commonly used as a problem-solving tool, particularly for characterization of CT-indeterminate liver lesions and when suspected pancreatic tumors are not visible on CT or when contrast-enhanced CT cannot be obtained (as in cases with severe allergy to iodinated intravenous contrast material). This preference for using MDCT as the main imaging tool in many hospitals and imaging centers is mainly due to the higher cost and lack of widespread availability of MRI compared to CT.** **See MRI Pancreatic Adenocarcinoma Protocol, PANC-A (4 of 8).**
- **MR cholangiopancreatography (MRCP) without IV contrast should not be utilized in the staging of pancreatic cancer, except in cases of renal failure or other contraindications to administration of gadolinium intravenous contrast.**

#4 The decision regarding resectability status should be made by consensus at multidisciplinary meetings/discussions following the acquisition of dedicated pancreatic imaging including complete staging. Use of a radiology staging reporting template is preferred to ensure complete assessment and reporting of all imaging criteria essential for optimal staging, which will improve the decision-making process.[1] **See Pancreatic Cancer Radiology Reporting Template, PANC-A (5 of 8)**

Continued on next page

[1]Al-Hawary MM, Francis IR, Chari ST, et al. Pancreatic ductal adenocarcinoma radiology reporting template: consensus statement of the Society of Abdominal Radiology and the American Pancreatic Association. Radiology 2014 Jan; 270(1):248-260.

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING

#5 The role of PET/CT (without iodinated intravenous contrast) scan remains unclear. Diagnostic CT or MRI with IV contrast as discussed above in conjunction with functional PET imaging can be used per institutional preference. PET/CT scan may be considered after formal pancreatic CT protocol in high-risk[2] patients to detect extra pancreatic metastases. It is not a substitute for high-quality, contrast-enhanced CT.

#6 EUS is not recommended as a routine staging tool. In select cases, EUS may be complementary to CT for staging.

#7 EUS-FNA is preferable to a CT-guided FNA in patients with resectable disease because of better diagnostic yield, safety, and potentially lower risk of peritoneal seeding with EUS-FNA when compared with the percutaneous approach. Biopsy proof of malignancy is not required before surgical resection, and a non-diagnostic biopsy should not delay surgical resection when the clinical suspicion for pancreatic cancer is high.

#8 Diagnostic staging laparoscopy to rule out metastases not detected on imaging (especially for body and tail lesions) is used in some institutions prior to surgery or chemoradiation, or selectively in patients who are at higher risk[2] for disseminated disease. Intraoperative ultrasound can be used as a diagnostic adjunct during staging laparoscopy.

#9 Positive cytology from washings obtained at laparoscopy or laparotomy is equivalent to M1 disease. If resection has been done for such a patient, he or she should be treated for M1 disease.

#10 For locally advanced/metastatic disease, the panel recommends serial CT (routine single portal venous phase or dedicated pancreatic protocol if surgery is still contemplated) or MRI of known sites of disease to determine therapeutic benefit. It is recognized that patients can demonstrate progressive disease clinically without objective radiologic evidence of disease progression.

Continued on next page

[2]Indicators of high-risk patients may include borderline resectable disease, markedly elevated CA 19-9, large primary tumors, or large regional lymph nodes.

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING

MDCT Pancreatic Adenocarcinoma Protocol[1]

Parameters	Details
Scan type	Helical (preferably 64-multidetector row scanner or more)
Section thickness	Thinnest possible (<3 mm). Preferably submillimeter (0.5–1 mm) if available
Interval	Same as section thickness (no gap)
Oral contrast agent	Neutral contrast (positive oral contrast may compromise the three-dimensional [3D] and maximum intensity projection [MIP] reformatted images)
Intravenous contrast	Iodine-containing contrast agents (preferably high concentration [>300 mg I/L]) at an injection rate of 3–5 mL/sec. Lower concentration contrast can be used if low Kv setting is applied.
Scan acquisition timing	Pancreatic parenchymal phase at 40–50 sec and portal venous phase at 65–70 sec, following the commencement of contrast injection
Image reconstruction and display	- Axial images and multiplanar reformats (in the coronal, and per institutional preference, sagittal plane) at 2–3 mm interval reconstruction - MIP or 3D volumetric thick section for vascular evaluation (arteries and veins)

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[1]Adapted from: Al-Hawary MM, Francis IR, Chari ST, et al. Pancreatic ductal adenocarcinoma radiology reporting template: consensus statement of the Society of Abdominal Radiology and the American Pancreatic Association. Radiology 2014 Jan; 270(1):248-260.

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING

MRI Pancreatic Adenocarcinoma Protocol[3]

Sequences	Plane	Slice thickness
T2-weighted single-shot fast spin-echo (SSFSE)	Coronal +/- axial	<6 mm
T1-weighted in-phase and opposed-phase gradient echo (GRE)	Axial	<6 mm
T2-weighted fat-suppressed fast spin-echo (FSE)	Axial	<6 mm
Diffusion-weighted imaging (DWI)	Axial	<6 mm
Pre and dynamic post IV contrast administration (gadolinium[4]) Three-dimensional [3D] T1-weighted fat-suppressed gradient-echo (in pancreatic, portal venous, and equilibrium phases)	Axial	Thinnest possible 2–3 mm (4–6 mm if overlapping)
T2-weighted MRCP (preferably three-dimensional [3D], fast relaxation fast spin-echo sequence [FRFSE])	Coronal	<3 mm

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[3]Sheridan MB, Ward J, Guthrie JA, et al. Dynamic contrast-enhanced MR imaging and dual-phase helical CT in the preoperative assessment of suspected pancreatic cancer: a comparative study with receiver operating characteristic analysis. AJR Am J Roentgenol 1999 Sep;173 (3):583-90.

[4]Unenhanced MRI can be obtained in cases of renal failure or contraindication to gadolinium intravenous contrast if enhanced CT cannot be obtained due to severe iodinated contrast allergy.

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING
PANCREATIC CANCER RADIOLOGY REPORTING TEMPLATE[1]

Morphologic Evaluation			
Appearance (in the pancreatic parenchymal phase)	☐ Hypoattenuating	☐ Isoattenuating	☐ Hyperattenuating
Size (maximal axial dimension in centimeters)	☐ Measurable	☐ Nonmeasurable (isoattenuating tumors)	
Location	☐ Head/uncinate (right of SMV)	☐ Body/tail (left of SMV)	
Pancreatic duct narrowing/abrupt cutoff with or without upstream dilatation	☐ Present	☐ Absent	
Biliary tree abrupt cutoff with or without upstream dilatation	☐ Present	☐ Absent	

Reporting Template continued on next page

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING
PANCREATIC CANCER RADIOLOGY REPORTING TEMPLATE[1]

Arterial Evaluation				
SMA Contact	☐ Present	☐ Absent		
Degree of solid soft-tissue contact	☐ ≤180	☐ >180		
Degree of increased hazy attenuation/stranding contact	☐ ≤180	☐ >180		
Focal vessel narrowing or contour irregularity	☐ Present	☐ Absent		
Extension to first SMA branch	☐ Present	☐ Absent		
Celiac Axis Contact	☐ Present	☐ Absent		
Degree of solid soft-tissue contact	☐ ≤180	☐ >180		
Degree of increased hazy attenuation/stranding contact	☐ ≤180	☐ >180		
Focal vessel narrowing or contour irregularity	☐ Present	☐ Absent		
CHA Contact	☐ Present	☐ Absent		
Degree of solid soft-tissue contact	☐ ≤180	☐ >180		
Degree of increased hazy attenuation/stranding contact	☐ ≤180	☐ >180		
Focal vessel narrowing or contour irregularity	☐ Present	☐ Absent		
Extension to celiac axis	☐ Present	☐ Absent		
Extension to bifurcation of right/left hepatic artery	☐ Present	☐ Absent		
Arterial Variant	☐ Present	☐ Absent		
Variant anatomy	☐ Accessory right hepatic artery	☐ Replaced right hepatic artery	☐ Replaced common hepatic artery	☐ Others (origin of replaced or accessory artery) _____
Variant vessel contact	☐ Present	☐ Absent		
Degree of solid soft-tissue contact	☐ ≤180	☐ >180		
Degree of increased hazy attenuation/stranding contact	☐ ≤180	☐ >180		
Focal vessel narrowing or contour irregularity	☐ Present	☐ Absent		

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING
PANCREATIC CANCER RADIOLOGY REPORTING TEMPLATE[1]

Venous Evaluation			
MPV Contact	☐ Present	☐ Absent	☐ Complete occlusion
Degree of solid soft-tissue contact	☐ ≤180	☐ >180	
Degree of increased hazy attenuation/stranding contact	☐ ≤180	☐ >180	
Focal vessel narrowing or contour irregularity (tethering or tear drop)	☐ Present	☐ Absent	
SMV Contact	☐ Present	☐ Absent	☐ Complete occlusion
Degree of solid soft-tissue contact	☐ ≤180	☐ >180	
Degree of increased hazy attenuation/stranding contact	☐ ≤180	☐ >180	
Focal vessel narrowing or contour irregularity (tethering or tear drop)	☐ Present	☐ Absent	
Extension	☐ Present	☐ Absent	
Other			
Thrombus within vein (tumor, bland)	☐ Present ☐ MPV ☐ SMV ☐ Splenic vein	☐ Absent	
Venous collaterals	☐ Present ☐ Around pancreatic head ☐ Porta hepatis ☐ Root of the mesentery ☐ Left upper quadrant	☐ Absent	

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PRINCIPLES OF DIAGNOSIS, IMAGING, AND STAGING
PANCREATIC CANCER RADIOLOGY REPORTING TEMPLATE[1]

Extrapancreatic Evaluation		
Liver lesions	☐ Present ☐ Suspicious ☐ Indeterminate ☐ Likely benign	☐ Absent
Peritoneal or omental nodules	☐ Present	☐ Absent
Ascites	☐ Present	☐ Absent
Suspicious lymph nodes	☐ Present ☐ Porta hepatis ☐ Celiac ☐ Splenic hilum ☐ Paraaortic ☐ Aortocaval ☐ Other_____	☐ Absent
Other extrapancreatic disease (invasion of adjacent structures)	☐ Present • Organs involved: _____	☐ Absent
Impression		
	Tumor size:_____	Tumor location: _____
Vascular contact	☐ Present • Vessel involved: _____ • Extent: _____	☐ Absent
Metastasis	☐ Present (Location _____)	☐ Absent

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CRITERIA DEFINING RESECTABILITY STATUS[1]

Resectability Status	Arterial	Venous
Resectable	No arterial tumor contact (celiac axis [CA], superior mesenteric artery [SMA], or common hepatic artery [CHA]).	No tumor contact with the superior mesenteric vein (SMV) or portal vein (PV) or ≤180° contact without vein contour irregularity.
Borderline Resectable[2]	Pancreatic head /uncinate process: • Solid tumor contact with CHA without extension to celiac axis or hepatic artery bifurcation allowing for safe and complete resection and reconstruction. • Solid tumor contact with the SMA of ≤180° • Presence of variant arterial anatomy (ex: accessory right hepatic artery, replaced right hepatic artery, replaced CHA and the origin of replaced or accessory artery) and the presence and degree of tumor contact should be should be noted if present as it may affect surgical planning. Pancreatic body/tail: • Solid tumor contact with the CA of ≤180° • Solid tumor contact with the CA of >180° without involvement of the aorta and with intact and uninvolved gastroduodenal artery [some members prefer this criteria to be in the unresectable category].	• Solid tumor contact with the SMV or PV of >180°, contact of ≤180° with contour irregularity of the vein or thrombosis of the vein but with suitable vessel proximal and distal to the site of involvement allowing for safe and complete resection and vein reconstruction. • Solid tumor contact with the inferior vena cava (IVC).
Unresectable[2]	• Distant metastasis (including non-regional lymph node metastasis) Head/uncinate process: • Solid tumor contact with SMA >180° • Solid tumor contact with the CA >180° • Solid tumor contact with the first jejunal SMA branch Body and tail • Solid tumor contact of >180° with the SMA or CA • Solid tumor contact with the CA and aortic involvement	Head/uncinate process • Unreconstructible SMV/PV due to tumor involvement or occlusion (can be due to tumor or bland thrombus) • Contact with most proximal draining jejunal branch into SMV Body and tail • Unreconstructible SMV/PV due to tumor involvement or occlusion (can be due to tumor or bland thrombus)

[1]Al-Hawary MM, Francis IR, Chari ST, et al. Pancreatic ductal adenocarcinoma radiology reporting template: consensus statement of the Society of Abdominal Radiology and the American Pancreatic Association. Radiology 2014 Jan; 270(1):248-260.
[2]Solid tumor contact may be replaced with increased hazy density/stranding of the fat surrounding the peri-pancreatic vessels (typically seen following neoadjuvant therapy); this finding should be reported on the staging and follow-up scans. Decision on resectability status should be made in these patients, in consensus at multidisciplinary meetings/discussions.

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PRINCIPLES OF SURGICAL TECHNIQUE

Pancreatoduodenectomy (Whipple technique)

The goals of surgical extirpation of pancreatic carcinoma focus on the achievement of an R0 resection, as a margin-positive specimen is associated with poor long-term survival.[1,2] Achievement of a margin-negative dissection must focus on meticulous perivascular dissection of the lesion in resectional procedures, recognition of the need for vascular resection and/or reconstruction, and the potential need for extra-pancreatic organ resection. Of course the biology of the cancer might not allow for an R0 resection even with the most meticulous surgery.

- Medial dissection of pancreatic head lesions is best achieved by complete mobilization of the portal and SMV from the uncinate process (assuming no evidence of tumor infiltration). Skeletalization of the lateral, posterior, and anterior borders of the superior mesenteric artery down to the level of the adventitia will maximize uncinate yield and radial margin.[3,4]
- In the absence of frank venous occlusion noted on preoperative imaging, the need for lateral venorrhaphy or complete portal or SMV resection and reconstruction to achieve an R0 resection may be suggested but is often not known until division of the pancreatic neck has occurred. Tethering of the carcinoma to the lateral wall of the PV is not uncommon and requires careful dissection to free the vein from the pancreatic head if in fact it is possible to do so. Differentiation of tumor infiltration into the vein wall from tumor-related desmoplasia is frequently impossible to ascertain. Data support an aggressive approach to partial or complete vein excision if tumor infiltration is suspected, although acceptance of this concept (particularly with respect to vein resection) is not universal.
- While further data with respect to arterial resection are clearly needed, judicious utilization of this technique would appear to be reasonable in very select populations.

Distal Pancreatectomy

The goals of left-sided resection are similar to those of pancreatoduodenectomy, although they are often more difficult to achieve due to the advanced stage at which most of these cancers are discovered.

- An R0 distal pancreatectomy for adenocarcinoma mandates en bloc organ removal beyond that of the spleen alone in up to 40% of patients.[5,6]
- Similar to the Whipple procedure, lateral venorrhaphy, vein excision and reconstruction, and dissection to the level of the celiac axis and SMA adventitia should be performed if complete tumor clearance can be achieved.[5,7]
- Spleen preservation is not indicated in adenocarcinoma.

[1]Bilimoria KY, Talamonti MS, Sener SF, et al. Effect of hospital volume on margin status after pancreaticoduodenectomy for cancer. J Am Coll Surg. Oct 2008;207(4):510-519.
[2]Winter JM, Cameron JL, Campbell KA, et al. 1423 pancreaticoduodenectomies for pancreatic cancer: A single-institution experience. J Gastrointest Surg. Nov 2006;10(9):1199-1210; discussion 1210-1191.
[3]Yeo TP, Hruban RH, Leach SD, et al. Pancreatic cancer. Curr. Probl. Cancer. Jul-Aug 2002;26(4):176-275.
[4]Nakeeb A, Lillemoe KD, Grosfeld JL. Surgical techniques for pancreatic cancer. Minerva Chir Apr 2004;59(2):151-163.
[5]Shoup M, Conlon KC, Klimstra D, at al. Is extended resection for adenocarcinoma of the body or tail of the pancreas justified? J Gastro Surg. Dec 2003;7(8):946-952; discussion 952.
[6]Christein JD, Kendrick ML, Iqbal CW, et al. Distal pancreatectomy for resectable adenocarcinoma of the body and tail of the pancreas. J Gastrointest Surg. Sep-Oct 2005;9(7):922-927.
[7]Strasberg SM, Linehan DC, Hawkins WG. Radical antegrade modular pancreatosplenectomy procedure for adenocarcinoma of the body and tail of the pancreas: ability to obtain negative tangential margins. J Am Coll Surg. Feb 2007;204(2):244-249.

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PATHOLOGIC ANALYSIS: SPECIMEN ORIENTATION, HISTOLOGIC SECTIONS, AND REPORTING

The primary purpose of pathologic analysis of the pancreatic specimen is to determine the pathologic stage of the tumor by evaluating the type, grade, size, and extent of the cancer.

Whipple Specimen

- **Specimen orientation**
  - **Specimen orientation and inking involves both the pathologist and surgeon as this will help to ensure accurate assessment of the size and extent of the tumor. There should be either direct communication between the surgeon and pathologist for proper orientation and margin identification, or the surgeon should identify the important margins with a clearly understood and documented method (eg, written on the pathology requisition); for example: stitch on posterior margin, safety pin on the retroperitoneal/uncinate margin.**
- **Margins**
  - **Definitions of the margins and uniformity of nomenclature are critical to accurate reporting.**
    - **◊ SMA (retroperitoneal/uncinate) Margin: The most important margin is the soft tissue directly adjacent to the proximal 3–4 cm of the superior mesenteric artery. This margin is often referred to as the "retroperitoneal margin" or "posterior margin," but has also been referred to as the "uncinate margin" or "mesenteric margin." More recently, this margin has been referred to as the "SMA margin" to correlate with its location on the specimen. Radial rather than en face sections of this margin will more clearly demonstrate how closely this margin is approached by tumor. The simple step of palpating the specimen can help guide the pathologist as to the best spot along the SMA margin to select for sampling.**
    - **◊ Posterior Margin: This margin is from the posterior caudad aspect of the pancreatic head that merges with the uncinate margin and that appears to be covered by loose connective tissue. Radial rather than en face sections of this margin will more clearly demonstrate whether it is involved by tumor. In some instances this margin can be included in the same section as the SMA margin section.**
    - **◊ Portal Vein Groove Margin: This is the smooth-surfaced groove on the posterior-medial surface of the pancreatic head that rests over the PV. Radial rather than en face sections of this margin will more clearly demonstrate whether it is involved by tumor and also will provide the distance of the tumor from the margin. As is true for the posterior margin, in some instances this margin can be included in the same section as the SMA margin section.**
    - **◊ Portal Vein Margins: If an en bloc partial or complete vein resection is added to the surgical specimen it should be marked separately. En face proximal and distal end margins of the vein should be separately submitted as Proximal Portal Vein Margin and Distal Portal Vein Margin. A section documenting tumor invasion into the vein wall should also be submitted. If feasible, this section should be a full thickness of the vein wall demonstrating the depth of tumor invasion, as this has been shown to have prognostic value.[1]**
    - **◊ Pancreatic Neck (transection) Margin: This is the en face section of the transected pancreatic neck. The section should be placed into the cassette with the true margin facing up so that the initial section into the block represents the true surgical margin.**
    - **◊ Bile Duct Margin: This is the en face section of the bile duct end. The section should be removed from the unopened duct and placed into the cassette with the true margin facing up so that the initial section into the block represents the true surgical margin.**
  - **Other margins analyzed in Whipple specimens include the proximal and distal enteric margins (en face sections) and anterior surface (closest representative). The anterior surface is not a true margin, but identification and reporting of this surface when positive may portend a risk of local recurrence, and therefore should be reported in all cases.[2-5]**
  - **Collectively, these pancreatic tissue surfaces constitute the circumferential transection margin. Designating the various specific margins with different colored inks will allow recognition on microscopy.**

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PATHOLOGIC ANALYSIS: SPECIMEN ORIENTATION, HISTOLOGIC SECTIONS, AND REPORTING

- **Histologic sectioning**
  - The approach to histologic sectioning is determined by the unique characteristics of the tumor, but is also influenced by institutional preferences, expertise, and experience. Options include axial, bi- or multi-valve slicing, and perpendicular sliding. Some experts in the field bisect the pancreas along probes placed in the bile and pancreatic ducts and then serially section along each half of the pancreas.
  - Axial slicing provides an overall assessment of the epicenter of the tumor relative to the ampulla, bile duct, duodenum, and pancreas, and all of the pancreatic circumferential tissue margins mentioned above.
  - There is no one correct way to dissect a Whipple specimen. The most important aspects of dissection are clear and accurate assessment of the margins.
  - It is currently unknown what constitutes an adequate margin in pancreatic carcinoma resection specimens. A standardized definition of this would allow better stratification of patients into adjuvant regimens following surgical extirpation. For instance, if less than 1-mm clearance is associated with an unacceptably high incidence of local recurrence, then strong consideration for postoperative radiation therapy (RT) might be indicated if not received preoperatively. Tumor clearance should be reported in millimeters for all margins described above to allow prospective accumulation of these important data for future analysis.
  - Attached organs resected with the specimen en bloc require serial sectioning to assess not only direct extension, but metastatic deposits as well. One section that demonstrates direct invasion of the organ and/or a separate metastatic deposit is required.
  - Consider frozen section analysis of the pancreatic neck and bile duct. To avoid cautery artifact that may confound the frozen section, assess the pancreatic neck and bile duct at time of surgery by frozen section approximately 5 mm from the transection margin. If tumor is located within 5 mm of margins, consider further excision of the pancreas and bile duct to ensure at least 5 mm of clearance.

Distal Pancreatectomy

- In left-sided resections the peripancreatic soft tissue margins and the pancreatic neck are assessed. Additionally, involvement of the splenic vessels should be documented and invasion of the spleen is important to determine, as direct tumor invasion constitutes a pT3 pathologic stage.
- Margin definitions are as follows:
  - Proximal Pancreatic (transection) Margin: A full en face section of the pancreatic body along the plane of transection. The section should be placed into the cassette with the true margin facing up so that the initial section into the block represents the true surgical margin. More than one block may be needed.
  - Anterior (cephalad) Peripancreatic (peripheral) Surface: This surface demonstrates the relationship between the tumor and the anterior or cephalad peripancreatic soft tissue and can be representative if grossly positive. Several such sections should be taken closest to the tumor to document absence of involvement; the exact number is dependent on the degree of ambiguity of gross involvement.
  - Posterior (caudad) Peripancreatic (peripheral) Margin: This margin demonstrates the relationship between the tumor and the posterior or caudad peripancreatic soft tissue and can be representative if grossly positive. Several such sections should be taken closest to the tumor to document absence of involvement; the exact number is dependent on the degree of ambiguity of gross involvement.

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PATHOLOGIC ANALYSIS: SPECIMEN ORIENTATION, HISTOLOGIC SECTIONS, AND REPORTING

Reporting

The NCCN Pancreatic Cancer Panel currently supports pathology synoptic reports from the College of American Pathologists (CAP). The proposal included herein is an abbreviated minimum analysis of pancreatic cancer specimens from the CAP recommendations. In addition to the standard TNM staging, other variables are included, all of which have prognostic implications in the evolution of this disease.[6,7]

Specimen type

- Tumor size (obtained from careful gross measurement of the largest dimension of the tumor in cm.)
- Histologic grade (G (x-4))
- Primary tumor extent of invasion (T (x-4))
- Regional lymph nodes (N (x-1))[a]
  - # Nodes recovered
  - # Nodes involved
- Metastases (M (0-1))
- Margins: (Involvement should be defined and surgical clearance measured in mm)
  - Whipple resection:
    - ◊ SMA (retroperitoneal/uncinate) Margin
    - ◊ Posterior Margin
    - ◊ Portal Vein Groove Margin
    - ◊ Pancreatic Neck (transection) Margin
    - ◊ Bile Duct Margin
    - ◊ Enteric Margins
    - ◊ Anterior Surface
  - Distal pancreatectomy:
    - ◊ Proximal Pancreatic (transection) Margin
    - ◊ Anterior (cephalad) Peripancreatic (peripheral) Surface (optional)
    - ◊ Posterior (caudad) Peripancreatic (peripheral) Margin
- Lymphatic (small vessel) Invasion (L)
- Vascular (large vessel) Invasion (V)
- Perineural Invasion (P)
- Additional pathologic findings
  - Pancreatic Intraepithelial Neoplasia
  - Chronic Pancreatitis

Final stage: G, T, N, M, L, V, P

[a]Every effort should be made to identify all regional lymph nodes within the pancreatectomy specimen (see Discussion).

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PATHOLOGIC ANALYSIS: SPECIMEN ORIENTATION, HISTOLOGIC SECTIONS, AND REPORTING

References

[1]Fukuda S, Oussoultzoglou E, Bachellier P, et al. Significance of the depth of portal vein wall invasion after curative resection for pancreatic adenocarcinoma. Arch surg Feb 2007;142(2):172-179; discussion 180.

[2]Verbeke CS. Resection margins and R1 rates in pancreatic cancer--are we there yet? Histopathol Jun 2008;52(7):787-796.

[3]The Royal College of Pathologists. Standards and minimum datasets for reporting cancers. Minimum dataset for the histopathological reporting of pancreatic, ampulla of Vater and bile duct carcinoma. The Royal College of Pathologists. 2002.

[4]Classification of pancreatic cancer. Japan Pancreas Society. 2nd ed. Tokyo: Kanehara; 2003.

[5]Hruban RH, Pitman MB, Klimstra DS. Tumors of the Pancreas. Atlas of Tumor Pathology, 4th series, fascicle 6. Washington, D.C.: American Registry of Pathology; Armed Forces Institutes of Pathology; 2007.

[6]Mitsunaga S, Hasebe T, Iwasaki M, et al. Important prognostic histological parameters for patients with invasive ductal carcinoma of the pancreas. Cancer Sci Dec 2005;96(12):858-865.

[7]Gebhardt C, Meyer W, Reichel M, Wunsch PH. Prognostic factors in the operative treatment of ductal pancreatic carcinoma. Langenbecks Arch Surg Jan 2000;385(1):14-20.

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PRINCIPLES OF PALLIATION AND SUPPORTIVE CARE[a]

Objectives: Prevent and ameliorate suffering while ensuring optimal quality of life

- **Biliary obstruction**
  - **Endoscopic biliary metal stent (preferred method)**
  - **Percutaneous biliary drainage with subsequent internalization**
  - **Open biliary-enteric bypass**

- **Gastric outlet obstruction**
  - **Good performance status**
    - ◊ **Gastrojejunostomy (open or laparoscopic) ± J-tube**
    - ◊ **Consider enteral stent[b]**
  - **Poor performance status**
    - ◊ **Enteral stent[b]**
    - ◊ **Venting percutaneous endoscopic gastrostomy (PEG) tube for gastric decompression**

- **Severe tumor-associated abdominal pain that is unresponsive to optimal, around-the-clock narcotic administration, or if patient experiences undesirable narcotic associated side effects (See NCCN Guidelines for Adult Cancer Pain)**
  - **EUS-guided celiac plexus neurolysis (fluoroscopic- or CT-guided if unavailable)**
  - **Consider palliative radiation with or without chemotherapy if not already given as part of primary therapy regimen. See Principles of Radiation Therapy (PANC-F).**

- **Depression, pain, and malnutrition (See NCCN Guidelines for Supportive Care)**
  - **Formal Palliative Medicine Service evaluation when appropriate**
  - **Nutritional evaluation when appropriate.**

- **Pancreatic exocrine insufficiency**
  - **Pancreatic enzyme replacement**

- **Thromboembolic disease**
  - **Low-molecular-weight heparin preferred over warfarin[c]**

[a]Palliative surgical procedures are best reserved for patients with a longer life expectancy.
[b]Placement of an enteral stent is particularly important for patients with poor performance status and should be done after biliary drainage is assured.
[c]A randomized trial examing the effects of prophylactic low-molecular-weight heparin showed a decrease in VTE but no effect on survival. (Pelzer U, Opitz B, Deutschinoff G, et al. Efficacy of prophylactic low–molecular weight heparin for ambulatory patients with advanced pancreatic cancer: Outcomes from the CONKO-004 trial. J Clin Oncol 2015;33:2028–2034.)

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PRINCIPLES OF RADIATION THERAPY

General Principles:

- Patients with pancreatic cancer are best managed by a multidisciplinary team.[1]
- Recommendations for RT for such patients are typically made based upon five clinical scenarios: 1) neoadjuvant/resectable; 2) borderline resectable; 3) locally advanced/unresectable; 4) adjuvant/resectable; and 5) palliative. For definitions of these scenarios, See Criteria Defining Resectability Status (PANC-B).
- Staging is optimally determined with modern contrast-enhanced abdominal CT (3-D CT) and/or MRI imaging with thin cuts through the pancreas along with an EUS.
- If patients present with biliary obstruction (jaundice/elevated direct bilirubin), plastic or metal stents should be placed prior to initiation of RT. A percutaneous drain can also be used if ERCP stent placement is unsuccessful.
- The role of laparoscopic evaluation prior to chemoradiation is controversial, although standard at some institutions.
- Ideally, patients should be treated on clinical trials when available. Radiation is typically given concurrently with chemotherapy, except in the palliative setting.

Standard Recommendations:

**Note: It is not known whether one regimen is necessarily more effective than another; hence, these are given as examples of commonly utilized regimens. However, other regimens based on similar principles are acceptable.

Neoadjuvant Resectable/Borderline Resectable:

- No standard treatment regimen currently exists for neoadjuvant resectable or borderline resectable pancreatic cancer. Neoadjuvant therapy for patients with resectable tumors should ideally be conducted in a clinical trial. Generally, use similar paradigms as for locally advanced unresectable disease.
  - Upfront fluoropyrimidine (CI-5-FU or capecitabine-based) chemoradiation.[2,3]
  - Upfront gemcitabine-based chemoradiation.[4]
  - Induction chemotherapy (2–6 cycles) followed by 5-FU- or gemcitabine-based chemoradiation.[5]
- Ideally, surgical resection should be attempted 4–8 weeks following chemoradiation. Surgery can be performed >8 weeks following chemoradiation; however, radiation-induced fibrosis may potentially make surgery more difficult.

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Note: All recommendations are category 2A unless otherwise indicated.
Clinical Trials: NCCN believes that the best management of any cancer patient is in a clinical trial. Participation in clinical trials is especially encouraged.

PRINCIPLES OF RADIATION THERAPY

Unresectable/Locally Advanced (non-metastatic):

- Induction chemotherapy followed by 5-FU or gemcitabine-based chemoradiation.[a,7,8]
- Upfront fluoropyrimidine (CI 5-FU or capecitabine)-based chemoradiation in select patients.
- Upfront gemcitabine-based chemoradiation in select patients.[9,10]

- Options include:
  - RT 45–54 Gy in 1.8–2.5 Gy fractions (doses higher than 54 Gy may be considered if clinically appropriate) or
  - 36 Gy in 2.4 Gy fractions.[11]
- Following chemoradiation, additional maintenance chemotherapy is sometimes used, especially if tumors are still unresectable.
- In cases where 1) it is highly unlikely that patients will become resectable (complete encasement of superior mesenteric/celiac arteries); 2) there are suspicious metastases; and 3) patients may not be able to tolerate chemoradiation, then it may be reasonable to start with chemotherapy (2–6 cycles) followed by definitive chemoradiation if there is no evidence of metastatic progression.
- If patients present with poorly controlled pain or local obstructive symptoms, it may be preferable to start with upfront chemoradiation.
- No standard total dose or dose per fraction has been established for SBRT; therefore, it should preferably be utilized as part of a clinical trial.[12]

Adjuvant:[b]

- Treatment options following pancreaticoduodenectomy or distal pancreatectomy include:
  - Gemcitabine or CI 5-FU (1 cycle) followed by CI 5-FU/RT followed by maintenance gemcitabine or CI 5-FU.[14]
  - Gemcitabine or bolus 5-FU/leucovorin or continuous infusion 5-FU.[15]
  - Gemcitabine or bolus 5-FU/leucovorin for 2–6 cycles followed by fluoropyrimidine- (CI 5-FU or capecitabine) based chemoradiation.[16]

RT 45–46 Gy in 1.8–2 Gy fractions to the tumor bed, surgical anastomoses (hepaticojejunostomy and gastrojejunostomy may be omitted if clinically appropriate), and adjacent lymph nodes, followed by an additional 5–9 Gy to the tumor bed and anastomoses, if clinically appropriate.[17]

Palliative:

- See Principles of Palliation and Supportive Care (PANC-E).
  - RT alone to the primary tumor plus a margin (typically 25–36 Gy in 2.4–5 Gy fractions) is reasonable for patients with metastatic disease who require local palliation for obstruction, pain, or bleeding.[18]
  - Palliative RT can also be considered for patients who are elderly and/or not candidates for definitive therapy because of comorbidities.
  - Metastatic sites causing pain may also be palliated with RT.

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[a]Based on preliminary data from the LAP-07 trial, there is no clear survival benefit with the addition of conventional chemoradiation following gemcitabine monotherapy. Chemoradiation may improve local control and delay the need for resumption therapy. (Huguet F, Hammel P, Vernerey D, et al. Impact of chemoradiation on local control and time without treatment in patients with locally advanced pancreatic cancer included in the international phase III LAP 07 study. J Clin Oncol 2014; 32:5s. Abstract 4001.)

[b]Adjuvant options listed apply only to patients who did not receive prior neoadjuvant therapy. For those who received prior neoadjuvant therapy, the adjuvant therapy options are dependent on the response to neoadjuvant therapy and other clinical considerations.

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PRINCIPLES OF RADIATION THERAPY

Radiation Therapy Treatment Planning Principles:

- Patients should undergo a CT simulation (thin slices through the pancreas/bed and locoregional nodal basins) with IV (assuming adequate kidney function) and oral contrast. For resected cases, preoperative CT scans and strategically placed surgical clips are used to determine the tumor bed, ideally with the surgeon's assistance. In the neoadjuvant, borderline, and locally advanced settings the pancreatic gross tumor volume (GTV) and pathologic nodes (minimum >1 cm) are contoured with assistance from structural (CT/MRI) and functional imaging (PET). [19,20]
- The planning target volume (PTV) should be defined per the ICRU-62 guidelines.[21] A GTV should be defined for intact pancreatic tumors. For adjuvant cases, a clinical target volume (CTV) includes high-risk peri-pancreatic lymph nodes, anastomoses (hepaticojejunostomy and gastrojejunostomy may be omitted if clinically appropriate), pancreatic tumor bed derived from presurgical imaging, and strategically placed surgical clips. CTV expansions are needed to include possible microscopic disease. Further expansion to PTV includes internal target volume (ITV) for target/breathing motion and additional patient setup error margin (SM).[22-24] Image guidance methods should be considered when constructing the PTV. Organs at risk (OARs) should also be contoured and evaluated in the dose-volume histogram (DVH). Elective nodal irradiation (ENI) is commonly used for adjuvant cases but is controversial for unresectable/neoadjuvant/borderline resectable cases.[11] Standard margin expansions for unresectable cases include the gross tumor and any pathologic lymph nodes (GTV) plus a 0.5–1.5 cm margin to target microscopic extension (CTV) and an additional 0.5–2 cm volume to account for tumor/breathing motion and patient setup errors (PTV). With these expansions, peripancreatic nodes are generally included.
- 3-D conformal RT (3D-CRT) or intensity-modulated RT (IMRT) with breathhold/gating techniques can result in improved PTV coverage with decreased dose to OARs. [25,26] With SBRT, smaller margins are used (0.2–0.5 cm) and the PTV does not cover locoregional elective nodal regions.[27] If small GTV margin expansions are used for CTV and PTV, breathing motion and setup error should be evaluated or controlled per the AAPM Task Group 76 guidelines.[28]
- IORT is delivered with electron beam RT (IOERT) or high-dose-rate brachytherapy (HDR-IORT). IORT is generally delivered in a single fraction and in combination with adjuvant or neoadjuvant chemoradiation. The role of IORT is controversial and should only be performed at specialized centers. It is sometimes used in cases where surgical resection may result in close or involved margins.[29]
- It is imperative to evaluate the DVH of the PTV and critical normal structures such as the liver, kidneys, spinal cord, liver, and bowel.
- (See Table 1, Normal Tissue Dose Volume Recommendations [PANC-F, 4 of 6]) While these examples of limits are empirical they differ based on dose per fraction, total dose delivered, and disease status (adjuvant vs. unresectable). Studies have shown that the tolerability of radiation is largely dependent on PTV size/ENI, types of concurrent systemic/targeted therapy, and whether conformal (3-D, IMRT, SBRT) vs. conventional radiation is used.

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PRINCIPLES OF RADIATION THERAPY

- **Fractionated RT is typically delivered as 30–55 Gy over ~3–6 weeks (1.8–3.0 Gy/fraction, using lower dose per fraction at higher cumulative doses while respecting normal tissue constraints) with concurrent 5-FU/capecitabine or gemcitabine as a radiosensitizer. Doses above 55 Gy may be possible in select cases; however, data are limited and normal tissue dose limits (see Table 1) should be maintained. For resected cases, 45 Gy is delivered to the tumor bed, surgical anastomosis (hepaticojejunostomy and gastrojejunostomy may be omitted if clinically appropriate), and regional lymph nodes. Additional radiation (~5–15 Gy) may be administered to the tumor bed/area of involved margins and anastomoses paying careful attention to dose to bowel and stomach. The use of high-energy photon beams is preferred. SBRT is often delivered in 1–5 fractions ranging from 5–25 Gy per fraction. IORT can be delivered in a single fraction alone (15–20 Gy) or in combination with external beam RT (EBRT) (10–20 Gy).**
- **Several clinical trials (RTOG) now refer to atlases to assist with contouring and adjuvant RT planning. (http://www.rtog.org/CoreLab/ContouringAtlases.aspx).**

Table 1: Normal Tissue Dose Volume Recommendations

Structure	Unresectable/Preoperative Recommendations[c]	Adjuvant/Resected Recommendations[d]
Kidney (right and left)	Not more than 30% of the total volume can receive ≥18 Gy. If only one kidney is functional, not more than 10% of the volume can receive ≥18 Gy.	If two functioning kidneys present, not more than 50% of the right and 65% of the left kidney should receive >18 Gy. For IMRT planning mean dose to bilateral kidneys should be ≤18 Gy. If only one kidney is present not more than 15% should receive ≥18 Gy and no more than 30% should receive ≥14 Gy.
Stomach, duodenum, jejunum	Max dose ≤55 Gy; not more than 30% of the volume can be between 45 and 55 Gy.	Max dose ≤55 Gy; <10% of each organ volume can receive between 50–53.99 Gy. <15% of each organ volume can receive 45–49.99 Gy.
Liver	Mean dose cannot exceed 30 Gy.	Mean liver dose ≤25 Gy.
Spinal cord	Max dose to a volume of at least 0.03 cc must be ≤45 Gy.	Max dose ≤45 Gy.

[c]Adapted from RTOG 0936 (3-D conformal, 1.8-50.5) and RTOG 1102 (IMRT, 2.2 to 55 Gy)
[d]Adapted from RTOG 0848 (3-D or IMRT)

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PRINCIPLES OF RADIATION THERAPY

Table 2: Commonly Used Radiation Therapy Abbreviations

3D-CRT	3-D Conformal Radiation Therapy
IMRT	Intensity-Modulated Radiation Therapy
SBRT	Stereotactic Body Radiation Therapy
SABR	Stereotactic Ablative Radiation Therapy
EBRT	External Beam Radiation Therapy
ENI	Elective Nodal Irradiation
IORT	Intraoperative Radiation Therapy
DVH	Dose-Volume Histogram
GTV	Gross Tumor Volume
CTV	Clinical Target Volume
IM	Internal Margin: Variations in shape/size of CTV due to respiration and adjacent structures
ITV	Internal Target Volume: encompasses the CTV and IM (ITV = CTV + IM)
PTV	Planning Target Volume
BED	Biologically Effective Dose
OAR	Organ At Risk
ABC	Airway Breathing Control
IGRT	Image-Guided Radiation Therapy
4DCT	Four-Dimensional Computed Tomography
CBCT	Cone Beam Computed Tomography

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PRINCIPLES OF RADIATION THERAPY
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PRINCIPLES OF CHEMOTHERAPY (1 of 3)

- **Systemic therapy is used in the neoadjuvant or adjuvant setting and in the management of locally advanced unresectable and metastatic disease.**
- **Goals of systemic therapy should be discussed with patients prior to initiation of therapy, and enrollment in a clinical trial is strongly encouraged.**
- **Close follow-up of patients undergoing chemotherapy is indicated.**

Metastatic

- **Acceptable chemotherapy combinations for patients with good performance status include:**
  - **FOLFIRINOX[1] (category 1) (preferred)**
  - **Gemcitabine + albumin-bound paclitaxel[2] (category 1) (preferred)**
  - **Gemcitabine + erlotinib[3] (category 1)[a]**
  - **Gemcitabine + capecitabine[4]**
  - **Gemcitabine + cisplatin[5] (Can be considered as an alternative to FOLFIRINOX in patients with possible hereditary cancers involving DNA repair mutations.)**
  - **Fixed-dose-rate gemcitabine, docetaxel, capecitabine (GTX regimen)[6] (category 2B)**
  - **Fluoropyrimidine + oxaliplatin (category 2B) (eg, 5-FU/leucovorin/oxaliplatin[7] or CapeOx[8])**
- **Acceptable monotherapy options for patients with poor performance status include:**
  - **Gemcitabine at 1000 mg/m^2 over 30 minutes, weekly for 3 weeks every 28 days (category 1).**
  - **Fixed-dose-rate gemcitabine (10 mg/m^2/min) may substitute for standard infusion of gemcitabine over 30 minutes (category 2B).**
  - **Capecitabine or continuous infusion 5-FU (category 2B)**
- **Second-line chemotherapy may consist of:**
  - **5-FU + leucovorin + liposomal irinotecan (category 1)[13] (for metastatic disease previously treated with gemcitabine-based therapy)**
  - **Gemcitabine-based therapy for those previously treated with fluoropyrimidine-based therapy**
  - **Fluoropyrimidine-based therapy for those previously treated with gemcitabine-based therapy**

Locally Advanced

- **Depending on performance status, mono- or combination systemic chemotherapy, as noted above, may be considered as initial therapy prior to chemoradiation for appropriate patients with locally advanced, unresectable disease[b]. Patients should be evaluated for recovery from hematologic and non-hematologic toxicity prior to initiation of chemoradiation. Patients who progress with metastatic disease are not candidates for chemoradiation unless required for palliative purposes.**

See Adjuvant, and Neoadjuvant PANC-G (2 of 3)
See References on PANC-G (3 of 3)

[a]Although this combination significantly improved survival, the actual benefit was small, suggesting that only a small subset of patients benefit.
[b]Based on preliminary data from the LAP-07 trial, there is no clear survival benefit with the addition of conventional chemoradiation following gemcitabine monotherapy.[12]

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PRINCIPLES OF CHEMOTHERAPY (2 of 3)

Adjuvant

- The CONKO 001 trial demonstrated significant improvements in disease-free survival and overall survival with use of postoperative gemcitabine as adjuvant chemotherapy versus observation in resectable pancreatic adenocarcinoma.[9]
- ESPAC-3 study results showed no significant difference in overall survival between 5-FU/leucovorin versus gemcitabine following surgery. When the groups receiving adjuvant 5-FU/leucovorin and adjuvant gemcitabine were compared, median survival was 23.0 months and 23.6 months, respectively.[10]
- The use of gemcitabine-based chemotherapy is frequently combined, sequentially, with 5-FU-based chemoradiotherapy.
- No significant differences were observed in the RTOG 97-04 study comparing pre- and post-chemoradiation 5-FU with pre- and post-chemoradiation gemcitabine for postoperative adjuvant treatment.[11]
- For patients with good performance status who relapse after receiving adjuvant therapy, FOLFIRINOX or gemcitabine + albumin-bound paclitaxel are options depending on the length of time since completion of adjuvant therapy.
- Recommended adjuvant therapy options apply to patients who did not receive prior neoadjuvant therapy. For those who received prior neoadjuvant therapy, the adjuvant therapy options are dependent on the response to neoadjuvant therapy and other clinical considerations.

Neoadjuvant

- There is limited evidence to recommend specific neoadjuvant regimens off-study, and practices vary with regard to the use of chemotherapy and chemoradiation. Acceptable regimens include FOLFIRINOX or gemcitabine + albumin-bound paclitaxel. Subsequent chemoradiation is sometimes included. Most NCCN Member Institutions prefer neoadjuvant therapy at a high-volume center.

See Metastatic and Locally Advanced PANC-G (1 of 3)

See References on PANC-G (3 of 3)

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PRINCIPLES OF CHEMOTHERAPY (3 of 3)
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NCCN Guidelines Version 1.2016 Staging Pancreatic Adenocarcinoma

NCCN Guidelines Index
Pancreatic Table of Contents
Discussion

Table 1

American Joint Committee on Cancer (AJCC) TNM Staging of Pancreatic Cancer (2010)

Because only a few patients with pancreatic cancer undergo surgical resection of the pancreas (and adjacent lymph nodes), a single TNM classification must apply to both clinical and pathologic staging.

Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ*
T1	Tumor limited to the pancreas, 2 cm or less in greatest dimension
T2	Tumor limited to the pancreas, more than 2 cm in greatest dimension
T3	Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery
T4	Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor)

* This also includes the "PanInIII" classification.

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis

Distant Metastases (M)

M0	No distant metastases
M1	Distant metastasis

Stage Grouping

Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage III	T4	Any N	M0
Stage IV	Any T	Any N	M1

Discussion

This discussion is being updated to correspond with the newly updated algorithm. Last updated 12/04/14

NCCN Categories of Evidence and Consensus

Category 1: Based upon high-level evidence, there is uniform NCCN consensus that the intervention is appropriate.

Category 2A: Based upon lower-level evidence, there is uniform NCCN consensus that the intervention is appropriate.

Category 2B: Based upon lower-level evidence, there is NCCN consensus that the intervention is appropriate.

Category 3: Based upon any level of evidence, there is major NCCN disagreement that the intervention is appropriate.

All recommendations are category 2A unless otherwise noted.

Table of Contents

Overview

During the year 2014 in the United States, an estimated 46,420 people will be diagnosed with pancreatic cancer, and approximately 39,590 people will die of pancreatic cancer.[1] This disease is the fourth most common cause of cancer-related death among U.S. men (after lung, prostate, and colorectal cancer) and women (after lung, breast, and colorectal cancer).[1] Its peak incidence occurs in the seventh and eighth decades of life.[1] Although incidence is roughly equal in both sexes, African Americans have a higher incidence of pancreatic cancer than white Americans.[2,3] Furthermore, the incidence of pancreatic cancer in the United States increased from 1999 to 2008, possibly because of the increasing prevalence of obesity, an aging population, and other unknown factors.[3-5] Mortality rates have remained largely unchanged.[6,7]

In these NCCN Guidelines for Pancreatic Adenocarcinoma, the diagnosis and management of adenocarcinomas of the exocrine pancreas are discussed; neuroendocrine tumors are not included (please see the NCCN Guidelines for Neuroendocrine Tumors, available at www.NCCN.org). These NCCN Guidelines are intended to assist with clinical decision-making, but they cannot incorporate all possible clinical variations and are not intended to replace good clinical judgment or individualization of treatments. Exceptions to the rule were discussed among the panel members during the process of developing and updating these guidelines. A 5% rule (omitting clinical scenarios that comprise less than 5% of all cases) was used to eliminate uncommon clinical occurrences or conditions from these guidelines. A recent study of 3706 patients treated for pancreatic cancer in large California hospitals showed that compliance with these NCCN Guidelines for Pancreatic Adenocarcinoma, defined very permissively, improves survival.[8]

As an overall guiding principle of these guidelines, the panel believes that decisions about diagnostic management and resectability of pancreatic cancer should involve multidisciplinary consultation at high-volume centers with use of appropriate imaging studies. In addition, the panel believes that increasing participation in clinical trials (only 4.6% of patients enroll in a pancreatic cancer trial[9]) is critical to making progress in this disease. Thus, the panel unanimously endorses participation in a clinical trial over standard or accepted therapy.

Literature Search Criteria and Guidelines Update Methodology

Prior to the update of this version of the NCCN Guidelines for Pancreatic Adenocarcinoma, an electronic search of the PubMed database was performed to obtain key literature in the field of pancreatic cancer published between July 28, 2013 and July 28, 2014, using the following search terms: (pancreatic cancer) OR (pancreatic adenocarcinoma) OR (pancreas adenocarcinoma) OR (pancreas cancer). The PubMed database was chosen because it remains the most widely used resource for medical literature and indexes only peer-reviewed biomedical literature.[10]

The search results were narrowed by selecting studies in humans published in English. Results were confined to the following article types: Clinical Trial, Phase II; Clinical Trial, Phase III; Clinical Trial, Phase IV; Practice Guideline; Guidelines; Randomized Controlled Trial; Meta-Analysis; Systematic Reviews; and Validation Studies.

The PubMed search resulted in 152 citations, and their potential relevance was examined. The data from key PubMed articles and articles from additional sources deemed as relevant to these Guidelines and discussed by the panel have been included in this version of the Discussion section (eg, e-publications ahead of print, meeting

abstracts). Recommendations for which high-level evidence is lacking are based on the panel's review of lower-level evidence and expert opinion.

The complete details of the Development and Update of the NCCN Guidelines are available on the NCCN website (www.NCCN.org).

Risk Factors and Genetic Predisposition

Although the increase in risk is small, pancreatic cancer is firmly linked to cigarette smoking.[11-16] An increased body mass index (BMI) is also associated with an increased risk for pancreatic cancer.[17-20] There is also some evidence that increased consumption of red/processed meat and dairy products is associated with an elevation in pancreatic cancer risk,[21,22] although other studies have failed to identify dietary risk factors for the disease.[15,23,24] Occupational exposure to chemicals such as beta-naphthylamine and benzidine is associated with increased risk for pancreatic cancer,[25] as is heavy alcohol consumption.[11,13,17,26] Recent data also suggest that low plasma 25-hydroxyvitamin D levels may increase the risk for pancreatic cancer.[27] Chronic pancreatitis has also been identified as a risk factor for pancreatic cancer,[28-31] with one study demonstrating a 7.2-fold increased risk for pancreatic cancer for patients with a history of pancreatitis.[32] Overall, further epidemiologic studies involving careful evaluation of these possible risk factors with adjustments for potential confounders are needed to clarify their impact on pancreatic cancer risk.

Diabetes and Pancreatic Cancer

The association between diabetes mellitus and pancreatic cancer is particularly complicated. Numerous studies have shown an association between new-onset non-insulin-dependent diabetes and the development of pancreatic cancer,[33-37] especially in those who are elderly, have a lower BMI, experience weight loss, or do not have a family history of diabetes.[38] In these short-onset cases of diabetes diagnosed prior to pancreatic cancer diagnoses, diabetes is thought to be caused by the cancer, although the physiologic basis for this effect is not yet completely understood.[39] A population-based study of 2122 patients with diabetes found that approximately 1% of patients diagnosed with diabetes who are age 50 years or younger will be diagnosed with pancreatic cancer within 3 years.[33]

Long-term diabetes, on the other hand, appears to be a risk factor for pancreatic cancer, as some studies have shown an association of pancreatic cancer with diabetes of 2- to 8-year duration.[40] However, certain risk factors such as obesity, associated with both diabetes and pancreatic cancer, may confound these analyses.[41] Furthermore, the use of diabetic medications has been reported to alter pancreatic cancer risk.[42] The use of insulin or sulfonylureas has been found to be associated with an increased risk for pancreatic cancer.[43-45] On the other hand, metformin may be associated with a reduced risk for pancreatic and other cancers.[43-48]

In addition, diabetes and diabetic medication may affect outcomes in patients with pancreatic cancer. Metformin use has been reported to result in higher pancreatic cancer survival in diabetics. A retrospective analysis of 302 patients with pancreatic cancer and diabetes treated at The University of Texas MD Anderson Cancer Center found that metformin use was associated with increased survival at 2 years (30.1% vs. 15.4%; $P = .004$) and increased overall survival (OS, 15.2 months vs. 11.1 months; $P = .009$).[49] The OS difference was significant only in patients without distant metastases and remained significant when insulin users were excluded. In contrast, data from a recent meta-analysis of >38,000 patients show that those with pancreatic cancer and diabetes have a significantly lower OS than those without diabetes (14.4

vs. 21.7 months; $P < 0.001$).[36] A similar result was seen in a prospective cohort study, in which the survival of 504 patients with and without diabetes who developed pancreatic cancer in the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial was compared.[50] After multivariable-adjustment, mortality was significantly higher in participants with diabetes compared to those without (HR, 1.52; 95% CI, 1.14–2.04; $P < .01$).

Genetic Predisposition

Pancreatic cancer is thought to have a familial component in approximately 10% of cases, and familial excess of pancreatic cancer is associated with high risk.[15,51-54] The genetic basis of this inherited predisposition is not known in most cases; however, some familial cancer syndromes are associated with an increased risk for pancreatic cancer (see *Table 1*, below).

Germline mutations in the *STK11* gene result in Peutz-Jeghers syndrome, in which individuals have gastrointestinal polyps and a highly elevated risk for colorectal cancer.[55-57] These individuals also have a highly elevated risk for developing pancreatic cancer, reported to be increased by as much as 132-fold.[58,59] Furthermore, *STK11* undergoes somatic mutation in approximately 5% of pancreatic cancers.[60]

As with non-hereditary forms of pancreatitis, familial pancreatitis is also associated with an increased risk for pancreatic cancer.[61] Several genes are associated with the familial form of pancreatitis, including *PRSS1*, *SPINK1*, and *CFTR*.[62] The increased risk for the development of pancreatic cancer in these individuals is estimated to be 26-fold to as high as 87-fold.[29,63-65]

Familial Malignant Melanoma syndrome (also known as Melanoma-Pancreatic Cancer syndrome or Familial Atypical Multiple Mole Melanoma syndrome [FAMMM]) is caused by germline mutation of the *CDKN2A* (p16INK4a/p14ARF) gene.[66] This syndrome is associated with a 20-fold to 47-fold increased risk for pancreatic cancer.[67,68] In addition, patients with Melanoma-Pancreatic Cancer syndrome may experience an earlier onset of pancreatic cancer than the general population.[69] In an unselected series of 225 patients with pancreatic cancer in Italy, 5.7% had mutations in *CDKN2A*.[70]

Lynch syndrome is the most common form of genetically determined colorectal cancer predisposition and is caused by germline mutations in DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, or *PMS2*).[71-76] Patients with Lynch syndrome also have an estimated 9- to 11-fold elevated risk for pancreatic cancer.[77,78]

An excess of pancreatic cancer is also seen in families with hereditary Breast-Ovarian Cancer syndrome, harboring *BRCA1* and *BRCA2* (breast cancer susceptibility gene-1 and -2) mutations, although the link with *BRCA2* is better established.[79-84] In a study of 187 Ashkenazi Jewish patients who had resections for pancreatic cancer, mutations in *BRCA1* and *BRCA2* were identified in 5.5% of patients.[81] Other studies of unselected patients with pancreatic cancer have detected *BRCA* mutations at a frequency of 4% to 7%.[85] The risk of pancreatic cancer is elevated 2- to 6-fold in these patients, and the age of onset is younger than average in the general population.[79,83,84]

BRCA1 and *BRCA2* are involved in the Fanconi DNA anemia/BRCA pathway. This pathway is responsible for the repair of DNA interstrand cross-links, and particular mutations in other Fanconi anemia/BRCA pathway genes, including in *PALB2*, *FANCC*, and *FANCG,* have also been identified as increasing pancreatic cancer susceptibility.[86-88] Additionally, whole-genome sequencing recently allowed for the identification of germline mutations in *ATM*, a DNA damage response

gene, in 2 kindreds with familial pancreatic cancer.[89] Further analyses then revealed *ATM* mutations in 4 of 166 individuals with familial pancreatic cancer.

As many as 80% of patients with a family history of pancreatic cancer have no known genetic cause.[51] A prospective registry-based study of 5179 individuals from 838 kindreds found that having just 1 first-degree relative with pancreatic cancer raises the risk for pancreatic cancer by 4.6-fold, whereas having 2 affected first-degree relatives raises the risk by about 6.4-fold.[90]

The panel emphasizes the importance of taking a thorough family history when seeing a new patient with pancreatic cancer. In particular, a family history of pancreatitis, melanoma, and cancers of the pancreas, colorectum, breast, and ovaries should be noted. A free online pancreatic cancer risk prediction tool, called PancPRO, is available and may help determine risk.[54] If a cancer syndrome is identified, at-risk relatives should be offered genetic counseling. With or without a known syndrome, individuals with a suspicious family history should be advised on risk-reducing strategies including smoking cessation and weight loss. In addition, the possibility of screening for pancreatic (see below) and other cancers should be discussed.

Premalignant Tumors of the Pancreas

Mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs) of the pancreas are cystic lesions that can be small and asymptomatic and are often discovered incidentally; MCNs have an ovarian-like stroma.[91-93] IPMNs can occur in the main duct and/or in the branch ducts. Lesions involving the main duct have a higher malignant potential than those in the branches, with the risk of malignancy at around 62%.[94] The risk of malignancy in MCNs is <15%.[94]

An international group of experts has established guidelines for the management of pancreatic IPMNs and MCNs,[94] as has a European group.[95] The international group strongly recommends resection in fit patients with main duct IPMNs.[94] For branch-duct IPMNs, surveillance is considered an appropriate option in older or unfit patients or for cysts lacking suspicious features. Branch-duct IPMNs that are ≥10 mm, have an enhancing solid component, or are in the head of the pancreas causing obstructive jaundice should be considered for resection.[94] Patients with resected IPMNs are followed with imaging studies to identify recurrences. For MCNs, the international group recommends resection for all fit patients, and recurrences are not observed.[94] The European group gives similar recommendations.[95]

Pancreatic Cancer Screening

Asymptomatic individuals at high risk for pancreatic cancer (ie, those with first-degree relatives with pancreatic cancer) were assessed using endoscopic ultrasound (EUS) in the Cancer of the Pancreas Screening 2 (CAPS2) project.[96] Preinvasive pancreatic neoplasms were detected in 10% of high-risk patients, suggesting that EUS may have a promising role in screening high-risk patients. The CAPS Consortium recently reported results of their CAPS3 study, in which 225 asymptomatic high-risk individuals were independently (in a blinded manner) screened once with CT, MRI, and EUS.[97] In this study, 42% of individuals were found to have an abnormality; 5 individuals underwent surgical interventions, 3 of whom had high-grade dysplasia in small IPMNs and intraepithelial neoplasias. When results of the 3 screening modalities were compared, EUS detected abnormalities in 42% of individuals, versus 33% and 11% for MRI and CT, respectively.

Interestingly, results from a prospective cohort study that followed high-risk individuals for an average of 4.2 years with annual MRI were

recently published.[98] Although 32% of 262 participants were found to have pancreatic abnormalities and some IPMNs and intraepithelial neoplasias were resected, 3 patients developed pancreatic adenocarcinoma (2 metastatic, 1 recurrent 30 months post-resection) despite screening. These results could be due to rapid malignant progression, but they are more likely a result of inadequate imaging by MRI.

The diagnostic yield of pancreatic cancer screening with EUS in asymptomatic individuals at high risk for familial disease was also investigated in the Netherlands,[99] while a German study used EUS plus MRI/magnetic resonance cholangiopancreatography (MRCP) in a similar high-risk population.[100] Although results from these trials seem promising overall, the malignant potential of some preinvasive pancreatic lesions and the impact of screening on survival are presently unclear. Results suggest that MRI/MRCP may be a useful adjunct or a noninvasive alternative to EUS for pancreatic cancer screening.

Newer screening methods to identify patients with early pancreatic cancer rather than those with preinvasive lesions may prove to be beneficial in the future. Examples of techniques being investigated are microRNA biomarkers in whole blood and serum metabolism profiling.[101-104] In addition, circulating cell-free DNA is being investigated as a possible biomarker for screening. One study showed that methylation patterns in cell-free plasma DNA can differentiate between pancreatitis and pancreatic cancer with a sensitivity of 91.2% and specificity of 90.8%.[105] In addition, CA 19-9 levels may be elevated in patients up to 2 years before a pancreatic cancer diagnosis, indicating that CA 19-9 has potential as a biomarker for screening high-risk patients.[106]

An international CAPS Consortium summit with 49 multidisciplinary experts was held in 2011 to develop consensus guidelines for pancreatic cancer screening.[107] The group recommends screening with EUS and/or MRI/MRCP for high-risk individuals, defined as first-degree relatives of patients with pancreatic cancer from familial kindreds; carriers of *p16* or *BRCA2* mutations with an affected first-degree relative; patients with Peutz-Jeghers syndrome; and patients with Lynch syndrome and an affected first-degree relative with pancreatic cancer. The group also concluded that more evidence is needed regarding optimal management of patients with detected lesions, the age to begin screening, and screening intervals.

Diagnosis and Staging

Ductal adenocarcinoma and its variants account for over 90% of pancreatic malignancies. The presenting symptoms of this disease can include weight loss, jaundice, floating stools, pain, dyspepsia, nausea, and depression; however, no early warning signs of pancreatic cancer have been established. As previously noted, sudden onset of adult type 2 diabetes in patients 50 years or older may be linked to a new diagnosis of pancreatic cancer; patients with long-standing diabetes may also develop pancreatic cancer (see *Diabetes and Pancreatic Cancer*, above). Thus, pancreatic carcinoma should be considered in diabetic patients with unusual manifestations, such as abdominal symptoms and continuous weight loss.

Unlike many other cancers, imaging is the primary means through which the stage of pancreatic cancer is determined. High-quality multi-phase imaging can help to preoperatively distinguish between patients eligible for resection with curative intent and those with unresectable disease. The criteria for defining resectable disease favor specificity over sensitivity to avoid denying surgery to patients with a potentially

resectable tumor.[108] All patients for whom there is clinical suspicion of pancreatic cancer or evidence of a dilated duct (stricture) should therefore undergo initial evaluation by CT performed according to a dedicated pancreas protocol.[109] In addition, the panel recommends imaging after neoadjuvant treatment to provide adequate staging and assessment of resectability status. Subsequent decisions regarding diagnostic management and resectability should involve multidisciplinary consultation, with use of appropriate studies to evaluate the extent of disease. The panel recommends that a multidisciplinary review ideally involve expertise from surgery, diagnostic imaging, interventional endoscopy, medical oncology, radiation oncology, and pathology.

The AJCC has developed staging criteria for adenocarcinoma of the pancreas that follow the tumor/node/metastasis (TNM) system.[110] Although the TNM staging criteria for pancreatic cancer in the 7th edition of the AJCC Cancer Staging Manual have taken into account the fact that tumors of the pancreas are evaluated preoperatively by CT or MRI to determine resectability status, these staging criteria also include information that can be determined only through postsurgical pathologic evaluation of resected tumor.[110,111] Recent validation of concordance between AJCC stage and OS has been provided through evaluation of 121,713 patients with pancreatic adenocarcinoma included in the National Cancer Data Base (NCDB).[111]

For clinical purposes, however, most NCCN Member Institutions use a clinical classification system based mainly on results of presurgical imaging studies. Following staging by pancreatic protocol CT (and EUS and/or MRI/MRCP or endoscopic retrograde cholangiopancreatography [ERCP] in some cases), liver function tests, and chest imaging, disease is classified as: 1) resectable; 2) borderline resectable (ie, tumors that are involved with nearby structures so as to be neither clearly resectable nor clearly unresectable with a high chance of an R1 resection); 3) locally advanced unresectable (ie, tumors that are involved with nearby structures to an extent that renders them unresectable despite the absence of evidence of metastatic disease); or 4) disseminated, and this system is used throughout the guidelines. See *Criteria for Resection* below for more detailed definitions.

Imaging Evaluations

Pancreatic Protocol CT and MRI

Multi-detector CT angiography, performed by acquiring thin, preferably sub-millimeter, axial sections using a dual-phase pancreatic protocol, with images obtained in the pancreatic and portal venous phase of contrast enhancement, is the preferred imaging tool for dedicated pancreatic imaging. Scan coverage can be extended to cover the chest and pelvis for complete staging as per institutional preferences. Multiplanar reconstruction is preferred as it allows precise visualization of the relationship of the primary tumor to the mesenteric vasculature as well as detection of subcentimeter metastatic deposits.[108,109,112] Studies have shown that 70% to 85% of patients determined by CT imaging to have resectable tumors were able to undergo resection.[108,113-117] However, the sensitivity of CT for small hepatic and peritoneal metastases is limited.

The difference in contrast enhancement between the parenchyma and adenocarcinoma is highest during the late arterial phase, thereby providing a clear distinction between a hypodense lesion in the pancreas and the rest of the organ. A multi-phasic pancreatic protocol also allows for selective visualization of important arterial (eg, celiac axis, superior mesenteric artery [SMA], peripancreatic arteries) and venous structures (eg, superior mesenteric vein [SMV], splenic vein, portal vein [PV]), thereby providing an assessment of vascular invasion by the tumor. All of this information can improve the prediction of

resectability. Software allowing for 3-D reconstruction of imaging data can provide additional valuable information on the anatomic relationship between the pancreatic tumor and the surrounding blood vessels and organs, and multiplanar reconstruction is preferred. However, further development of this technology may be needed before it is routinely integrated into clinical practice.[116]

Patients commonly present to the oncologist with a non-pancreas protocol CT already performed. The panel feels that if the CT scan is of high quality, it can be sufficient. If not, a pancreas protocol CT is recommended. Such selective reimaging was shown to change the staging and management of patients with pancreatic adenocarcinoma in 56% of cases retrospectively reviewed at one institution.[118]

Pancreas protocol MRI can be a helpful adjunct to CT in the staging of pancreatic cancer, particularly for characterization of CT-indeterminate liver lesions and when suspected pancreatic tumors are not visible on CT or in cases of contrast allergy.[119,120]

Recently, a multidisciplinary expert consensus group defined standardized language for the reporting of imaging results.[109] Such uniform reporting can help improve the accuracy and consistency of staging to determine optimal treatment strategies for individual patients and can allow cross-study and cross-institutional comparisons for research purposes. Use of the template also ensures a complete assessment and reporting of all imaging criteria essential for optimal staging and can therefore aid in determining optimal management. The use of the radiology staging reporting template is thus preferred by the panel.

Endoscopic Ultrasound

NCCN Member Institutions vary in the use of additional staging technologies, such as EUS. The role of EUS in staging is felt to be complementary to CT, providing additional information for patients whose initial scans show no lesion or whose lesions have questionable involvement of blood vessels or lymph nodes.[121-124] In particular, EUS may provide assessment of certain types of vascular invasion.[125,126] It is the consensus of the panel that while the accuracy of EUS in assessing the involvement of certain veins (eg, PV) is high, this technique is less accurate in imaging tumor invasion of the SMA.[127] Therefore, EUS is not recommended as a routine staging tool.

EUS is also used to discriminate between benign and malignant strictures or stenosis, because severe stenosis and marked proximal dilatation most often indicate malignancy.[128] EUS can also be used to evaluate periampullary masses, separating invasive from noninvasive lesions. In addition, EUS plays a role in better characterizing cystic pancreatic lesions due to the ability to aspirate the cyst contents for cytologic, biochemical, and molecular analysis. On EUS, malignant cystic lesions may present as a hypoechoic cystic/solid mass or as a complex cyst, and they are frequently associated with a dilated main pancreatic duct. Some therapeutic interventions can also be done with EUS (eg, celiac neurolysis, removal of ascites). Because this procedure is operator dependent, some divergence in use may occur because of differing technical capabilities and available expertise.

Endoscopic Retrograde Cholangiopancreatography and MRI/Magnetic Resonance Cholangiopancreatography

ERCP is a technique that combines endoscopic and fluoroscopic procedures and is generally limited to therapeutic interventions.[129] In the guidelines, ERCP with duct brushing cytology is recommended as clinically indicated for patients without a mass in the pancreas and

without evidence of metastatic disease who require biliary decompression and who undergo additional imaging with EUS to help establish a diagnosis.[130] Thus from a therapeutic standpoint, ERCP allows for stent placement and can be used to palliate biliary obstruction when surgery is not elected or if surgery must be delayed.

MRI/MRCP is considered to be equivalent to EUS/ERCP in the diagnostic setting; brushings can be obtained with either. MRI/MRCP can also be performed with secretin to increase secretion of pancreatic juices from the proximal pancreas for better delineation of a pancreatic duct that has a subtle stricture.[131]

PET/CT

The utility of PET/CT for upstaging patients with pancreatic cancer has also been evaluated. In a retrospective study, the use of PET/CT following a standard CT protocol showed increased sensitivity for detection of metastatic disease when compared with the standard CT protocol or PET/CT alone.[132] The sensitivity of detecting metastatic disease for PET/CT alone, standard CT alone, and the combination of PET/CT and standard CT were 61%, 57%, and 87%, respectively. In this study, the clinical management of 11% of patients with invasive pancreatic cancer was changed as a result of PET/CT findings. Nevertheless, the role of PET/CT in this setting is evolving and has not yet been established.[133,134] PET/CT is not a substitute for high-quality contrast-enhanced CT, although it can be considered as an adjunct to a formal pancreatic CT protocol in high-risk patients. Indicators of high risk for metastatic disease may include borderline resectable disease, markedly elevated carbohydrate antigen (CA) 19-9, large primary tumors, large regional lymph nodes, and patients who are very symptomatic.

Laparoscopy

Laparoscopy is another potentially valuable diagnostic tool for staging; it can identify peritoneal, capsular, or serosal implants or studding of metastatic tumor on the liver that may be missed even with the use of a pancreatic CT protocol.[135-137] The yield of laparoscopy is dependent on the quality of preoperative imaging and the likelihood of metastatic disease. A key goal is to avoid unnecessary laparotomy, which can be accomplished in an estimated 23% of patients in whom curative intent surgery is planned,[136] although routine use of staging laparoscopy is controversial. The panel does not consider staging laparoscopy to be a substitute for poor-quality preoperative imaging.

Some evidence provides support for a selective approach to staging laparoscopy (ie, it is performed if the presence of occult metastatic disease is suggested by high-quality imaging or certain clinical indicators).[138] For example, preoperative serum CA 19-9 levels >100 U/mL or >215 U/mL (see discussion of *Biomarkers*, below) have been associated with a greater likelihood of advanced disease and an increased probability of a positive finding on staging laparoscopy.[139,140] In a recent prospective review of 838 patients who were diagnosed with resectable pancreatic tumors on imaging evaluation between 1999 and 2005, 14% were found to have unresectable disease (21% yield if only pancreatic adenocarcinoma was considered) following subsequent laparoscopy.[141] Characteristics associated with an increased laparoscopic yield of unresectable disease include the location of the tumor, tumor histology, the presence of weight loss and jaundice, and the facility conducting the imaging evaluation.

Diagnostic staging laparoscopy to rule out metastases not detected on imaging (especially for patients with body and tail lesions) is used routinely in some NCCN Member Institutions prior to surgery or

chemoradiation, or selectively in patients who are at higher risk for disseminated disease (ie, borderline resectable disease; markedly elevated CA 19-9; large primary tumors; large regional lymph nodes; highly symptomatic). Thus the panel believes that staging laparoscopy can be considered for patients staged with resectable pancreatic cancer who are considered to be at increased risk for disseminated disease and for patients with borderline resectable disease prior to administration of neoadjuvant therapy. The panel considers positive cytology from washings obtained at laparoscopy or laparotomy to be equivalent to M1 disease.[142]

Biopsy

Although a pathologic diagnosis is not required before surgery, it is necessary before administration of neoadjuvant therapy and for patients staged with locally advanced, unresectable pancreatic cancer or metastatic disease. A pathologic diagnosis of adenocarcinoma of the pancreas is often made using fine-needle aspiration (FNA) biopsy with either EUS guidance (preferred) or CT. EUS-FNA is preferable to CT-guided FNA in patients with resectable disease because of better diagnostic yield, safety, and potentially lower risk of peritoneal seeding with EUS-FNA when compared with the percutaneous approach.[143-145] Additional risks of CT-directed FNA biopsy include the potential for greater bleeding and infection because of the need to traverse vessels and bowel. EUS-FNA also gives the benefit of additional staging information at the time of biopsy.

EUS-FNA is highly accurate and reliable for determining malignancy. A recent retrospective analysis of 317 patients with EUS-FNA results from 2 institutions found that 97% of cases deemed malignant were in fact malignant on clinical follow-up.[146] In contrast, 13% of cases that were classified as benign were actually malignant. In rare cases when an EUS-FNA cannot be obtained from a borderline resectable or unresectable patient, other acceptable methods of biopsy exist. For instance, intraductal biopsies can be obtained via endoscopic cholangioscopy.[147] A percutaneous approach[144] or a laparoscopic biopsy[148] are other alternatives. Pancreatic ductal brushings or biopsies can also be obtained at the time of ERCP, often revealing malignant cytology consistent with pancreatic adenocarcinoma.

If a biopsy does not confirm malignancy, at least 1 repeat biopsy should be performed; EUS-FNA with or without a core biopsy at a center with multidisciplinary expertise is preferred. Alternative diagnoses including autoimmune pancreatitis should be considered (see *Differential Diagnoses*, below). A positive biopsy is required before administration of chemotherapy. However, it is important to reiterate that biopsy proof of malignancy is not required before surgical resection for clearly resectable or borderline resectable patients and that a nondiagnostic biopsy should not delay surgical resection when the clinical suspicion for pancreatic cancer is high. The NCCN Pancreatic Adenocarcinoma Panel strongly recommends that all diagnostic and surgical management decisions involve multidisciplinary consultation.

Evolving changes in molecular analyses of pancreatic cancer have led some institutions to attempt to procure additional tumor-rich, formalin-fixed, paraffin-embedded tissue to bank for future genomic studies. Several methods can be used to obtain such samples, including core biopsy, but the panel believes that core biopsies should not replace EUS-FNA, but rather can be done in addition to EUS-FNA.

Differential Diagnoses

Chronic pancreatitis and other benign conditions are possible differential diagnoses of patients suspected of having pancreatic cancer.[149-153] Autoimmune pancreatitis, a rare form of chronic

pancreatitis also known as lymphoplasmacytic sclerosing pancreatitis, is a heterogeneous disease that can present with clinical and radiologic characteristics of pancreatic cancer, such as jaundice, weight loss, an elevated CA 19-9 level, and the presence of diffuse pancreatic enlargement, a pancreatic ductal stricture, or a focal pancreatic mass.[151,154-156] The classic appearance of the pancreas on abdominal CT in patients with diffuse pancreatic involvement is a sausage-shaped enlargement of the organ with a capsule-like peripheral rim surrounding the pancreas, although focal enlargement of the pancreas is observed in some cases.[155] Cardinal histologic features of autoimmune pancreatitis include prominent lymphocytic infiltration of the pancreatic parenchyma with associated fibrosis.

In addition, fine-needle aspirates can be misinterpreted as malignant or suspicious for malignancies.[157,158] As a benign disease that can be effectively treated with corticosteroids, autoimmune pancreatitis must be distinguished from pancreatic cancer to avoid unnecessary surgery and prevent delay in the initiation of appropriate treatment.[157-160]

The finding of increased serum immunoglobulin (Ig) G levels is supportive of a diagnosis of autoimmune pancreatitis, although an elevated level of serum IgG4 specifically is the most sensitive and specific laboratory indicator.[161] A recent study found that IgG4 levels of >1.0 g/L combined with CA 19-9 levels of <74 U/mL distinguished patients with autoimmune pancreatitis from those with adenocarcinoma with 94% sensitivity and 100% specificity.[162] Jaundiced patients with locally advanced disease should be reviewed for autoimmune pancreatitis, and IgG4 levels should be assessed.

Autoimmune pancreatitis can, however, be negative for IgG4, thus closely mimicking pancreatic adenocarcinoma when there is a large pancreatic mass. For patients with borderline resectable disease and cancer not confirmed after 2 or 3 biopsies, a second opinion is recommended. Alternative diagnoses should be considered, especially autoimmune pancreatitis, and a short course of steroid treatment may be an appropriate first approach. If no response is seen, the patient should undergo laparotomy for removal of the mass.

Biomarkers

Many tumor-associated antigens have been studied in connection with pancreatic adenocarcinoma, including carcinoembryonic antigen (CEA), pancreatic anti-oncofetal antigen, tissue polypeptide antigen, CA 125, and CA 19-9. The panel recognizes the importance of identifying biomarkers to personalize therapy in this difficult disease, and they emphasize the need for collection and sharing of tissue to help accelerate the discovery of predictive and prognostic biomarkers (see *Future Clinical Trials: Recommendations for Design*, below).

CA 19-9

The best validated and most clinically useful biomarker is CA 19-9, a sialylated Lewis A blood group antigen. CA 19-9 is commonly expressed and shed in pancreatic and hepatobiliary disease and in many malignancies; thus, it is not tumor-specific. However, the degree of increase in CA 19-9 levels may be useful in differentiating pancreatic adenocarcinoma from inflammatory conditions of the pancreas (see *Differential Diagnoses*, below).[163] CA 19-9 has potential uses in diagnosis, in screening, in staging, in determining resectability, as a prognostic marker after resection, and as a predictive marker for response to chemotherapy.[164]

CA 19-9 is a good diagnostic marker, with sensitivity of 79% to 81% and specificity of 82% to 90% in symptomatic patients, but its low positive predictive value makes it a poor biomarker for screening.[165]

Preoperative CA 19-9 levels correlate with both AJCC staging and resectability and thus can provide additional information for staging and determining resectability, along with information from imaging, laparoscopy, and biopsy.[166-168]

CA 19-9 also seems to have value as a prognostic and a predictive marker for pancreatic cancer in various settings. In resectable disease, for instance, low postoperative serum CA 19-9 levels or a serial decrease in CA 19-9 levels following surgery have been found to be prognostic for survival for patients undergoing resection.[165,166,168-174] In a prospective study of patients undergoing surgery with curative intent, median survival for the group of patients with post-resectional CA 19-9 levels of <180 U/mL was significantly higher compared with the group with higher levels of CA 19-9 following surgery (HR, 3.53; $P < .0001$).[170]

Also in the resectable setting, data from an analysis of 260 consecutive patients support the predictive role of postoperative CA 19-9 levels for benefit of adjuvant therapy.[173] Among patients with CA 19-9 levels of <90 U/mL, those who received adjuvant therapy (mostly gemcitabine-based) had a longer disease-free survival (DFS) than those who did not (26.0 months vs. 16.7 months; $P = .011$). In contrast, patients with higher CA 19-9 levels did not appear to benefit from adjuvant therapy, with DFS of 16.2 months and 9.0 months for those receiving or not receiving adjuvant therapy, respectively ($P = .719$). In this same study, the 11 patients with post-adjuvant therapy CA 19-9 levels <37 U/mL did not die of pancreatic cancer, while the 8 patients with increased CA 19-9 levels post-adjuvant therapy had a median DFS of 19.6 months, suggesting a possible prognostic benefit of post-adjuvant therapy CA 19-9 levels in this setting.

In the neoadjuvant/borderline resectable setting, a recent study of 141 patients treated at MD Anderson Cancer Center found that post-treatment CA 19-9 levels were a good prognostic marker in patients receiving neoadjuvant therapy with or without subsequent resection.[175] This study found that a normalization of CA 19-9 to <40 U/mL was associated with improvements in OS in non-resected (15 months vs. 11 months; $P = .02$) and resected (38 months vs. 26 months; $P = .02$) patients.

In the advanced disease setting, data support the role of CA 19-9 as a prognostic marker.[169,176,177] In a prospective study of patients with advanced pancreatic cancer, pretreatment CA 19-9 serum levels were shown to be an independent prognostic factor for survival.[176] In addition, the change in CA 19-9 levels during chemotherapy in patients with advanced disease has been shown to be useful for evaluating the benefit of treatment, although the data are not entirely consistent.[176-181] For example, a recent study that pooled individual patients' data from 6 prospective trials found that a decline in CA 19-9 levels from baseline to after surgery and 2 rounds of adjuvant therapy were associated with a better outcome.[169] In fact, increases of <5% in CA 19-9 were also associated with improved outcomes compared to patients with larger increases (OS, 10.3 months vs. 5.1 months; $P = .002$).

It is important to note that CA 19-9 may be undetectable in Lewis antigen-negative individuals.[182] Furthermore, CA 19-9 may be falsely positive in cases of biliary infection (cholangitis), inflammation, or biliary obstruction (regardless of etiology) and do not necessarily indicate cancer or advanced disease.[183,184] Preoperative measurement of CA 19-9 levels (category 3) is therefore best performed after biliary decompression is complete and bilirubin is normal. If biliary decompression is not performed in a jaundiced patient, CA 19-9 levels can be assessed (category 3), but they do not represent an accurate baseline.

The panel recommends measurement of serum CA 19-9 levels prior to surgery (category 3), following surgery immediately prior to administration of adjuvant therapy, and for surveillance (category 2B). The panel emphasizes the importance of obtaining a CA 19-9 measurement immediately before the therapeutic intervention to have an accurate baseline from which to follow response. Of note, a number of different methods are commercially available for quantifying this tumor-associated antigen. Measurements of serum levels of CA 19-9 using one testing method cannot be extrapolated to results obtained using a different procedure.

hENT1

A recent development in the field of advanced pancreatic cancer involves a potential predictive marker. Gemcitabine is a prodrug that must be taken into cells via a nucleoside transporter.[185] Human equilibrative nucleoside transporter 1 (hENT1) is a nucleoside transporter that has been studied as a predictor for response to gemcitabine. Preliminary clinical data have shown that hENT1 expression may in fact predict response to gemcitabine.[186-191]

hENT1 has been validated in 2 retrospective analyses as a predictive biomarker for benefit from gemcitabine. A recent retrospective analysis of core tissue from patients treated on the adjuvant gemcitabine ESPAC-3 trial found that hENT1 expression was predictive of response to gemcitabine but not to 5-FU.[187] Median survival for patients treated with gemcitabine was 17.1 months versus 26.2 months for those with low versus high hENT1 expression, respectively (P = .002). In the 5-FU group, median survival was 25.6 months versus 21.9 months for the low and high hENT1 groups, respectively (P = .36). A similar analysis was performed on samples of patients treated on RTOG 9704.[186] As with the ESPAC-3 study, hENT1 expression was associated with OS (HR, 0.40; 95% CI, 0.22–0.75; P = .03) and DFS (HR, 0.39; 95% CI, 0.21–0.73; P = .003) in patients receiving gemcitabine, but hENT1 expression was not associated with OS (HR, 0.78; 95% CI, 0.47–1.27; P = .31) and DFS (HR, 0.72; 95% CI, 0.45–1.16; P = .18) in the group given 5-FU.

Thus, hENT1 appears to be an excellent predictive biomarker in the adjuvant setting based on the assay used in both of these studies (IHC with the 10D7G2 antibody). Other separate retrospective analyses of results from the adjuvant CONKO-001 trial and the AIO-PK0104 trial were unable to confirm these results using a different antibody for the IHC analysis (SP120).[192,193]

Unfortunately, hENT1 could also not be validated in the metastatic setting in the LEAP trial, which also used the SP120 different assay to determine hENT1 expression. Results from the phase II, randomized, open-label LEAP trial, which compared a lipid-conjugated form of gemcitabine that does not require hENT1 for cell entry (CO-1.01) with gemcitabine in patients with metastatic disease with high versus low expression of hENT1, found that hENT1 expression was not predictive for outcomes in patients treated with gemcitabine.[194] Trial results also found no differences in OS between the 2 treatments in patients with low hENT2 expression (HR, 0.99; 95% CI, 0.75–1.33).

Further studies based on hENT1 expression using the 10D7G2 assay are handicapped by the fact that no commercial source of the antibody and no CLIA-approved testing are available.

Systemic Therapy Approaches

Systemic therapy is used in all settings of pancreatic adenocarcinoma. It is important that biopsy confirmation of pancreatic adenocarcinoma be obtained before treatment in all cases (see Table 2, below). At least 2 or 3 negative or indeterminate biopsies should be obtained before

entertaining alternative diagnoses (see *Differential Diagnoses,* above). A second opinion should also be obtained in such a case. Occasionally, other cancer types are confirmed, and the patient should be treated according to the appropriate NCCN Guideline. The data supporting the regimens used in pancreatic cancer are described below.

Gemcitabine Monotherapy

For patients with locally advanced or metastatic disease, gemcitabine has been established as providing clinical benefit and a modest survival advantage over treatment with bolus 5-FU.[195] The panel recommends gemcitabine monotherapy as one option for front-line therapy for patients with metastatic disease (category 1) or locally advanced disease and a good performance status. Because the approved indications for gemcitabine include the relief of symptoms, the panel also recommends gemcitabine monotherapy as a reasonable option for symptomatic patients with metastatic or locally advanced unresectable disease with poor performance status (category 1).

Gemcitabine monotherapy also has category-1 evidence supporting its use in the adjuvant setting. In the large phase III CONKO-001 trial, in which 368 patients without prior chemotherapy or RT were randomly assigned to adjuvant gemcitabine versus observation following macroscopically complete resection, an intention-to-treat (ITT) analysis of the data showed that the primary endpoint of increased DFS was met (13.4 months vs. 6.9 months; $P < .001$, log rank).[196] Final results from this study showed median OS to be improved significantly for patients in the gemcitabine arm (22.8 months vs. 20.2 months; HR, 0.76; 95% CI, 0.61–0.95; $P = .01$).[197] An absolute survival difference of 10.3% was observed between the two groups at 5 years (20.7% vs. 10.4%).[197]

Fixed-Dose-Rate Gemcitabine

Studies have suggested that the infusion rate of gemcitabine may be important for its efficacy. Gemcitabine is a prodrug, which must be phosphorylated for antitumor activity. Clinical studies have shown that administering gemcitabine at a fixed dose rate (FDR) maximizes intracellular concentrations of the phosphorylated forms of gemcitabine.[198] In a randomized phase II trial of patients with locally advanced or metastatic pancreatic cancer, the infusion of gemcitabine at an FDR led to better survival compared with gemcitabine delivered at a higher dose, over 30 minutes.[199] In the phase III randomized ECOG-6201 trial of patients with advanced pancreatic cancer, median survival was increased in the group receiving FDR gemcitabine vs. standard gemcitabine (6.2 months vs. 4.9 months; $P = .04$), although this outcome did not satisfy the protocol-specified criteria for superiority.[200] When gemcitabine is considered for the treatment of advanced pancreatic cancer, the NCCN Panel views FDR gemcitabine (10 mg/m^2/min) as a reasonable alternative to the standard infusion of gemcitabine over 30 minutes (category 2B).

FDR gemcitabine is incorporated into some commonly used gemcitabine-based regimens (eg, GEMOX [gemcitabine, oxaliplatin]; GTX [gemcitabine, docetaxel, and capecitabine]). See *Gemcitabine Combinations*, below.[201,202] The combination of FDR gemcitabine and capecitabine has also been found to be active and well-tolerated.[203]

Gemcitabine Combinations

The NCCN Panel acknowledges that, historically, combination chemotherapy did not appear to be superior to monotherapy in the era of 5-FU-based therapy. However, because gemcitabine is superior to bolus 5-FU in the advanced setting when efficacy endpoints of survival and relief from symptoms are used, it is now often combined with other

chemotherapeutic agents for patients with good performance status. Gemcitabine has been investigated in combination with potentially synergistic agents (such as cisplatin, oxaliplatin, capecitabine, 5-FU, and irinotecan) or in a multidrug combination (eg, cisplatin, epirubicin, gemcitabine, 5-FU).[200-202,204-214] Two recent meta-analyses of randomized controlled trials found that gemcitabine combinations give a marginal benefit in OS over gemcitabine monotherapy in the advanced setting, with a significant increase in toxicity.[215,216]

Combinations recommended in the advanced setting are discussed below. The panel does not consider the combination of gemcitabine plus docetaxel[217] or gemcitabine plus irinotecan[214,217,218] to meet the criteria for inclusion in the guidelines. In addition, gemcitabine plus sorafenib is not recommended. The recent multi-center, double-blind, placebo-controlled, randomized phase III BAYPAN trial compared gemcitabine plus either sorafenib or placebo in chemotherapy-naïve patients with advanced or metastatic disease.[219] This trial did not meet its primary endpoint of progression-free survival (PFS) in its 104 patients (5.7 months vs. 3.8 months; $P = .90$). Gemcitabine combinations are currently being studied in the adjuvant setting.

Of note, results from several studies have indicated that the benefit of gemcitabine combination chemotherapy is predominantly seen in patients with good performance status.[208,209,211]

Gemcitabine Plus Albumin-Bound Paclitaxel

Albumin-bound paclitaxel is a nanoparticle form of paclitaxel. In a publication of a phase I/II trial, 67 patients with advanced pancreatic cancer received gemcitabine plus albumin-bound paclitaxel. At the maximum tolerated dose, the partial response rate was 48%, with an additional 20% of patients demonstrating stable disease for ≥16 weeks. The median OS at this dose was 12.2 months.[220]

Based on these results, the large, open-label, international, randomized phase III MPACT trial was initiated in 861 patients with metastatic pancreatic cancer and no prior chemotherapy.[221] Participants were randomized to receive gemcitabine plus albumin-bound paclitaxel or gemcitabine alone. The trial met its primary endpoint of OS (8.7 months vs. 6.6 months; $P < .0001$; HR, 0.72).[221,222] The addition of albumin-bound paclitaxel also improved other endpoints, including 1-year survival, 2-year survival, response rate, and PFS. The most common grade 3 or higher adverse events attributable to albumin-bound paclitaxel were neutropenia, fatigue, and neuropathy. Updated results of the MPACT trial show that long-term survival is possible with gemcitabine plus albumin-bound paclitaxel, as 3% of patients from that arm were alive at 42 months, whereas no patients were alive from the control arm at that time.[222]

For the 2013 guidelines, the panel upgraded the combination of gemcitabine plus albumin-bound paclitaxel from a category 2B to a category 1 recommendation for the treatment of patients with metastatic disease and good performance status based on these results. It is listed as a preferred option in this setting. By extrapolation of the data, the panel recommends this combination in the locally advanced, good performance status setting as well (category 2A). The panel also notes that this combination is an acceptable option in the neoadjuvant/borderline resectable setting.

Gemcitabine Plus Erlotinib and Other Targeted Therapeutics

Although phase II trial results of gemcitabine combined with new targeted drugs (eg, bevacizumab, cetuximab) were encouraging,[223,224] results of phase III studies of combinations of gemcitabine with a biologic agent have indicated that only the combination of gemcitabine plus erlotinib is associated with a statistically significant increase in survival when compared to gemcitabine alone.[225-229] Results of the

CALGB phase III trial, which evaluated gemcitabine and bevacizumab (an anti-vascular endothelial growth factor [VEGF] antibody) compared with gemcitabine plus placebo in patients with locally advanced or metastatic pancreatic cancer, and the Southwest Oncology Group (SWOG) phase III randomized trial, which assessed cetuximab (which targets the epidermal growth factor receptor [EGFR]) plus gemcitabine versus gemcitabine alone, did not reveal improvements in survival upon addition of the biologic agent.[226,227] In a phase III trial comparing gemcitabine and erlotinib with or without bevacizumab in patients with metastatic pancreatic cancer, bevacizumab did not improve OS, although a significant improvement in PFS was observed with the addition of bevacizumab to the gemcitabine/erlotinib combination.[229] A randomized phase III trial of another VEGF inhibitor, axitinib, in combination with gemcitabine also failed to show any improvement in OS of patients with advanced pancreatic adenocarcinoma.[228] Similarly, the VEGF trap ziv-aflibercept in combination with gemcitabine did not extend OS in a phase III trial in the metastatic setting.[230]

In contrast, in the phase III, double-blind, placebo-controlled NCIC CTG PA.3 trial of 569 patients with advanced or metastatic pancreatic cancer randomly assigned to receive erlotinib (which is an inhibitor of EGFR tyrosine kinase) plus gemcitabine versus gemcitabine alone, patients in the erlotinib arm showed statistically significant improvements in OS (HR, 0.82; $P = .038$) and PFS (HR, 0.77; $P = .004$) when compared to patients receiving gemcitabine alone.[225] Median survival was 6.24 months and 1-year survival was 23%, compared with 5.91 months and 17% in the control arm. Adverse events, such as rash and diarrhea, were increased in the group receiving erlotinib, but most were grade 1 or 2.[225] This trial, other trials, and community experience show that occurrence of grade 2 or higher skin rash is associated with better response and OS of patients receiving erlotinib.[225,231,232]

The NCCN Panel recommends gemcitabine-erlotinib combination therapy as another option for patients with locally advanced or metastatic disease and good performance status (category 1). However, the panel notes that although this combination significantly improved survival, the actual benefit was small, suggesting that only a small subset of patients benefit.

Gemcitabine Plus Cisplatin

Data regarding the survival impact of combining gemcitabine with a platinum agent are conflicting, and results of randomized controlled trials have not provided support for use of gemcitabine plus cisplatin in the treatment of patients with advanced pancreatic cancer. Three phase III trials evaluating the combination of gemcitabine with cisplatin versus gemcitabine alone in patients with advanced pancreatic cancer failed to show a significant survival benefit for the combination over the single agent.[205,206,209]

Nevertheless, selected patients may benefit from this regimen because patients with breast and ovarian cancers who are carriers of a *BRCA* mutation[233-235] and selected patients with inherited forms of pancreatic cancer[31] may have disease that is particularly sensitive to a platinum agent. A retrospective study from Johns Hopkins University School of Medicine of patients with metastatic pancreatic cancer and a family history of breast, ovarian, or pancreatic cancers suggested that response to gemcitabine and cisplatin was superior even with one affected relative.[236] Patients with a family history of pancreatic cancer alone demonstrated a large survival advantage when treated with platinum-based chemotherapy (6.3 vs. 22.9 months; HR, 0.34; 95% CI, 0.15–0.74; $P < .01$).[236] Furthermore, in a recent report, 5 of 6 patients with known *BRCA* mutations and metastatic pancreatic adenocarcinoma treated with a platinum-based regimen at Memorial Sloan Kettering Cancer Center showed a radiographic partial

response.[237] Thus, gemcitabine plus cisplatin may be a good choice in selected patients with disease characterized by hereditary risk factors (eg, *BRCA* or *PALB2* mutations). The panel recommends gemcitabine plus cisplatin for patients with metastatic disease, especially those with possible hereditary cancers, as a category 2A recommendation.

Gemcitabine Plus Fluoropyrimidine

A number of randomized trials have investigated the combination of gemcitabine with a fluoropyrimidine in patients with advanced pancreatic cancer. The ECOG E2297 trial compared gemcitabine monotherapy with gemcitabine and bolus 5-FU/leucovorin in patients with advanced pancreatic cancer; no statistically significant survival advantage was observed for patients receiving the combination regimen.[204] A randomized study in 533 patients with advanced disease found that PFS and objective response rates were significantly improved in patients receiving gemcitabine plus capecitabine when compared with gemcitabine alone, although a trend toward an improvement in OS for the combination arm did not reach statistical significance.[207] Similarly, results from another smaller phase III trial evaluating this combination did not demonstrate an OS advantage for overall study population receiving the combination of gemcitabine with capecitabine, although a post-hoc analysis showed OS to be significantly increased in the subgroup of patients with good performance status.[211] A recent meta-analysis of 8 randomized controlled trials, including >2000 patients, found that OS was significantly improved when a fluoropyrimidine was added to gemcitabine.[238] In particular, OS was better in patients receiving gemcitabine plus capecitabine than in patients receiving gemcitabine alone (HR, 0.87; $P = .03$).

Although there are concerns about dosing and toxicity of capecitabine in a U.S population, results from a recent study suggest that a biweekly regimen of fixed-dose gemcitabine in combination with capecitabine is both effective and well tolerated in patients with advanced disease.[203]

The NCCN Panel considers gemcitabine-based combination therapy with capecitabine to be a reasonable option (category 2A) for patients with locally advanced or metastatic disease and a good performance status who are interested in pursuing more aggressive therapy outside a clinical trial.

GTX Regimen

The panel includes the combination of gemcitabine, docetaxel, and capecitabine (GTX regimen) as a category 2B recommendation for the treatment of patients with advanced disease and good performance status. In a report of 35 patients with metastatic pancreatic cancer treated with this regimen, the authors reported an overall response rate of 29% (all had partial responses), with an additional 31% of patients exhibiting a minor response or stable disease.[202] The median survival was 11.2 months for all patients and 13.5 months for patients exhibiting a partial response. This regimen demonstrated significant toxicities, however, with 14% of patients having grade 3/4 leukopenia, 14% having grade 3/4 thrombocytopenia, and 9% having grade 3/4 anemia. A recent retrospective case-review study at The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins found similar results, with a median OS of 11.6 months and grade 3 or greater hematologic and non-hematologic toxicity rates of 41% and 9%, respectively.[239]

5-FU/Leucovorin

5-FU with leucovorin is listed in the guidelines as a category 1 option in the adjuvant setting. Results from the European Study Group for Pancreatic Cancer (ESPAC)-1 trial, reported by Neoptolemos and colleagues, suggested that 5-FU/leucovorin is superior to observation.[240] In addition, results from the ESPAC-3 trial of bolus 5-

FU/leucovorin versus gemcitabine following surgery showed no difference in median OS between the arms (23.0 months and 23.6 months, respectively).[241]

Leucovorin Shortage

There is currently a shortage of leucovorin in the United States. There are no specific data to guide management under these circumstances, and all proposed strategies are empiric. The panel recommends several possible options to help alleviate the problems associated with this shortage. One is the use of levo-leucovorin, which is commonly used in Europe. A dose of 200 mg/m^2 of levo-leucovorin is equivalent to 400 mg/m^2 of standard leucovorin. Another option is for practices or institutions to use lower doses of leucovorin for all doses in all patients, since the panel feels that lower doses are likely to be as efficacious as higher doses, based on several studies. The QUASAR study found that 175 mg leucovorin gave similar survival and 3-year recurrence rates as 25 mg leucovorin when given with bolus 5-FU to patients as adjuvant therapy following R0 resections for colorectal cancer.[242] Another study showed no difference in response rate or survival in patients with metastatic colorectal cancer receiving bolus 5-FU with either high-dose (500 mg/m^2) or low-dose (20 mg/m^2) leucovorin.[243] Also, the Mayo Clinic and North Central Cancer Treatment Group (NCTTG) determined that there was no therapeutic difference between the use of high- (200 mg/m^2) or low- (20 mg/m^2) dose leucovorin with bolus 5-FU in the treatment of advanced colorectal cancer, although 5-FU doses were different in the 2 arms.[244] Finally, if none of the above options are available, treatment without leucovorin would be reasonable. For patients who tolerate this without grade II or higher toxicity, a modest increase in 5-FU dose (in the range of 10%) may be considered.

FOLFIRINOX

In 2003, a French group reported the results of an open phase I study to assess the feasibility of a combination therapy consisting of 5-FU/leucovorin plus oxaliplatin and irinotecan (FOLFIRINOX) for the treatment of patients with metastatic solid tumors.[245] Their study included 2 patients with pancreatic cancer, and the regimen showed anti-tumor activity. A subsequent multicenter phase II trial specifically for patients with advanced pancreatic adenocarcinoma demonstrated promising response rates.[246] A later randomized phase II trial showed a response rate of >30% to FOLFIRINOX in patients with metastatic pancreatic cancer.[247]

Results from the randomized phase III PRODIGE trial evaluating FOLFIRINOX versus gemcitabine in patients with metastatic pancreatic cancer and good performance status showed dramatic improvements in both median PFS (6.4 months vs. 3.3 months; $P < .001$) and median OS (11.1 months vs. 6.8 months; $P < .001$), in favor of the group receiving FOLFIRINOX.[248] Because of these strong results, the panel added FOLFIRINOX as a preferred, category 1 recommendation for first-line treatment of good performance status patients with metastatic pancreatic cancer in 2011. It is listed as a category 2A recommendation for patients with locally advanced unresectable disease by extrapolation. The panel also lists this regimen as an acceptable option in the neoadjuvant/borderline resectable setting.

There are, however, some concerns about the toxicity of the FOLFIRINOX regimen. In the PRODIGE trial, some of the grade 3/4 toxicity rates that were significantly greater in the FOLFIRINOX group than in the gemcitabine group were 45.7% for neutropenia, 12.7% for diarrhea, 9.1% for thrombocytopenia, and 9.0% for sensory neuropathy.[248] Despite the high levels of toxicity, no toxic deaths have

been reported.[246-248] Furthermore, the PRODIGE trial determined that, despite this toxicity, fewer patients in the FOLFIRINOX group than in the gemcitabine group experienced a degradation in their quality of life at 6 months (31% vs. 66%, $P < .01$).[248] A more detailed analysis of the quality of life of patients in this trial has been published and shows that FOLFIRINOX maintained and even improved quality of life more than gemcitabine did.[249]

The panel appreciates that toxicity of FOLFIRINOX can be managed with a variety of approaches. For example, a group from Memorial Sloan Kettering Cancer Center reported good activity and acceptable toxicity of first-line FOLFIRINOX at 80% dose intensity with routine growth factor support in carefully selected patients with metastatic or locally advanced disease.[250] Median OS was 12.5 months in the metastatic setting and 13.7 months in patients with locally advanced disease.

Capecitabine and Continuous Infusion 5-FU

The panel lists capecitabine monotherapy and continuous infusion 5-FU as first-line treatment options for patients with locally advanced unresectable or metastatic disease (category 2B). They are also recommended as options in the adjuvant settings (category 2A for continuous infusion 5-FU and category 2B for capecitabine). The capecitabine recommendation is supported by a randomized phase III trial from the Arbeitsgemeinschaft Internistische Onkologie (AIO) group in which OS was similar in patients with advanced pancreatic cancer receiving capecitabine plus erlotinib followed by gemcitabine monotherapy or gemcitabine plus erlotinib followed by capecitabine monotherapy.[251]

Note that the capecitabine dose recommended by the panel (1000 mg/m^2 PO twice daily) is less than the dose described by Cartwright and colleagues, because the higher dose has been associated with increased toxicity (eg, diarrhea, hand and foot syndrome).[252]

Fluoropyrimidine Plus Oxaliplatin

The combination of a fluoropyrimidine (5-FU/leucovorin or capecitabine) with oxaliplatin is listed as a possible first-line treatment for metastatic or locally advanced disease (category 2B). The panel bases these recommendations on the randomized phase III CONKO-003 trial (5-FU/leucovorin/oxaliplatin vs. best supportive care) and on a phase II study (CapeOx).[253,254] Both of these studies only enrolled patients who had received 1 prior chemotherapy regimen, but the panel feels the extrapolation to first-line therapy is appropriate (category 2B).

Possible Role of Maintenance Therapy in Advanced Disease

With the success of more effective regimens in patients with advanced disease, questions have been raised about how best to manage the treatment-free interval prior to disease progression. Options include stopping treatment, dropping the most toxic agents, and using different agents for maintenance therapy.

A recent randomized phase II trial (PACT-12) had intriguing results that suggest maintenance therapy with the angiogenesis inhibitor sunitinib after a full course of first-line treatment may have a benefit in some patients with metastatic disease.[255] Patients without evidence of progression after 6 months of initial therapy (n=55; mostly gemcitabine combinations) were randomized to sunitinib or observation. Median OS was 9.2 months in the observation group versus 10.6 months in the sunitinib group (HR, 0.71; 95% CI, 0.40–1.26; $P = .11$). The small sample size precludes strong conclusions; however, the 1- and 2-year survival rates were 36% and 7% in the observation arm compared with 41% and 23% in the sunitinib arm, suggesting that a subset of patients derive significant benefit. Anti-angiogenic agents have not been

successful in the treatment of pancreatic cancer to date. However, results of the PACT-12 trial suggest that there may in fact be a role for these compounds in this disease. Angiogenesis inhibitors may be more useful after more effective first-line treatments. Clearly additional trials in this important area are needed.

Second-Line Systemic Therapy in the Advanced Setting

A recent systematic review of clinical trials that assessed the efficacy of second-line therapy after gemcitabine in pancreatic cancer concluded that, while data are very limited, evidence suggests an advantage of additional chemotherapy over best supportive care.[256] For patients with advanced disease who have received prior gemcitabine-based therapy, fluoropyrimidine-based chemotherapy regimens are acceptable second-line options.[253,254,257] Gemcitabine-based therapy can be given to those previously treated with fluoropyrimidine-based therapy.

Results from the phase III CONKO-003 trial presented in 2008 showed significant improvements in both median PFS (13 weeks vs. 9 weeks; *P* = .012) and median OS (20 weeks vs. 13 weeks; *P* = .014) when oxaliplatin was added to 5-FU/leucovorin,[258,259] making this regimen the standard approach for second-line therapy for patients without prior exposure to fluoropyrimidine-based therapy at that time. Final results of the trial were published in 2014.[260] The median OS in the OFF arm was 5.9 months (95% CI, 4.1–7.4), whereas it was 3.3 months (95% CI, 2.7–4.0) in the 5-FU/LV arm, for a significant improvement in the hazard ratio (HR, 0.66; 95% CI, 0.48–0.91; *P* = .01).

However, results from the open-label phase III PANCREOX trial show that the addition of oxaliplatin to 5-FU/LV in second-line treatment may be detrimental.[261] In this trial, 108 patients with advanced pancreatic cancer who progressed on gemcitabine-based treatment were randomized to receive second-line mFOLFOX6 or infusional 5FU/LV. No difference was seen in median PFS (3.1 vs 2.9 months; *P* = .99), but median OS was worse in those in the FOLFOX arm (6.1 vs. 9.9 months; *P* = .02). Furthermore, the addition of oxaliplatin resulted in increased toxicity, with rates of grade 3/4 adverse events of 63% in the FOLFOX arm and of 11% in the 5-FU/LV arm.

The AIO-PK0104 trial also assessed second-line therapy in a randomized crossover trial and found capecitabine to be efficacious after progression on gemcitabine/erlotinib in patients with advanced disease.[262] In this trial, capecitabine/erlotinib followed by gemcitabine gave similar outcomes to the aforementioned sequence.

Chemoradiation Approaches

In patients with pancreatic cancer, radiation is usually given concurrently with gemcitabine- or fluoropyrimidine-based chemotherapy. Chemotherapy is used as a radiosensitizer, increasing the toxicity of radiation to tumor cells. Although the mechanism of radiosensitization is not entirely clear, it is postulated that gemcitabine and fluoropyrimidines decrease the number of tumor cells in the S phase of the cell cycle, a stage at which cells are resistant to radiation damage.[263]

Chemoradiation is sometimes used for pancreatic cancer in the adjuvant setting, because of its potential to decrease the likelihood of local recurrence. It is also sometimes used in the locally advanced setting, namely in those patients who do not progress during initial chemotherapy. Chemoradiation is also often incorporated into neoadjuvant regimens, although randomized trials demonstrating the role of chemoradiation in this setting have not been done. Chemoradiation can also be given as second-line therapy in patients with locally advanced unresectable disease or in resected patients if it was not previously given and if the primary site is the sole site of

progression. Finally radiation, without chemotherapy, is used in the metastatic setting as palliation for pain refractory to narcotic therapy. Varying levels of evidence support the use of chemoradiation in each setting, as discussed in more detail below.

Adjuvant Chemoradiation

In 1985, the Gastrointestinal Tumor Study Group (GITSG) initially reported that the median survival of patients undergoing pancreatoduodenectomy could be prolonged almost 2-fold by postoperative chemoradiation.[264,265] In this study, patients were randomly assigned to either observation or RT combined with an intermittent bolus of 5-FU after resection. A standard split course of 4,000 cGy was used. 5-FU, 500 mg/m^2 daily for 3 days, was given concurrently with each 2,000-cGy segment of RT. The 5-FU regimen was then continued weekly for a full 2 years. In addition to a prolonged median survival, chemoradiation also resulted in a 2-year actuarial survival of 42%, compared with 15% in the control group.[264]

Other studies have also shown an advantage to adjuvant chemoradiation over observation after resection. EORTC conducted a phase III trial (40891) in patients with both ampullary and pancreatic adenocarcinoma assessing adjuvant radiotherapy and 5-FU versus observation alone after surgery. They found that the benefit of therapy was small in a subset of patients with pancreatic adenocarcinoma and was not statistically significant.[266] At a median follow-up of 11.7 years, no statistically significant differences were observed in the different study arms with respect to PFS or OS for the subset of patients with pancreatic cancer.[267]

More contemporary studies have compared different regimens incorporating chemoradiation. The Radiation Therapy Oncology Group study RTOG 9704 was a phase III study that evaluated postoperative adjuvant treatment of resected pancreatic adenocarcinoma using either gemcitabine or fluorouracil for 3 weeks before and 12 weeks after 5-FU-based chemoradiation for both groups.[268] This trial, which utilized daily fractionated radiotherapy, included prospective quality assurance of all patients, including central review of preoperative CT imaging and radiation fields.[269] Results of this study showed that, for patients with tumors of the pancreas head (representing 388 of the 451 patients enrolled in the trial), there was a non-statistically significant increase in OS in the gemcitabine arm compared with the 5-FU arm (median and 3-year survival of 20.5 months and 31% vs. 16.9 months and 22%; P = .09); this benefit became more pronounced on multivariate analysis (HR, 0.80; 95% CI, 0.63–1.00; P = .05). The recently published 5-year analysis of RTOG 9704 showed that there was in fact no difference in OS between the two groups, although patients with tumors in the head of the pancreas showed a trend toward improved OS with gemcitabine (P = .08) upon multivariate analysis.[270]

The Role of Radiation in Adjuvant Regimens

The majority of the data comparing chemotherapy to chemoradiation in the adjuvant setting do not generally show an advantage to the addition of radiation. Results of ESPAC-1 suggested that the addition of radiation to adjuvant 5-FU chemotherapy may be unnecessary and perhaps even harmful (OS, 13.9, 21.6, and 19.9 months for chemoradiation, chemotherapy, and chemotherapy plus chemoradiation, respectively),[240] although the ESPAC-1 trial has been criticized for lack of attention to quality control for RT.[271-273] A phase II study by GERCOR randomized patients to adjuvant gemcitabine or adjuvant gemcitabine-based chemoradiation.[274] No differences were seen in OS (24.4 months vs. 24.3 months) or DFS (10.9 months vs. 11.8 months) between the groups, but with only 45 patients in each arm no P values were reported. In addition, the multicenter, open-label,

randomized phase III CapRI trial recently found that adjuvant chemoradiation with 5-FU, cisplatin, and interferon alfa-2b (IFN α-2b) followed by 5-FU chemotherapy gave outcomes no better than adjuvant treatment with 5-FU alone.[275]

A 2012 meta-analysis of 15 prospective, randomized trials found that adjuvant chemoradiation did not improve DFS, 2-year survival, or OS (odds ratio, 0.99; $P = .93$) compared to surgery alone, while adjuvant chemotherapy improved all 3 outcomes (odds ratio for OS, 1.98; $P < .001$).[276] A 2013 meta-analysis of 9 trials found similar results, with HRs for death compared to no adjuvant treatment of 0.62 for 5-FU (95% CI, 0.42–0.88), 0.68 for gemcitabine (95% CI, 0.44–1.07), 0.91 for chemoradiation (95% CI, 0.55–1.46), 0.54 for chemoradiation plus 5-FU (95% CI, 0.15–1.80), and 0.44 for chemoradiation plus gemcitabine (95% CI, 0.10–1.81).[277]

However, a population-based assessment of outcomes of patients in the NCDB with pancreatic cancer resected from 1998 to 2002 found the opposite result: chemoradiation gave better OS than chemotherapy in a performance-status–matched comparison to no adjuvant treatment (HR, 0.70; 95% CI, 0.61–0.80 vs HR, 1.04; 95% CI, 0.93–1.18).[278] A multi-institutional pooled analysis of 955 consecutive patients who had R0-1 resections for pancreatic cancer also supports the supposition that adjuvant chemoradiation improved survival compared to chemotherapy alone (OS, 39.9 months vs. 27.8 months; $P < .001$).[279]

To definitively clarify the role of chemoradiation following gemcitabine monotherapy in the adjuvant setting, RTOG is conducting trial 0848 (ClinicalTrials.gov NCT01013649). Patients without evidence of progressive disease after 5 cycles of gemcitabine-based chemotherapy are being randomized to 1 additional round of chemotherapy or 1 additional round of chemotherapy followed by chemoradiation with capecitabine or 5-FU. The primary endpoint is OS, and the trial is estimated to be completed in 2020.

Benefit of Adjuvant Chemoradiation in Patient Subsets

It has been suggested that subsets of patients (eg, patients with R1 resections or positive lymph nodes) may be more likely to benefit from adjuvant chemoradiation.

Studies that have looked at R0 or R1 subsets of patients have found mixed results. For instance, patients treated in the ESPAC-1 trial did not derive a benefit from the addition of radiation to adjuvant chemotherapy, irrespective of margin status.[280] In contrast, results from a prospectively collected database of 616 patients with resected pancreatic cancer at the Johns Hopkins Hospital found that adjuvant chemoradiation benefited both the R0 and R1 subsets compared to observation alone.[281] The Mayo Clinic performed a retrospective review of 466 patients who had R0 resections for pancreatic adenocarcinoma, and found an OS benefit of adjuvant chemoradiation over observation.[282] In addition, a retrospective review of resected >1200 patients from the Johns Hopkins Hospital and the Mayo Clinic who received adjuvant 5-FU-based chemoradiation or were observed following resection found that chemoradiation improved outcomes regardless of margin status (R0: RR, 0.61; 95% CI, 0.47–0.77, $P < .001$. R1: RR, 0.52; 95% CI, 0.36–0.74; $P < .001$).[283] A meta-analysis of 4 randomized controlled trials found evidence for an increased survival benefit of adjuvant chemoradiation in the R1 subset (HR for death, 0.72; 95% CI, 0.47–1.10) over the R0 subset (HR for death, 1.19; 95% CI, 0.95–1.49).[284] Fewer analyses have looked at the role of chemoradiation in resected patients with positive lymph nodes. One retrospective review compared outcomes of 94 patients who underwent distal pancreatectomy at the Johns Hopkins Hospital and either received adjuvant chemoradiation or were just observed following resection.[285] An exploratory subset

analysis suggested that patients with positive lymph nodes derived greater benefit from adjuvant chemoradiation than those with negative nodes. In addition, a meta-analysis of 4 randomized controlled adjuvant trials found that chemoradiation had a similar lack of benefit in lymph node-positive and -negative patients.[286]

Chemoradiation for Locally Advanced Disease

Chemoradiation is a conventional option for the management of unresectable locoregional pancreatic cancer, although the utility of chemoradiation in this population of patients is controversial.[287] It is mainly used in selected patients who do not develop metastatic disease during initial chemotherapy.

The role of chemoradiation in locoregional pancreatic cancer was initially defined in a trial conducted in locally advanced disease by GITSG.[265] In this study, the combination of bolus 5-FU and split-course radiation (total dose, 4000 cGy) was compared with radiation alone or with 6000 cGy combined with 5-FU. A nearly 2-fold increase in median survival (42.2 vs. 22.9 weeks) was observed with the regimen of bolus 5-FU and 4000 cGy compared with radiation alone. Subsequent generations of studies have sought to optimize the use of 5-FU, and most contemporary studies no longer use split-course radiation.[288] Gemcitabine has also been used as a radiation sensitizer in the locally advanced setting.[289-293] Evidence suggests that concurrent gemcitabine and radiation can yield similar or better outcomes when compared with 5-FU-based chemoradiation in the setting of locally advanced disease.[288,292,294,295] The use of capecitabine as a radiosensitizer has also been assessed in this setting and appears to be effective.[296]

A recent meta-analysis identified 15 randomized controlled trials (1128 patients) that compared chemoradiation to either chemotherapy or radiation in the locally advanced setting.[297] Whereas combined modality therapy significantly improved survival compared to radiation alone, survival was the same when compared to those receiving chemotherapy alone. Increased toxicity was observed in the chemoradiation group.

Upfront Chemoradiation in Locally Advanced Disease

Results of 2 early randomized trials comparing upfront chemoradiation to chemotherapy in locally advanced disease were contradictory.[298,299] Three phase II trials also assessed the upfront chemoradiation approach in locally advanced pancreatic adenocarcinoma, with median survival rates ranging from 8.2 to 9 months.[289,300-302] Results from small, single-arm trials of upfront chemotherapy followed by chemoradiation in locally advanced disease have been discussed.[303]

The more recent phase III randomized ECOG-4201 trial, which assessed gemcitabine compared with gemcitabine plus RT followed by gemcitabine alone in patients with locally advanced, unresectable pancreatic cancer, was closed early due to poor accrual. However, an ITT analysis of data for the 74 patients enrolled in this study showed that median OS was significantly longer in the chemoradiation therapy arm of the study (11.1 months vs. 9.2 months; $P = .017$).[304] However, the poor accrual rate decreased its statistical power, there was no difference in PFS, and the confidence intervals for OS overlapped between the two groups of patients, leading some to state that the results do not rise to the level of evidence required to determine standard of care.[305]

The benefit of chemotherapy versus chemoradiation was also addressed in the phase III FFCD-SFRO study from France, in which patients with locally advanced pancreatic cancer were randomly assigned to receive either gemcitabine alone or an intensive induction regimen of chemoradiation with 5-FU plus cisplatin followed by

gemcitabine maintenance treatment.[306] In this study, gemcitabine alone was associated with a significantly increased OS rate at 1 year compared with chemoradiation (53% vs. 32%; HR, 0.54; 95% CI, 0.31–0.96; *P* = .006). This study was stopped before the planned accrual, because an interim analysis revealed that patients in the chemoradiation arm had a lower survival rate. Also, patients in the chemoradiation arm experienced severe toxicity and were more likely to receive a shorter course of maintenance therapy with gemcitabine, suggesting that the observed differences in survival were most likely attributable to the extreme toxicity of this particular chemoradiation regimen.

Thus, the role of upfront chemoradiation in the setting of locally advanced pancreatic cancer is still undefined. The panel points out that if patients present with poorly controlled pain or local invasion with bleeding, it may be preferable to start with upfront chemoradiation therapy.[289,291]

Chemoradiation Following Chemotherapy in Locally Advanced Disease

Starting with 2 to 6 cycles of systemic chemotherapy followed by chemoradiation therapy is an option for selected patients with unresectable disease and good performance status who have not developed metastatic disease.[307-309] This sequence is especially recommended in cases where: 1) it is highly unlikely that the patient will become resectable (ie, complete encasement of superior mesenteric/celiac arteries); 2) there are suspicious metastases; or 3) the patient may not be able to tolerate chemoradiation. Employing an initial course of chemotherapy may improve systemic disease control in these cases. In addition, the natural history of the disease can become apparent during the initial chemotherapy, thus allowing the selection of patients most likely to benefit from subsequent chemoradiation. For example, a retrospective analysis of outcomes from the GERCOR studies indicated that first-line treatment with chemotherapy may be a useful strategy for selecting patients with locally advanced disease who are more likely to benefit from subsequent chemoradiation therapy.[307]

However, preliminary data from the international phase III LAP 07 trial showed no clear survival benefit (the primary outcome measure) with the addition of conventional chemoradiation following gemcitabine monotherapy.[310] In this study, 269 patients with disease control after induction gemcitabine-based chemotherapy were randomized to additional chemotherapy or to chemoradiation with capecitabine. Median OS was 16.5 months in the chemotherapy arm versus 15.3 months in the chemoradiation arm (HR, 1.03; 95% CI, 0.79–1.34; *P* = .83). Differences were noted in other potentially meaningful outcomes such as time to reinitiation of therapy (159 days in the chemoradiation arm vs. 96 days in the control arm; *P* = .05) and local tumor progression (34% in the chemoradiation arm vs. 65% in the control arm; *P* < .0001).[311] Because there are now more active chemotherapy regimens than gemcitabine monotherapy, additional studies are planned to assess the role of radiation after more active chemotherapy.

Advanced Radiation Techniques

IMRT is increasingly being applied for therapy of locally advanced pancreatic adenocarcinoma and in the adjuvant setting with the aim of increasing radiation dose to the gross tumor while minimizing toxicity to surrounding tissues.[312-316] A retrospective treatment planning study evaluated the dose escalation that might have been possible in 15 patients with locally advanced, unresectable pancreatic adenocarcinoma if IMRT had been used instead of 3-D conformal planning.[316] While the authors concluded that the IMRT plans would allow for significant increase in target volume dose with substantial dose reductions to local organs at risk, there is no clear consensus on the

appropriate maximum dose of radiation when IMRT is used. Results of a recent study demonstrated that IMRT resulted in reduced grade 3/4 toxicities when the authors made a cross-study comparison of toxicities in patients who received a similar 5-FU-based regimen with 3-D conformal radiation in the RTOG 9704 trial.[268,317] Comparing the 2 trials, rates of grade 3/4 nausea and vomiting were 0% vs. 11% (P = .024), and rates of grade 3/4 diarrhea were 3% vs. 18% (P = .017),[317] suggesting that IMRT may be well tolerated and allow for higher radiation doses to the tumor.[317] There is no clear consensus on the appropriate maximum dose of radiation when IMRT technique is used.

Stereotactic body radiotherapy (SBRT) is another technique aimed at increasing dose to the gross tumor while sparing radiation to nearby healthy tissue.[318-323] Retrospective analysis of 77 patients with unresectable disease demonstrated that while SBRT gave effective local control, it gave no improvement to OS and was associated with significant toxicities.[318] However, another retrospective review of 71 patients reported a median OS of 10.3 months with only 3 patients (4%) experiencing grade 3 toxicity.[321] No standard total dose or dose per fraction has been established for SBRT, and the panel currently recommends that SBRT only be utilized as part of a clinical trial.

Intraoperative radiation therapy (IORT) can allow for higher doses of radiation because sensitive structures can be excluded from the radiation fields. IORT is sometimes administered to patients with borderline resectable disease who have received maximal neoadjuvant therapy to sterilize close or involved margins at the time of surgery, although data in this setting are lacking. It is also sometimes used when a patient is found to be unresectable at the time of surgery and in cases of locally recurrent disease. Most studies of IORT in patients with locally advanced pancreatic cancer found that while local control may be improved, no change in survival is evident with use of IORT because of the high frequency at which metastatic disease develops.[324-327] Some groups, however, believe that IORT can offer benefits in very carefully selected patients with non-metastatic disease.[328-330] Overall, there is no clear established role for IORT in patients with pancreatic cancer,[331] and the panel believes it should only be performed at specialized centers.

Management of Metastatic Disease

The primary goals of treatment for metastatic pancreatic cancer are palliation and lengthened survival. Survival benefits are usually limited to patients with adequate performance status (ECOG 0-1, with good pain management, patent biliary stent, and adequate nutritional intake). Systemic therapy is therefore recommended for patients with metastatic disease and good performance status, as described in *Systemic Therapy Approaches*, above, and in the guidelines.

Patients who present with poor performance status may benefit from the administration of gemcitabine (category 1 recommendation), but comfort-directed measures are always paramount (see *Palliative and Supportive Care*, below, and the NCCN Guidelines for Supportive Care, available at www.NCCN.org). An alternative option for these patients is palliative and best supportive care.

Before initiating cytotoxic therapy, an open dialogue regarding the goals and side effects of treatment should take place and, if needed, adjunctive strategies can be used (see *Palliative and Supportive Care*, below). Of note, debilitated patients with advanced disease may have abrupt changes in clinical status. Therefore, if treatment is begun, it should proceed with close follow-up. Patients may experience sudden onset of bleeding or thromboembolism, rapidly escalating pain, biliary stent occlusion, cholangitis, or other infections. Moreover, clinically meaningful tumor progression may develop quickly, and tumor-related symptoms may be inappropriately attributed to chemotherapy or other

causes. For instance, patients who complain of intractable nausea and vomiting may have gastric outlet obstruction rather than chemotherapy-induced emesis. Peritoneal carcinomatosis may manifest as ascites or in its more subtle form, as abdominal bloating, as decreased oral intake, and as constipation.

For patients who do well on initial therapy, a chemotherapy holiday is appropriate, or maintenance therapy can be considered (see, *Possible Role of Maintenance Therapy*, above). After progression, second-line therapy is possible, especially in patients who maintain a good performance status (see *Second-Line Systemic Therapy in the Advanced Setting*, above).

Management of Locally Advanced Disease

As in the metastatic setting, the primary goals of treatment of patients with unresectable, locoregional pancreatic cancer are palliation and lengthened survival. Also, as in metastatic disease, patients with locally advanced disease are treated with systemic therapy based on their performance status. Gemcitabine (category 1) and palliative and best supportive care are options for patients with poor performance status, whereas patients with good performance status can be treated with more intensive therapy (eg, FOLFIRINOX [category 2A], gemcitabine/albumin-bound paclitaxel [category 2A]) or with gemcitabine monotherapy (category 2A), as described in *Systemic Therapy Approaches*, above, and in the guidelines.

Historically, most studies in the locally advanced setting used gemcitabine monotherapy. However, there is an increasing emphasis on understanding the role of modern, more active regimens in locoregional unresectable disease. The experience with FOLFIRINOX in 22 patients with locally advanced pancreatic cancer at the Massachusetts General Hospital Cancer Center through February 2012 was recently reported.[332] An overall response rate of 27% was observed, and the median PFS was 11.7 months. Five patients (23%) were able to undergo R0 resections, although 3 of these patients experienced distant recurrence by 5 months. It was also reported that 32% of patients receiving FOLFIRINOX required ≥1 hospitalization or visit to the emergency department during treatment.

Other studies and case reports addressing the use of chemotherapy with or without chemoradiation in patients with locally unresectable disease have noted that the opportunity for curative intent resection occasionally arises.[332-341] The panel believes that patients with a significant response to chemotherapy and/or chemoradiation may be considered for surgical resection, but acknowledges that such conversions are rare in patients with true locally advanced disease. Following resection, these patients have similar survival rates as those initially determined to be resectable.[342]

The use of chemoradiation following chemotherapy in locally advanced disease is discussed above (See *Chemoradiation for Locally Advanced Disease*).

Management of Resectable and Borderline Resectable Disease

Surgical Management

Surgical resection is the only potentially curative technique for managing pancreatic cancer. However, more than 80% of patients present with disease that cannot be cured with surgical resection.[343] Early concerns about high mortality associated with various pancreatic resection procedures[344] have now been lessened by studies demonstrating an acceptably low (<5%) mortality in experienced centers (see *Effect of Clinical Volume*, below).[345] Even under the most optimal clinical trial conditions, however, the median survival of resected

patients following adjuvant therapy ranges from 20.1 to 23.6 months.[196,240,241,268] Negative margin status (ie, R0 resection), tumor DNA content, tumor size, and absence of lymph node metastases are the strongest prognostic indicators for long-term patient survival.[346-348] With respect to margin status, there is evidence for the converse statement—the survival benefits of an R1 resection may be comparable to definitive chemoradiation without surgery.[349-351]

Criteria for Resection

The NCCN Panel recommends that decisions about diagnostic management and resectability always involve multidisciplinary consultation at high-volume centers with use of appropriate high-quality imaging studies to evaluate the extent of disease. Although it is clear that patients with visceral, peritoneal, or pleural metastases or with metastases to nodes beyond the field of resection derive no benefit from resection, institutions differ in their approaches to patients with locoregional disease involvement (pancreas and peripancreatic lymph nodes).

Based on their clinical experience with the primary management of pancreatic tumors, an expert consensus group developed criteria to define tumor resectability so as to improve patient selection for surgery and increase the likelihood of an R0 resection.[108,352] Other groups have also put forth definitions of resectability of pancreatic cancer.[353-355] A more restrictive definition of borderline resectable pancreatic tumors has also been described.[356] This definition uses degrees of contact (eg, interface between tumor and SMA measuring <180˚ of vessel wall circumference) rather than subjective terms such as abutment and impingement. The panel endorses this definition for use in clinical trials. Using any of these sets of criteria, tumors are classified as resectable; borderline resectable; or unresectable (ie, locally advanced or metastatic disease).

The NCCN Pancreatic Adenocarcinoma panel has adapted the criteria put forth by other groups and lists its recommended criteria for defining resectability status in the guidelines. The consensus of the panel is that patients should be selected for surgery on the basis of curative intent as determined by the probability of obtaining negative (R0) resection margins. Overall, the likelihood of attaining negative margins is the key criterion for consideration when determining whether a patient is a potential candidate for resection.[355,357] In this context, a borderline resectable lesion can be defined as one in which there is a higher likelihood of an incomplete resection. Patients at high risk for positive surgical margins are not considered to be good candidates for an upfront resection. Furthermore, the panel recommends that patient factors be considered when deciding whether a patient is a surgical candidate. Age of the patient, comorbidities, performance status, and frailty are all things to be discussed during the multidisciplinary review. Please refer to the NCCN Guidelines for Senior Adult Oncology for further discussion of the treatment of older patients.

Primary Surgery for Pancreatic Cancer

The nature and extent of the surgery for resectable tumors depend on the location and size of the tumor. Because tumors of the body and tail cause symptoms late in their development, they are usually advanced at diagnosis and are rarely resectable. When tumors in the pancreatic tail are resectable, distal pancreatectomy, in which the surgeon removes the tail and body of the pancreas, as well as the spleen, is commonly performed. If the cancer diffusely involves the pancreas or is present at multiple sites within the pancreas, a total pancreatectomy may be required, where the surgeon removes the entire pancreas, part of the small intestine, a portion of the stomach, the common bile duct, the gallbladder, the spleen, and nearby lymph nodes. Patients with tumors in the head of the pancreas, who usually present because of

jaundice, are treated with open or laparoscopic pancreatoduodenectomy (ie, the Whipple procedure).[358,359]

If the tumor is found to be unresectable during surgery, the panel recommends biopsy confirmation of adenocarcinoma at this time, if a biopsy was not performed previously. If a patient with jaundice is found to be unresectable at surgery, then the panel recommends stenting or biliary bypass at that time. In addition, gastrojejunostomy can be considered if appropriate regardless of jaundice (category 2B for prophylactic gastrojejunostomy). Celiac plexus neurolysis can also be performed, especially when indicated by pain in a patient with jaundice (category 2B for a non-jaundiced patient). See *Severe Tumor-Associated Abdominal Pain*, below, for more details about these procedures.

Pancreatoduodenectomy (Whipple Procedure)

Achievement of a margin-negative dissection must focus on meticulous perivascular dissection of the lesion in resectional procedures, recognition of the need for vascular resection and/or reconstruction, and the potential need for extra-pancreatic organ resection. Of course, the biology of the cancer might not allow for an R0 resection even with the most meticulous surgery.

Medial dissection of pancreatic head lesions is best achieved by complete mobilization of the PV and SMV from the uncinate process (assuming no evidence of tumor infiltration). Further, skeletonization of the lateral, posterior, and anterior borders of the SMA down to the level of the adventitia will maximize uncinate yield and radial margin (see Figure 1).[360,361] Optimal dissection and skeletonization of the SMA can be achieved using ultrasonic or thermal dissectors (Harmonic scalpel or LigaSure). Division of the retroperitoneal tissues between the uncinate process and the SMA with a stapler or a clamp and cut technique may leave up to 43% of the soft tissue between the uncinate process and the SMA in situ and results in suboptimal clearance and increases the risk of an R1 resection.[362,363]

The panel recommends analysis of the pancreatic neck and bile duct at time of surgery by frozen section. Frozen sections should be taken approximately 5 mm from the transection margin, with the clean cut side facing up, to avoid cautery artifact that may confound analysis and result in false negatives. If tumor is located within 5 mm of margins, further excision of the pancreas should be considered to ensure at least 5 mm of clearance.

In the absence of frank venous occlusion noted on preoperative imaging, the need for lateral venorrhaphy or complete PV or SMV resection and reconstruction to achieve an R0 resection may be suggested, but it is often not known until division of the pancreatic neck has occurred. Tethering of the carcinoma to the lateral wall of the PV is not uncommon and requires careful dissection to free the vein from the pancreatic head if it is possible to do so. Differentiation of tumor infiltration into the vein wall from tumor-related desmoplasia is frequently impossible to ascertain. The liberal use of partial or complete vein resection when vein infiltration is suspected during Whipple procedures has been studied.[364-366] On evaluation of excised vein specimens, only 60% to 70% had histologic evidence of frank tumor involvement, and R0 resections were still not obtainable in 10% to 30% of patients despite increasing the magnitude of the operative procedure. However, if an R0 resection is obtained with vein excision, longevity appears similar to those with R0 resections without venous involvement, with no significant increase in morbidity and mortality. These data support an aggressive approach to partial or complete vein excision if tumor infiltration is suspected, although acceptance of this concept (particularly with respect to vein resection) is not universal.

Although numbers are more limited, similar findings have been noted with respect to hepatic arterial resection and reconstruction.[366,367] Others, however, have noted poor short- and long-term outcomes with arterial resection.[368,369] While further data with respect to arterial resection are clearly needed, judicious utilization of this technique would appear to be reasonable in very select populations.

A recent population-based study of 10,206 patients from the Nationwide Inpatient Sample from years 2000 through 2009 found that vascular reconstruction (about 90% venous and 10% arterial) is associated with a higher risk of intraoperative and postoperative complications.[369] No difference in mortality was seen.

Distal Pancreatectomy

The goals of left-sided resection are similar to those of pancreatoduodenectomy, although they are often more difficult to achieve because of the advanced stage at which most of these cancers are discovered. Spleen preservation is not indicated in distal pancreatectomy for adenocarcinoma, and an R0 distal pancreatectomy for adenocarcinoma mandates en bloc organ removal beyond that of the spleen alone in up to 40% of patients.[370,371] In addition, similar to the Whipple procedure, lateral venorrhaphy, vein excision and reconstruction, and dissection to the level of the celiac axis and SMA adventitia should be performed if complete tumor clearance can be achieved.[371,372] Utilization of these radical resections is associated with an increase in blood loss, transfusion requirements, operating time, length of stay, and morbidity, but mortality remains rare.[370-372] Encouragingly, tumor clearance (R0 resection) has been reported in up to 72% to 91% of patients, with long-term survival equivalent to those having standard resection for more localized disease.[371,372] Local recurrence, however, remains problematic even with pathologically negative margins.[372]

There is an increasing role for laparoscopic distal pancreatectomy. Results from 172 patients treated at the Mayo Clinic found significant benefits in the patients who had laparoscopic versus open resections in blood loss, the need for blood transfusions, and the length of hospital and intensive care unit stays without any difference in oncologic outcomes.[373] In addition, results from a meta-analysis of 4 studies of 665 total patients suggest that the laparoscopic method is safe and results in shorter hospital stays.[374] Furthermore, results from a population-based, retrospective cohort study that included 8957 patients showed similarly that the laparoscopic approach can decrease complication rates and shorten hospital stays.[375]

Portal Vein Resection

Vascular invasion has been a conventional contraindication to pancreatic resection. Early attempts at resection and reconstruction of the SMA and SMV in the 1970s were associated with poor results in a few patients who underwent "regional" pancreatectomy.[376] Both autologous and synthetic grafts were used for arterial and venous reconstructions. As morbidity from pancreatoduodenectomy decreased, a subset of patients was identified who were in need of resection of the SMV wall to achieve negative margins during removal of their tumors. Thus, in the 1990s, there was renewed interest in vein resection for complete resections. The group from the University of Texas MD Anderson Cancer Center has championed this approach, demonstrating that vein resection and reconstruction can allow for complete resection and is not associated with increased morbidity or mortality when compared with patients who did not require vein resection.[377] Furthermore, long-term outcome is not significantly worse for patients undergoing venous resection during pancreatoduodenectomy compared to patients who receive standard pancreatoduodenectomy.[378]

Although compelling, this approach has not been universally accepted. During the 1990s, several studies reported operative mortality of 0% to 16.5%, 3-year Kaplan-Meier survival of 12% to 23%, and median survival of 5 to 14 months in patients receiving vein resection.[379-382] One study found that properly selected patients with adenocarcinoma of the pancreatic head who required vein resection (n = 141) had a median survival of approximately 2 years that did not differ from those having standard pancreatoduodenectomy and was superior to historical patients believed to have locally advanced disease who did not receive surgical treatment.[366] A meta-analysis of 22 retrospective studies (2890 patients) found that vein resection resulted in perioperative morbidity and mortality equal to that of standard resection, but R0 resection rates were lower in that group.[383] In a recent multi-institutional database analysis of 492 patients undergoing pancreaticoduodenectomy, R0 resection rates were no different between the 14% who had vein resection compared to those without venous involvement (66% vs. 75%; *P* = NS).[384] Nevertheless, a few groups have recommended caution and only use vein resection for selected patients.

Pylorus Preservation

Reconstruction options for the stomach after pancreatoduodenectomy center on preservation of the pylorus. Traverso and Longmire[385] reported the modern use of pylorus preservation in 1978. The hypothesis was that preservation would improve emptying and provide nutritional benefit, but the benefits have been inconsistent to date. Yeo et al reported no adverse effects of pylorus preservation[386]; however, van Berge Henegouwen et al reported longer nasogastric drainage times.[387] In several randomized and nonrandomized studies,[388-393] the pylorus-preserving procedure seemed to be associated with shorter surgical duration. No consistent data suggest that pylorus preservation leads to a better quality of life or nutritional status in patients after resection. Thus, pylorus-preserving pancreatoduodenectomy remains an unproven but certainly acceptable alternative to classic pancreatoduodenectomy performed with antrectomy.

Pancreatic Anastomosis

Efforts have focused on preventing pancreatic leaks and fistulas, which are morbid and potentially lethal complications of pancreatoduodenectomy. Pancreaticojejunostomy has traditionally been the standard reconstruction and is the major focus of morbidity and mortality after pancreatoduodenectomy because of leaks, abscess formation, and fistulas from this anastomosis. A randomized study at Johns Hopkins Hospital found no difference in fistula rates after pancreaticojejunostomy and pancreaticogastrostomy.[394] However, a more recent multicenter, randomized, superiority trial compared the outcomes of 329 patients undergoing pancreaticoduodenectomy with either pancreaticojejunostomy or pancreaticogastrostomy.[395] A significant difference was seen in the primary outcome measure of postoperative fistulas, which occurred in 19.8% of patients in the pancreaticojejunostomy group and 8.0% of patients in the pancreaticogastrostomy group (OR, 2.86; 95% CI, 1.38–6.17; *P* = .002). An increase in grade ≥3a postoperative complications was seen, however, in the pancreaticogastrostomy group (24% vs. 21%). Criticisms of this trial have been published.[396] Although a meta-analysis of 4 randomized controlled trials (676 patients) concluded that pancreaticogastrostomy is associated with a lower risk of fistula formation than pancreaticojejunostomy (RR, 0.41; 95% CI, 0.21–0.62),[397] the optimal approach to anastomosis remains undefined.[398]

Surgeons have also examined various other options for the pancreaticojejunal anastomosis; end-to-end, end-to-side, duct-to-mucosa, and invaginating techniques have all proven to be safe and effective.[399,400] Results of a prospective trial show that pancreatic fistula

can be almost entirely avoided by a technique that combines placement/tying of sutures under magnification with meticulous attention to blood supply.[401] Stents used in the 1930s and 1940s continue to be used today, but data suggest that they do not decrease leak rates.[402]

In addition to technical modifications, octreotide has been examined for its ability to decrease postoperative pancreaticojejunal leaks in patients undergoing pancreatic resections. However, octreotide did not decrease fistula rates when assessed in 2 prospective, randomized, double-blind, placebo-controlled studies (at the University of Texas MD Anderson Cancer Center and Johns Hopkins Hospital).[403,404] Pasireotide, in contrast, significantly decreased the rate of grade ≥3 fistula, leak, or abscess in a single-center, double-blind, randomized controlled trial of 300 patients (9% in pasireotide group vs. 21% in placebo group; RR, 0.44; 95% CI, 0.24–0.78; P = .006).[405] Finally, the use of fibrin glue sealant does not appear to decrease the rate of pancreatic fistulas.[406]

Extended Lymphadenectomy

The role of lymph node dissection as a component of pancreatoduodenectomy has been explored. In the 1970s and 1980s, pathology and autopsy studies demonstrated a high incidence of nodal metastasis (sometimes as high as 80%), leading some groups to propose a more aggressive lymphadenectomy in an attempt to regionally control disease.[407,408] A standard lymphadenectomy in patients undergoing pancreatoduodenectomy entails removal of nodes at the duodenum and pancreas and on the right side of the hepatoduodenal ligament, the right side of the SMA, and the anterior and posterior pancreatoduodenal lymph nodes.[409] An extended lymphadenectomy is most commonly performed in the United States by removing not only the nodes removed in the standard procedure, but also the soft tissue in the retroperitoneum from the hilum of the right kidney to the left lateral border of the aorta on the right side, and from the PV to the origin of the inferior mesenteric artery on the left.[410]

Several prospective, randomized trials have addressed the role of lymphadenectomy in patients undergoing pancreatoduodenectomy. The Italian Multicenter Lymphadenectomy Group reported on a series of 81 patients randomly assigned to pancreatoduodenectomy with or without extended lymph node resection. Although the statistical power was low, this study did not support the concept that an extended lymphadenectomy was a good prognostic factor.[411] A larger randomized prospective trial was performed at Johns Hopkins Hospital from 1996 through 2001 to evaluate the role of extended lymph node dissections.[412] The group of patients who received the regional lymphadenectomy in addition to pancreatoduodenectomy had longer operation times, but overall median survival did not differ between the 2 groups at 1, 3, and 5 years.[412-414] Recently, a randomized multicenter trial in Japan came to similar conclusions.[415] Furthermore, multiple systematic literature reviews and meta-analyses of randomized controlled trials comparing pancreatoduodenectomy with standard versus extended lymphadenectomy support the conclusion that the extended procedure does not have any impact on survival.[416-418] In addition, patients undergoing extended lymphadenectomy have increased rates of postoperative diarrhea compared to patients undergoing the standard resection.[419]

The information to date thus does not show any survival advantage to performing a regional lymphadenectomy in addition to the standard pancreatoduodenectomy.[420] At this point in time, data suggest that nodal metastases are a marker of systemic disease and that their removal is unlikely to alter OS. One exception might be in the situation of an otherwise R0 resection with clinically positive adenopathy outside the standard field of dissection. Overall, outside of a clinical trial, a

regional lymphadenectomy should not be considered as a routine part of the Whipple procedure, although consideration can be given to sampling of the aortocaval and common hepatic artery nodes, as those with positive nodes in these positions have inferior prognoses.[421,422]

Preoperative Biliary Drainage

The main goals of preoperative biliary drainage are to alleviate the symptoms of pruritus and cholangitis and to potentially make surgery less morbid by improving liver function preoperatively. Although controversial, several studies have suggested that pancreatoduodenectomy is associated with higher perioperative mortality when done in the setting of hyperbilirubinemia.[423-425] Stenting of the biliary system can improve symptoms and liver function, but it is not clear whether these changes can decrease the mortality rate of the Whipple procedure. Several prospective and retrospective studies have failed to show decreased mortality in patients with preoperative biliary drainage.[426-432] A retrospective analysis from a prospective database of 593 patients treated with pancreatoduodenectomy at MD Anderson Cancer Center found that self-expandable metal stents did not affect postoperative complications, 30-day mortality, length of stay, anastomotic leak, margin status, or determination of unresectability during resection, although more wound infections and longer operative times were observed in this group.[433] In contrast, a multicenter, randomized trial comparing preoperative biliary drainage with surgery alone for 202 patients with cancer of the pancreatic head characterized by obstructive jaundice showed a nearly 2-fold increase in the rate of serious complications in the stented group (74% vs. 39%; relative risk in the surgery alone group, 0.54; 95% CI, 0.41–0.71; $P < .001$). However, no significant differences in surgery-related complications, length of hospital stay, or mortality were observed.[434]

Based on these reports, most groups who perform resection without neoadjuvant treatment advocate selective use of decompression only in patients who are symptomatic, septic, coagulopathic, have renal insufficiency, or in whom surgical resection is significantly delayed. The panel includes in this group patients who present with jaundice and potentially resectable disease if symptoms of cholangitis or fever are present or if they have significant pruritus and an expected delay to surgery of >1 week.

For patients with jaundice undergoing neoadjuvant induction therapy before pancreatic resection, biliary decompression is necessary before initiation of therapy and appears to be well tolerated with minimal increase in perioperative morbidity. The University of Texas MD Anderson Cancer Center reported on its experience with more than 300 patients, 57% of whom had preoperative biliary drainage as part of a neoadjuvant chemoradiation program.[435] It was found that wound complications were significantly increased in the drainage group; however, no other association was found for sepsis, fistulae, or death. Placement of a stent is thus required prior to administration of neoadjuvant therapy for patients with jaundice.[436-439]

The panel notes that stents are an evolving technology. The choice of stents includes plastic and metal; fully covered, partially covered, or uncovered; rigid; or self-expanding (also see the discussion on stents in *Palliation and Supportive Care*, below). While any stent can become occluded, several groups have reported better patency with metal stents.[437-439] Metal stents are generally viewed as more permanent than plastic stents. Covered metal stents may give more durable patency, since the cover prevents tumor ingrowth,[440] but the reported differences between covered and uncovered stents are not dramatic.[440,441] Furthermore, migration is more of an issue with covered stents.[441] This issue has led to the introduction of partially covered stents,[442] though

these stents may still migrate in a substantial number of patients.[443,444] Most metal stents used today are self-expanding. Their small initial diameters make them easy to place, and their placement rarely requires dilation.[442] Several panel members reported that their institutions use plastic stents in patients with short life expectancies (<3 months).[442] A clinical trial is currently recruiting patients to compare metal and plastic stents for preoperative biliary decompression in patients with pancreatic cancer (ClinicalTrials.gov NCT01191814). In the absence of level-1 data, the panel consensus is that short, self-expanding, metal stents are preferred because they are easy to place without dilation, are unlikely to interfere with the subsequent resection, and have a longer patency time than plastic stents. The panel cautions against placement of a metal stent prior to tissue proof of malignancy.

Effect of Clinical Volume

Several studies have examined the effect of institutional volume on patient outcomes. The fundamental premise was that the decreasing morbidity and mortality seen in the 1980s and 1990s were the direct result of large, single-institution experiences. Moreover, the concern was that if surgeons performed pancreatoduodenectomy less frequently, patients might have increased morbidity and mortality. A group from Memorial Sloan Kettering Cancer Center examined the issue in 1995 and found that in a cohort of almost 2000 patients, high-volume centers in New York State had significantly less mortality than low-volume centers (4% vs. 12.3%).[445] High volume was defined as more than 50 cases per year, and this relationship correlated in a regression analysis. Of note, 75% of the cases in New York State were performed in low-volume centers. Several other studies have assessed regional outcomes with pancreatoduodenectomy from U.S. hospitals.[446-450] These studies have reported decreased mortality, hospital length of stay, and overall cost at higher-volume centers (or with surgeons who perform the resections frequently) when compared with low-volume centers. Interestingly, this effect was also seen in reports from Canada and the Netherlands.[451-453]

The definitions of high and low volume varied among all these studies. However, a striking difference was seen when the mortality rates from pancreatoduodenectomy in very-low-volume (0–1 procedure/year) and low-volume (1–2 procedures/year) hospitals were compared with rates in higher-volume hospitals (>5 procedures/year).[454] In-hospital mortality rates at these very-low-volume and low-volume hospitals were significantly higher than at high-volume hospitals (16% and 12%, respectively, vs. 4%; $P < .001$). The importance of hospital volume in improving survival after pancreatic cancer surgery is even more marked when pancreatoduodenectomy is compared to other major surgeries. In a retrospective analysis of data from the national Medicare claims database and the Nationwide Inpatient Sample, hospitals performing 6 to 16 and >16 procedures per year were classified as “high” and “very-high” volume centers.[455] In this study, 6 or more pancreatic resections were performed at only 6.3% of hospitals. The largest difference in operative mortality between very-low-volume (16.3%) and high-volume (3.8%) centers was seen for pancreatoduodenectomy, as compared to major surgery at any other site, further reinforcing the magnitude of the effect that high-volume centers can have specifically on pancreatic cancer outcomes.

Furthermore, a study involving 301,033 patients with pancreatic adenocarcinoma included in the NCDB that evaluated the treatment patterns of 1667 hospitals over a 19-year period showed that patients were more likely to receive multimodality therapy at academic institutions considered to be high-volume hospitals.[456] In addition, a recent systematic review showed that margin status correlates with hospital volume, with negative margin rates ranging from 55% in low-

volume centers to 76% for very-high-volume centers (P = .008).[457] This review also found that 5-year survival rates were higher in high-volume centers. In contrast, hospital readmission after pancreatoduodenectomy appears to be more of a function of patient characteristics than hospital or surgeon volume.[458]

The NCCN Panel recommendation is that pancreatic resections should be done at institutions that perform a large number (at least 15–20) of pancreatic resections annually.

Pathology

Progress in treating pancreatic adenocarcinoma is encumbered by a lack of uniformity among treating physicians in defined areas that include pathologic analysis and reporting.[459] A more standardized approach in this area could maximize the chances of a more complete and consistent pathology report that is similar among pathologists in the same institution and among institutions around the world. Ultimately, a more consistent approach to patient assessment, surgical technique, and pathologic evaluation of the resected pancreatic specimen from gross examination to pathologic report will provide better communication among the various treating physicians. It will also provide a clear and specific understanding of the individual patient's malignancy, including critical margin status, which will then allow a more accurate comparison of the existing and evolving treatment regimens for this lethal disease.

Specimen Orientation, Sectioning, Pathologic Analysis, and Reporting

The primary purpose of pathologic analysis of the pancreatic specimen is to determine the pathologic stage of the tumor by evaluating the type, grade, size, and extent of the cancer. Pathology synoptic reports (protocols) are useful for reporting results from examinations of surgical specimens; these reports assist pathologists in providing clinically useful and relevant information. In 2004, the Commission on Cancer (CoC) of the American College of Surgeons mandated the use of specific checklist elements of the protocols as part of its Cancer Program Standards for Approved Cancer Programs. The pathology synoptic reports from the College of American Pathologists (CAP) comply with the CoC requirements, and the latest revisions to the CAP Pancreatic (Exocrine) protocol were issued in October 2013.[460] The NCCN Pancreatic Adenocarcinoma Panel currently supports the CAP pathology synoptic reports. The proposal included in the guidelines (see *Pathologic Analysis: Specimen Orientation, Histologic Sections, and Reporting* in the Guidelines) is an abbreviated *minimum* analysis of pancreatic cancer specimens from the CAP recommendations. In addition to the standard TNM staging, other variables are included, all of which have prognostic implications in the evolution of this disease.[461,462]

Lymph Node Counts and Lymph Node Ratio

The CAP recommendations include a count of the number of lymph nodes recovered and the number of involved nodes.[460] Recent retrospective database analyses have found that patients with N0 disease have a better prognosis with an increasing number of examined lymph nodes.[463-465] These results suggest that a significant portion of patients with N0 disease might be understaged. Based on these data, groups have recommended the minimum number of lymph nodes examined to be from 11 to 17 to provide optimal staging and to serve as a quality indicator.[463,465,466] The panel believes that every effort should be made to identify all regional lymph nodes within the pancreatectomy specimen.

For patients with N1 disease, lymph node ratio (positive node/nodes examined) appears to be related to prognosis.[463-470] For instance, in one analysis, patients with <15% of examined positive nodes had a 5-year

survival rate of 21.7%, while those with >15% positive nodes had a 5.2% 5-year survival rate (P = .0017).[468]

Whipple Specimen

Specimen orientation and inking involves both a pathologist and surgeon, as this will help to ensure accurate assessment of the size and extent of the tumor. There should be either direct communication between the surgeon and pathologist for proper orientation and margin identification, or the surgeon should identify the important margins with a clearly understood and documented method (ie, written on the pathology requisition). For example, a stitch can be placed on the posterior margin and a safety pin on the retroperitoneal/uncinate margin.

One of the impediments to comparison of data across institutions is the variability in the names given to various margins. Definitions of the margins and uniformity of nomenclature are critical to accurate reporting. The panel's recommended definitions are included in the *Pathologic Analysis: Specimen Orientation, Histologic Sections, and Reporting* section in the guidelines. Margins defined include the SMA (retroperitoneal/uncinate) margin, the posterior margin, the PV groove margin, the proximal and distal PV margins, the pancreatic neck (transection) margin, and the bile duct margin (see Figure 2). Other margins analyzed in Whipple specimens include the proximal and distal enteric margins (en face sections) and the anterior surface (closest representative). The anterior surface is not a true margin, but identification and reporting of this surface when positive may portend a risk of local recurrence, and so should be reported in all cases.[459,471-473] Collectively, these pancreatic tissue surfaces constitute the circumferential transection margin. Designating the various specific margins with different colored inks will allow recognition on microscopy.

The approach to histologic sectioning of a Whipple specimen is determined by the unique characteristics of the tumor, but is also influenced by institutional preferences, expertise, and experience. There is no one correct way to dissect a Whipple specimen. Options include axial, bi- or multi-valve slicing, and perpendicular slicing (see Figure 3). Some experts in the field bisect the pancreas along probes placed in the bile and pancreatic ducts and then serially section along each half of the pancreas. Axial slicing provides an overall assessment of the epicenter of the tumor relative to the ampulla, bile duct, duodenum and pancreas, and all of the pancreatic circumferential tissue margins (see Figure 4).

The most important aspects of dissection are clear and accurate assessment of the margins. It is currently unknown what constitutes an adequate margin in pancreatic carcinoma resection specimens. A standardized definition of this would allow better stratification of patients into adjuvant regimens following surgical extirpation. For instance, if less than 1-mm clearance is associated with an unacceptably high incidence of local recurrence, then strong consideration for postoperative radiation therapy (RT) might be indicated if not received preoperatively. The panel strongly recommends reporting tumor clearance in millimeters for all margins (as noted in the *Pathologic Analysis: Specimen Orientation, Histologic Sections, and Reporting* section of the guidelines) to allow prospective accumulation of these important data for future analysis.

A recent retrospective review compared the outcomes of 169 patients with R0 resections of close margins (within 1 mm) to 170 patients with wider margins (>1 mm) and found an improvement in OS with wider margins (35 months vs. 16 months; P < .001).[474] In fact, the close-margin R0 patients had a median survival time similar to that of the R1 population (16 months vs. 14 months; P = .6). Consistent with these

results, another recent retrospective review of 285 patients found that those with R1 resections, defined as tumor ≤1 mm from the margin, had a significantly worse local recurrence-free survival than those with R0 resections (HR, 4.27; 95% CI, 2.07–8.81).[475,476] Finally, a recent study, which used a standardized pathologic protocol that involved multicolor inking and careful evaluation of multiple margins distances, found that patients with R1 resections (tumor at 0 mm) had a median survival of 17.7 months, while those with R0 resections had a median survival of 32.9 months (P = .10).[477] Together, these results suggest that an appropriate definition of a negative margin may be >1 mm.

Attached organs resected with the specimen en bloc require serial sectioning to assess not only direct extension, but metastatic deposits as well.

Distal Pancreatectomy Specimen

In left-sided resections, the peripancreatic soft tissue margins and the pancreatic neck are assessed (see Figure 5). Additionally, involvement of the splenic vessels should be documented, and invasion of the spleen is important to determine, because direct tumor invasion constitutes a pT3 pathologic stage. Frozen section analysis of the pancreatic neck is recommended. Definitions of the proximal pancreatic (transection) margin, the anterior (cephalad) peripancreatic (peripheral) surface, and the posterior (caudad) peripancreatic (peripheral) margin are included in the guidelines (see *Pathologic Analysis: Specimen Orientation, Histologic Sections, and Reporting* in the guidelines).

Perioperative Therapy

Even with R0 resections, recurrence rates are very high in this disease. Therefore, additional therapy is required for all patients with resected pancreatic adenocarcinoma.

Postoperative (Adjuvant) Therapy

Results of many trials have shown that adjuvant therapy improves outcomes over observation following resection (see sections on *Systemic Therapy Approaches* and on *Chemoradiation Approaches*, above). While results of RTOG 9704 cannot be directly compared with the results of the CONKO-001, ESPAC-1, or ESPAC-3 trials because of differences in treatment design, timing of imaging, and patient characteristics (eg, patients enrolled in CONKO-001 were more likely to be lymph node-negative and to have positive resection margins than those in RTOG 9704; and CONKO-001 excluded patients with high postoperative CA 19-9 or CEA levels[196]), it is interesting to note that median OS for patients in the gemcitabine arm of CONKO-001 (22.8 months), the gemcitabine-containing arm of RTOG 9704 (20.5 months), the bolus 5-FU/leucovorin arm of ESPAC-1 (20.1 months), and the gemcitabine and 5-FU/leucovorin arms of the ESPAC-3 study (23.6 and 23.0 months) are remarkably similar.

Based on the data discussed above, no definite standard has been established in the adjuvant treatment of pancreatic cancer at this time. Gemcitabine- or fluoropyrimidine-based chemoradiation with additional gemcitabine, continuous infusion 5-FU, or 5-FU/leucovorin chemotherapy and chemotherapy alone with gemcitabine (category 1), 5-FU/leucovorin (category 1), or continuous infusion 5-FU are listed in the guidelines as options for adjuvant treatment. It was the consensus of the panel that when chemotherapy alone is the choice of adjuvant therapy, gemcitabine is preferred over 5-FU/leucovorin for most patients due to its more favorable toxicity profile. In the adjuvant setting, capecitabine monotherapy is also listed in the guidelines (category 2B). The panel considers capecitabine to be a reasonable alternative to 5-FU/leucovorin only in this setting as a last choice in patients for whom other options are inappropriate or unacceptable.

Regardless of the therapy being considered it is important to evaluate the patient for extent of disease prior to therapy, because some patients have early recurrence within the first few weeks following surgery. In addition, the panel recommends restaging a patient with imaging following systemic chemotherapy if chemoradiation is planned. While no studies have demonstrated superiority of giving chemoradiation before versus after chemotherapy in the adjuvant setting, when patients have a margin-positive resection, upfront chemoradiation followed by systemic chemotherapy is an appropriate option.

A recent retrospective analysis of data from patients in the ESPAC-3 trial found that completion of the full course of chemotherapy was an independent prognostic factor for survival, but that time to treatment initiation after surgery was not.[478] These results suggest that delaying chemotherapy until patients adequately recover could possibly improve outcomes. The panel therefore recommends that adjuvant treatment be initiated within 12 weeks, after adequate recovery from surgery.

Ongoing clinical trials in the adjuvant setting include ESPAC-4 (www.controlled-trials.com/ISRCTN96397484), which is comparing gemcitabine with capecitabine to gemcitabine alone; RTOG 0848 (ClinicalTrials.gov NCT01013649), which is assessing gemcitabine with or without subsequent chemoradiation; a phase II study comparing FOLFIRINOX with albumin-bound paclitaxel (ClinicalTrials.gov NCT02243007); the APACT study (ClinicalTrials.gov NCT01964430), comparing albumin-bound paclitaxel with gemcitabine; and the IMPRESS trial, which is comparing gemcitabine (with or without chemoradiation) with and without algenpantucel-L immunotherapy (ClinicalTrials.gov NCT01072981).

Preoperative (Neoadjuvant) Therapy

The standard approach to therapy in patients with resectable disease has been postoperative treatment, with median survivals in the range of 20.1 to 23.6 months under the most optimal clinical trial conditions.[196,240,241,268] However, it is becoming increasingly apparent that patients with borderline resectable disease, who are at higher risk for R1 resections, are potentially in need of a different management approach. Contemporary approaches to perioperative treatment have focused on neoadjuvant therapy for patients with borderline resectable disease with the goal of improving OS.[337,340] Neoadjuvant therapy is also sometimes used in resectable patients, especially in those with high-risk features. The putative benefits of neoadjuvant therapy include increasing the likelihood that a higher proportion of resectable patients will receive chemotherapy and/or radiation; the potential to downsize tumors so as to increase the likelihood of a margin-free resection (ie, conversion to resectable status); the potential to select for surgery those patients with more stable disease or disease that is more responsive to therapy; and the treatment of micrometastases at an earlier stage.[339,341,355,479] Moreover, surgery following neoadjuvant treatment appears to be safe.[480,481]

EUS-FNA is the preferred method of obtaining histologic confirmation of disease, and such confirmation is necessary before administering neoadjuvant therapy. A repeat biopsy should be performed in cases where the initial biopsy results do not confirm cancer. In addition, staging laparoscopy, performed to evaluate for the possible presence of metastatic disease, can be considered before neoadjuvant therapy. Furthermore, patients for whom neoadjuvant therapy is planned should be assessed for jaundice, and placement of a stent (preferably a short, self-expanding metal stent, as discussed in *Preoperative Biliary Drainage,* above) is recommended prior to initiation of neoadjuvant therapy in patients with jaundice.[437-439]

There is insufficient evidence to recommend specific neoadjuvant regimens, and practices vary with regards to chemotherapy and chemoradiation. Acceptable regimens include FOLFIRINOX or gemcitabine/albumin-bound paclitaxel. Subsequent chemoradiation is sometimes included. Studies of these regimens without chemoradiation are in progress. The role of chemoradiation with more active chemotherapy regimens also needs to be tested.

Abdominal (pancreas protocol), pelvic, and chest imaging should be repeated following neoadjuvant therapy, and staging laparoscopy can be considered at this time if not previously performed. Surgical resection should only be attempted if there is a high likelihood of achieving an R0 resection. Surgery is ideally performed 4 to 8 weeks after therapy. Surgery can be performed more than 8 weeks following therapy, but radiation-induced fibrosis may potentially make surgery more difficult. Importantly, results from retrospective studies suggest that radiographic response does not correlate with pathologic response.[482,483] Therefore, if no apparent tumor shrinkage is observed after neoadjuvant treatment and no extrapancreatic progressive disease is evident, surgery should still be attempted.

Neoadjuvant Therapy in Borderline Resectable Disease

Patients with borderline resectable disease have the options of upfront resection (category 2B) with adjuvant therapy or neoadjuvant therapy followed by restaging and resection in patients without disease progression precluding surgery. The use of neoadjuvant therapy in the setting of borderline resectable disease has been a highly debated topic. However, although there is no high-level evidence supporting its use, most NCCN Member Institutions now prefer an initial approach involving neoadjuvant therapy, as opposed to immediate surgery, for patients with borderline resectable disease. In fact, the panel down-graded its recommendation for upfront resection in borderline cases to category 2B in the 2014 version of these guidelines.

Several trials have shown that preoperative treatment of borderline resectable pancreatic adenocarcinoma can be effective and well-tolerated.[484-489] A phase I/II trial of neoadjuvant therapy in borderline resectable disease allowed 4 of 26 patients (15%) to be resected.[488] A randomized phase II trial comparing 2 different neoadjuvant regimens in borderline resectable disease was terminated early due to poor accrual, but 5 of 21 patients (24%) were resected.[487] A recent multi-institutional phase II trial found that full-dose gemcitabine, oxaliplatin, and radiation given preoperatively to patients with resectable (n=23), borderline resectable (n=39), or unresectable disease (n=6) found the approach to be feasible with an overall R0 resection rate of 53%.[486] In this study, 63% of all evaluable patients underwent resection, with 84% of those patients achieving an R0 resection.

In 2 retrospective reviews, 31% to 35% of borderline resectable patients who completed neoadjuvant therapy had R0 resections.[490,491] A systematic review and meta-analysis of 19 cohort studies found that unresectable patients (including both borderline and unresectable patients) undergoing neoadjuvant chemoradiation therapy had similar 1-year survival outcomes as patients who were initially deemed resectable.[492] In this study, 40% of treated patients were ultimately resected.

It is important to note that no randomized phase III trials have compared the approach of neoadjuvant therapy in borderline resectable disease compared to the approach of taking these patients to surgery without initial therapy, and the best regimens to use in the borderline neoadjuvant setting are unknown. Several phase II clinical trials are currently underway to determine the R0 resection rate following

neoadjuvant chemotherapy in patients with borderline resectable or unresectable locally advanced disease (eg, ClinicalTrials.gov NCT00557492). In addition, the Alliance A021101 trial (NCT01821612) is a single-arm pilot study evaluating the safety and efficacy of FOLFIRINOX before capecitabine-based chemoradiation and surgery in this population.[356] Initial results in patient series suggest that neoadjuvant regimens including FOLFIRINOX are a promising approach in patients with borderline resectable disease.[493,494] Additional randomized trials are needed.

Neoadjuvant Therapy in Resectable Disease

A number of studies have evaluated the use of neoadjuvant chemoradiation in patients with resectable disease.[339,340,495-503] A retrospective review of the collective experience at the University of Texas MD Anderson Cancer Center suggested that the use of preoperative chemoradiation therapy in patients with resectable disease is advantageous.[496] The authors suggest that preoperative therapy gives a selection advantage because approximately 25% of patients who are restaged after therapy are found to have progressive disease and are therefore spared the morbidity of a surgical procedure that would not benefit them.[496] In this analysis of 132 consecutive patients, the authors reported that combined preoperative chemoradiation and pancreatoduodenectomy yielded a median survival of 21 months, and 32% of patients were alive without evidence of disease at a median follow-up of 14 months.[496] The MD Anderson group has continued to champion this approach both for its ability to select patients for resection and for cost-effectiveness.[504] Other potential advantages of the neoadjuvant approach in resectable patients have also been described, including sterilization of the field before resection potentially reducing spread during surgery; increased rates of R0 resections; decreased incidence of pancreatic fistulas; prevention of delays or reductions of adjuvant therapy after surgery; and improved delivery of chemotherapy and radiosensitizing oxygenation.[481,505,506]

Although most studies investigating the neoadjuvant experience in patients with resectable pancreatic cancer are retrospective, several small phase II studies have been published.[481,505,507,508] In a randomized phase II trial evaluating the safety and efficacy of gemcitabine-based chemotherapy regimens as neoadjuvant therapy for patients with resectable pancreatic cancer, more patients receiving gemcitabine with cisplatin were able to undergo resection compared with those in the gemcitabine-only arm.[501]

In a prospective trial, preoperative radiation with concurrent gemcitabine was administered to 86 patients with resectable disease, and patients were restaged 4 to 6 weeks following completion of neoadjuvant treatment.[498] Although all patients were able to complete neoadjuvant therapy, at the time of restaging, only 73 (85%) patients were able to undergo surgery; the majority of the remaining patients were precluded from undergoing a pancreatoduodenectomy due to the presence of more advanced disease. Similar results were observed in another phase II trial involving preoperative gemcitabine/cisplatin followed by gemcitabine-based chemoradiation.[438] In this study, which enrolled 90 patients, 79 patients were able to complete neoadjuvant therapy, and 52 patients underwent surgery. Again, the main reason patients were precluded from surgery was the finding of more advanced disease at restaging following completion of neoadjuvant therapy. A cross-study comparison of these results suggests that inclusion of preoperative chemotherapy prior to initiation of gemcitabine-based chemoradiation did not improve survival.[479] These results provide support for restaging patients with abdominal (pancreas protocol), pelvic, and chest imaging and diagnostic laparoscopy before committing them to laparotomy after neoadjuvant therapy.

Although evidence suggests that there may be a better chance of margin-negative resection with preoperative therapy,[509] results of randomized trials addressing this issue are needed. A recent randomized phase II trial, which was terminated early because of slow accrual, compared gemcitabine/cisplatin neoadjuvant chemoradiation with upfront surgery; both arms received adjuvant chemotherapy.[510] With only 66 patients eligible for analysis, no significant differences were seen in R0 resection rate (52% vs. 48%), (y)pN0 rate (39% vs. 30%), or OS (25.0 months vs. 18.9 months), although all results favored the neoadjuvant arm and no safety issues were noted. The phase III NEOPA trial, with OS as the primary endpoint, is currently recruiting patients with resectable pancreatic cancer to compare neoadjuvant gemcitabine chemoradiation therapy to upfront surgery in this population (ClinicalTrials.gov NCT01900327).[511] A phase II trial with R0 resection as the primary endpoint is also ongoing (ClinicalTrials.gov NCT01389440).

At this time, the panel does not recommend neoadjuvant therapy for most resectable patients, except in a clinical trial. For selected patients who appear technically resectable but have poor prognostic features (ie, borderline resectable disease; markedly elevated CA 19-9; large primary tumors; large regional lymph nodes; highly symptomatic), however, consideration can be given to neoadjuvant therapy after biopsy confirmation, although a clinical trial is still preferred.

Adjuvant Treatment After Neoadjuvant Therapy

For patients who received neoadjuvant treatment, data supporting additional therapy after surgery are lacking. The consensus of the panel is that patients who have received neoadjuvant chemoradiation or chemotherapy may be candidates for additional chemotherapy following surgery and multidisciplinary review. When chemotherapy is given, the choice of regimen may be based on response seen to neoadjuvant therapy.

Adjuvant chemotherapy or adjuvant chemoradiation should only be considered for pre-treated patients who have adequately recovered from surgery and have no evidence of recurrence or metastatic disease; treatment should ideally be initiated within 4 to 8 weeks. It is recommended that the patient undergo a pretreatment baseline assessment following surgery, including CT scan and CA 19-9 level, to evaluate for the presence of metastatic disease before adjuvant chemoradiation is initiated. Further, the panel recommends restaging a patient with imaging following systemic chemotherapy, if it will precede chemoradiation.

Surveillance of Resected Patients

Although data on the role of surveillance in patients with resected pancreatic adenocarcinoma are very limited,[512-514] recommendations are based on the consensus that earlier identification of disease may facilitate patient eligibility for investigational studies or other forms of treatment. The panel recommends history and physical examination for symptom assessment every 3 to 6 months for 2 years, then annually. CA 19-9 determinations and follow-up CT scans every 3 to 6 months for 2 years after surgical resection are category 2B recommendations, because data are not available to show that earlier treatment of recurrences, following detection by increased tumor marker levels or CT scan, leads to better patient outcomes. In fact, a recent analysis of the SEER-Medicare database showed no significant survival benefit for patients who received regular surveillance CT scans.[515]

Management of Recurrent Disease After Resection

As cross-sectional body imaging has improved, small-volume metastatic disease or local recurrence is being detected in patients with resected pancreatic cancer who are otherwise maintaining good functional status. As many as 50% of them will continue to maintain a sufficiently good performance status to consider second-line therapy.[516] These patients will, however, ultimately progress.

For patients experiencing a recurrence of disease following resection, the panel recommends consideration of confirmatory biopsy (category 2B). In all cases of recurrent disease, a clinical trial is the preferred option; palliative and best supportive care without additional therapy should also be an option, especially for patients with poor performance status. Alternatively, chemoradiation can be considered in patients with local disease recurrence only, if not previously administered, or an alternative chemotherapy regimen can be given. For patients for whom there is evidence of metastatic disease (with or without a local recurrence), treatment decisions are influenced by the length of time from completion of adjuvant therapy to the detection of metastases. If adjuvant therapy was completed less than 6 months prior to development of metastatic disease, the panel recommends that an alternative chemotherapy option be administered. When this period is greater than 6 months, systemic therapy as previously administered or an alternative systemic regimen is recommended. Recommended regimens for patients with previous adjuvant treatment and good performance status are gemcitabine/albumin-bound paclitaxel and FOLFIRINOX.

Management of Isolated Pulmonary Metastases

Some patients have isolated lung metastases after resection of localized pancreatic adenocarcinoma. A growing body of evidence in this population suggests that these patients have a prolonged survival compared to patients with metastases in other locations.[517,518] Preliminary data also suggest that pulmonary metastasectomy may be advantageous in this population.[519] More data are needed before recommendations can be made regarding the management of pulmonary metastases of pancreatic cancers.

Palliative and Supportive Care

A significant subset of patients with pancreatic cancer will require substantial palliative interventions that are, in many respects, unique to the disease. The multidisciplinary management of symptoms due to biliary obstruction, gastric outlet obstruction, and cancer-related pain is of primary importance. The main objective of palliative care is to prevent and ameliorate suffering while ensuring optimal quality of life. Palliative surgical procedures are best reserved for patients with longer life expectancies.

Biliary Obstruction

Approximately 65% to 75% of patients with pancreatic cancer develop symptomatic biliary obstruction.[520] For patients diagnosed with unresectable disease and biliary obstruction upon initial evaluation, the best palliation is provided by an endoscopic biliary stent, especially when anticipated survival is limited. In most cases, a permanent self-expanding metal stent (SEMS) is recommended unless biliary bypass is performed (also see the discussion on stents in *Preoperative Biliary Drainage*, above). Stent occlusion that causes recurrent cholangitis is a well-known complication of plastic (temporary) biliary stents and typically occurs within 3 months of insertion. Metal stents are wider in diameter than plastic stents (ie, less likelihood of blockage) and become embedded in the bile duct, whereas plastic stents are more likely to become occluded but can be replaced. Results of a randomized,

controlled trial of 100 patients at a single center randomly assigned to receive either a plastic stent or a covered self-expanding metal stent inserted endoscopically indicated that median patency times were 1.8 and 3.6 months (P = .002), respectively.[521] A meta-analysis comparing metal and plastic biliary stents placed endoscopically in patients with pancreatic adenocarcinoma characterized by biliary obstruction showed similar results.[522] This study suggested that the risk of recurrent biliary obstruction was lower for the metal stents (RR, 0.52; 95% CI, 0.39–0.69), although no significant differences in technical/therapeutic success, complications, or 30-day mortality were found. Another recent randomized trial showed that covered SEMS had longer patency than uncovered SEMS in the setting of biliary obstruction due to pancreatic cancer, because covered stents prevented the ingrowth of tumor.[523]

When a biliary stent cannot be placed (often because the endoscope cannot be advanced past the neoplasm that is obstructing the gastric outlet), percutaneous biliary drainage with subsequent internalization may be necessary. An alternative is to sequentially dilate the duodenum endoscopically, place a metallic biliary stent, and then place an enteral stent.[524] Durable palliation of biliary obstruction can often be achieved with an expandable metallic biliary endoprosthesis (eg, Wallstent, Boston Scientific) in this situation.[524]

For patients with jaundice and potentially resectable disease who are found to have unresectable tumors following laparotomy, an open biliary-enteric bypass provides durable palliation of biliary obstruction and can be combined with procedures that palliate symptoms resulting from gastric outlet obstruction and cancer-related pain. The panel recommends stenting or an open biliary-enteric bypass with or without gastrojejunostomy (category 2B for prophylactic gastrojejunostomy[525,526]) and with or without celiac plexus neurolysis [527-529] (category 2B in non-jaundiced patients). Please see *Gastric Outlet Obstruction* and *Severe Tumor-Associated Abdominal Pain* below for more detailed information on these procedures. Bypass of the common bile duct (choledochojejunostomy) or common hepatic duct (hepaticojejunostomy) to the jejunum is preferred to bypass of the gallbladder (cholecystojejunostomy) since choledochojejunostomy/hepaticojejunostomy provide more durable and reliable palliation of biliary obstruction.[520]

Biliary decompression is also required for jaundiced patients with disease progression precluding surgery with or without neoadjuvant therapy. Here, stenting or biliary bypass is recommended, with or without gastrojejunostomy (category 2B for prophylactic gastrojejunostomy[525,526]) and with or without celiac plexus neurolysis (category 2B). One final circumstance requiring biliary drainage is in jaundiced patients with locally advanced or metastatic disease (those for whom surgical resection will not be attempted). In this situation, a SEMS is preferred unless biliary bypass was performed at the time of laparoscopy or laparotomy. If cancer has not been biopsy-confirmed in the setting of locally advanced disease in a jaundiced patient, brushings can be obtained at the time of stent placement.

Gastric Outlet Obstruction

Symptomatic gastric outlet obstruction occurs in 10% to 25% of patients with pancreatic cancer.[520] Patients with locally advanced or metastatic disease and a short life expectancy or poor performance status who develop gastric outlet obstruction may be palliated with an endoscopically placed enteral stent after biliary drainage is assured.[524] An alternative for these patients with poor performance status is percutaneous endoscopic gastrostomy (PEG) tube placement. For a fit patient with a life expectancy greater than 3 to 6 months (ie, locally advanced disease) who develops gastric outlet obstruction, an open or

laparoscopic gastrojejunostomy (duodenal bypass) with or without a jejunostomy (J) tube should be considered since it may provide more durable and effective palliation of gastric outlet obstruction than an enteral stent.[530-532] Nevertheless, placement of an enteral stent is also an option for these patients.

For patients with potentially resectable disease who undergo a laparotomy and are found to have unresectable disease, a prophylactic gastrojejunostomy should be performed for those deemed to be at risk of developing symptomatic gastric outlet obstruction (category 2B). The role of prophylactic gastrojejunostomy in otherwise asymptomatic patients who are found to have unresectable cancers at the time of laparotomy has been evaluated. Two randomized controlled trials have investigated the role of prophylactic gastrojejunostomy for unresectable periampullary cancer, the majority arising from the head of the pancreas.[525,526] In both studies, approximately 20% of patients who did not undergo a prophylactic gastrojejunostomy developed late gastric outlet obstruction that required therapy. A recent meta-analysis found similar results, with development of gastric outlet obstruction in 2.5% of patients in the prophylactic gastrojejunostomy group and 27.8% of those not receiving gastrojejunostomy.[533] In both studies, prophylactic retrocolic gastrojejunostomy significantly decreased the incidence of late gastric outlet obstruction but did not extend the length of stay or increase complication rates, such as delayed gastric emptying.

Severe Tumor-Associated Abdominal Pain

Most patients with locally advanced or metastatic pancreatic cancer experience cancer-related pain.[529] General principles for cancer-related pain management can be found in the NCCN Guidelines for Adult Cancer Pain (available at www.NCCN.org). Because advanced pancreatic cancer often infiltrates the retroperitoneal nerves of the upper abdomen, celiac plexus neurolysis should be considered (category 2B, except when indicated by pain in a jaundiced patient who is found unresectable at surgery, for which the recommendation is a category 2A). In several randomized controlled trials, celiac plexus neurolysis significantly improved pain relief in patients with advanced pancreatic cancer.[527,529,534] In a recent study of 96 patients with pain related to suspected pancreatic cancer, half were randomized to EUS-guided celiac plexus neurolysis at the time of EUS if unresectable adenocarcinoma was confirmed.[528] These patients reported better pain relief at 3 months (P = .01), suggesting that early EUS-guided celiac plexus neurolysis may be beneficial. A recent meta-analysis of 7 randomized controlled trials concluded that celiac plexus neurolysis improved pain scores at 4 weeks but not at 8 weeks in patients with pancreatic cancer.[535] Minimally invasive techniques including EUS-guided (preferred if available) and percutaneous fluoroscopic- or CT-guided celiac plexus neurolysis are recommended, but laparoscopic, thoracoscopic, and open approaches can also be used.

In selected patients with severe local back pain refractory to narcotic therapy, palliative RT may be considered, even in the setting of metastatic disease, if not already given as part of primary therapy. In such cases, radiation is given with or without concurrent chemotherapy to the primary tumor plus a margin (typically 25–36 Gy in 2.4–5 Gy fractions), or radiation alone is given to the metastatic site.

Pancreatic Exocrine Insufficiency

Exocrine enzyme insufficiency in pancreatic cancer is caused by tumor-induced damage to the pancreatic parenchyma and/or blockage of the pancreatic duct, or by surgical removal of pancreatic tissue, and results in an inadequate production of digestive enzymes.[536,537] This deficiency in pancreatic enzymes results in inadequate absorption of fat,

carbohydrates, and proteins, leading to steatorrhea, abdominal cramps, weight loss, and malnutrition.[538] Oral pancreatic exocrine enzyme replacement therapy is recommended for patients with pancreatic cancer who have symptoms of exocrine enzyme deficiency. Because pancreatic exocrine insufficiency occurs in up to 94% of patients undergoing pancreatic surgery,[539,540] therapy may be initiated without diagnostic tests. Enteric-coated mini-microspheres containing preparations of pancreatic enzymes are taken orally (25,000–75,000 units of lipase for a main meal and 10,000–25,000 units of lipase for a snack, depending on fat content), with half of the dose taken at the start of the meal and half taken in the middle of the meal.[538] For patients not responding to this therapy, doses of the enzyme preparation can be increased, and inhibition of gastric secretion with a proton pump inhibitor can also be considered.[538,539] Patients with a clinical suspicion of pancreatic exocrine insufficiency despite appropriate replacement may need a more thorough nutritional evaluation.

Thromboembolic Disease

The risk of developing venous thromboembolic disease is substantially increased in patients with pancreatic cancer.[541,542] The panel recommends low-molecular-weight heparin (LMWH) as preferred therapy over warfarin for patients with pancreatic cancer who develop a venous thromboembolism (VTE). Support for this recommendation comes from results of 2 large, prospective, randomized clinical trials: CLOT and CONKO 004. In the CLOT study, an approximately 2-fold decrease in the incidence of recurrent VTE at 6 months was observed in patients with advanced or metastatic cancer diagnosed with a VTE who were treated with the LMWH, dalteparin, compared with those treated with an oral anticoagulant.[543] In the CONKO 004 trial, VTE- and chemotherapy-naïve patients with advanced pancreatic cancer were randomized to receive palliative chemotherapy with or without the LMWH, enoxaparin.[544] The risk of developing symptomatic VTE was significantly lower for patients in the LMWH arm of the study with no significant increase in bleeding observed in this group compared to those not receiving enoxaparin. Please see the NCCN Guidelines for Venous Thromboembolic Disease, available at www.NCCN.org, for more information.

Depression, Pain, and Malnutrition

For many patients, a diagnosis of pancreatic cancer may result in significant psychosocial distress, including anxiety, depression, and sleep disturbances.[545] In fact, the suicide rate in male patients with pancreatic cancer is reportedly 11 times that of the general population.[546] Empathetic discussion about the natural history of this disease and its prognosis and the provision of support and counseling both by the primary oncology team and specialized services may help to alleviate this distress. The panel recommends that patients be screened and evaluated for depression and other psychosocial problems following the NCCN Guidelines for Distress Management (available at www.NCCN.org).

Because pain and malnutrition are also prevalent in patients with pancreatic cancer, the panel recommends that patients with locally advanced or metastatic pancreatic cancer receive a nutritional evaluation and a formal evaluation by a Palliative Medicine Service, when appropriate. Additional resources are detailed in the NCCN Guidelines for Palliative Care and the NCCN Guidelines for Adult Cancer Pain (available at www.NCCN.org).

Future Clinical Trials: Recommendations for Design

In 2007, a meeting was convened by the National Cancer Institute's Gastrointestinal Cancer Steering Committee in recognition of the failure

of a number of phase III trials to show clinically significant benefit for patients with pancreatic cancer and to address the importance of integrating basic and clinical knowledge in the design of clinical trials in pancreatic cancer. Meeting participants included representatives from industry, government, and the community, as well as academic researchers and patient advocates. Several important themes emerging from this meeting are summarized below, and the recommendations put forward by the committee are endorsed by the NCCN Pancreatic Adenocarcinoma Panel.[547]

- With the emergence of new agents to treat pancreatic cancer, particularly biologics, clinical trial strategies incorporating principles of molecular biology and new imaging methods as well as results from preclinical studies are important.
- For patients enrolled in clinical trials, banking of tumor tissue samples should be required along with paired blood and serum samples.
- Biomarkers that serve as surrogate markers of the anticancer effects of investigational agents should be sought, and assays to measure such biomarkers should be well validated.
- Clinical trials should enroll homogeneous patient populations with respect to disease stage (ie, separate trials for patients with locally advanced disease and metastatic disease) and patient performance status. Criteria for selecting study populations should take into account the putative differential efficacy of the agent (ie, vaccines in patients with early-stage disease).
- Phase III trials should not be initiated in the absence of clinically meaningful efficacy and safety signals in the phase II setting.
- Phase II and III clinical trials should have a primary endpoint of OS.
- Quality control standards for preoperative imaging interpretation, pathologic assessment of tumor specimens, and surgical selection criteria are critical when evaluating adjuvant therapies.

A 2011 consensus report from a group of European experts came to many of the same conclusions.[548] Additionally, the group states that FOLFIRINOX can be considered as a new standard treatment option in selected patients in future clinical trials, but that gemcitabine should remain the standard for most patients. An international expert panel also met to discuss current and future pancreatic cancer research and came to similar conclusions.[516] In addition, the Intergroup Pancreatic Cancer Task Force's Tissue Acquisition Working Group has made recommendations regarding the prospective collection and sharing of tissue to accelerate the discovery of predictive and prognostic biomarkers.[549] These recommendations include centralization of biorepositories and mandatory collection of tissue (when there is sufficient material), blood, serum, and plasma in all phase III trials.

ASCO also recently convened a working group to discuss designs for pancreatic cancer clinical trials that would accomplish meaningful clinical improvements.[550] This group concluded OS should be the primary endpoint of first-line, metastatic pancreatic cancer trials. They also concluded that trials should aspire to a 3- to 4-month improvement in OS in gemcitabine-eligible and gemcitabine/albumin-bound paclitaxel-eligible patients and a 4- to 5-month improvement in OS for FOLFIRINOX-eligible patients to give results with true clinical impact.

To determine appropriate historic controls for single arm phase II trials based on gemcitabine, an algorithm has been developed, based on an

analysis of a database of cooperative group trials, that can be used to calculate historic benchmarks for OS and PFS.[551]

Neoadjuvant Clinical Trials

For neoadjuvant trials, study populations should be well defined and standardized. The panel endorses use of a restrictive definition of borderline resectable disease in clinical trials, such as that defined in a recent Intergroup trial.[356] Endpoints should also be standardized and could include resection rates, R0 resection rates, local recurrence rates, pathologic response rates, DFS, and OS.[552]

Summary

Resection remains the only chance for a cure for pancreatic adenocarcinoma, and most resectable patients should undergo surgery without delay, followed by adjuvant therapy. Borderline resectable patients and select resectable patients can undergo neoadjuvant therapy in the hopes of improving the chances for an R0 resection or can immediately undergo surgery (category 2B). Additional therapy is an option for those patients whose disease recurs following surgery. Patients with locally advanced unresectable disease and good performance status can undergo chemotherapy and chemoradiation with second-line therapy if performance status is maintained after progression. Good performance status patients presenting with metastatic disease can undergo chemotherapy and can undergo second-line therapy if performance status is maintained after progression. Specific palliative measures are recommended for patients with advanced pancreatic adenocarcinoma characterized by biliary or gastric obstruction, severe abdominal pain, or other tumor-associated manifestations of the disease.

Overall, in view of the relatively high likelihood of poor outcomes for patients with all stages of pancreatic cancer, the NCCN Panel recommends that investigational options be considered in all phases of disease management.

Table 1: Selected Genetic Syndromes with Associated Pancreatic Cancer Risk

Syndrome	Gene	Estimated cumulative risk of pancreatic cancer	Estimated increased risk compared to general population
Peutz-Jeghers Syndrome	*STK11*	11%–36% by age 65–70 years[59]	132-fold[58]
Familial Pancreatitis	*PRSS1, SPINK1, CFTR*	40%–53% by age 70–75 years[63-65]	26-fold to 87-fold[29,63-65]
Melanoma-Pancreatic Cancer Syndrome	*CDKN2A*	17% by age 75 years[68]	20-fold to 47-fold[67,68]
Lynch Syndrome	*MLH1, MSH2 (MSH6)*	4% by age 70 years[77]	9-fold to 11-fold[77,78]
Hereditary Breast-Ovarian Cancer Syndrome	*BRCA1, BRCA2*	1.4%–1.5% (women) and 2.1%–4.1% (men) by age 70[79,84]	2.4-fold to 6-fold[79,83,84]
Familial Pancreatic Cancer	Unknown in most families (family X is an exception)*	≥3 first-degree relatives with pancreatic cancer: 7%–16% by age 70[51] 2 first-degree relatives with pancreatic cancer: 3% by age 70[51]	≥3 first-degree relatives with pancreatic cancer: 32-fold[90] 2 first-degree relatives with pancreatic cancer: 6.4-fold[90] 1 first-degree relative with pancreatic cancer: 4.6-fold[90]

*One family (family X) with a mutation in the *palladin* (*PALLD*) gene has been identified.[553]

Table 2: Potential Indications for Various Therapies in the Treatment of Pancreatic Adenocarcinoma

Regimen	Resectable (adjuvant)	Borderline Resectable (neoadjuvant)	Locally Advanced	Metastatic (good performance status)
Gemcitabine	√ (category 1)		√ (category 1 for poor performance status)	√ (category 1 for good and poor performance status)
Gemcitabine/Albumin-Bound Paclitaxel		√	√	√ (category 1; preferred)
Gemcitabine/Erlotinib			√	√ (category 1; survival benefit is small)
Gemcitabine/Cisplatin			√ (especially if possible hereditary cancer)	√ (especially if possible hereditary cancer)
Gemcitabine/Capecitabine			√	√
Fixed-dose-rate gemcitabine			√	√ (category 2B)
GTX [Fixed-dose-rate gemcitabine/docetaxel/capecitabine]			√ (category 2B)	√ (category 2B)
5-FU/Leucovorin	√ (category 1)			
FOLFIRINOX		√	√	√ (category 1; preferred)
Capecitabine	√ (category 2B)		√ (category 2B)	√ (category 2B)
Continuous Infusion 5-FU	√		√ (category 2B)	√ (category 2B)
Fluoropyrimidine/Oxaliplatin (eg, FOLFOX, CapeOx)			√ (category 2B)	√ (category 2B)
Radiation	√ (fluoropyrimidine- or gemcitabine-based)	√ (subsequent chemoradiation is sometimes included)	√ (in select patients without systemic metastases; fluoropyrimidine- or gemcitabine-based)	√ (palliative only)

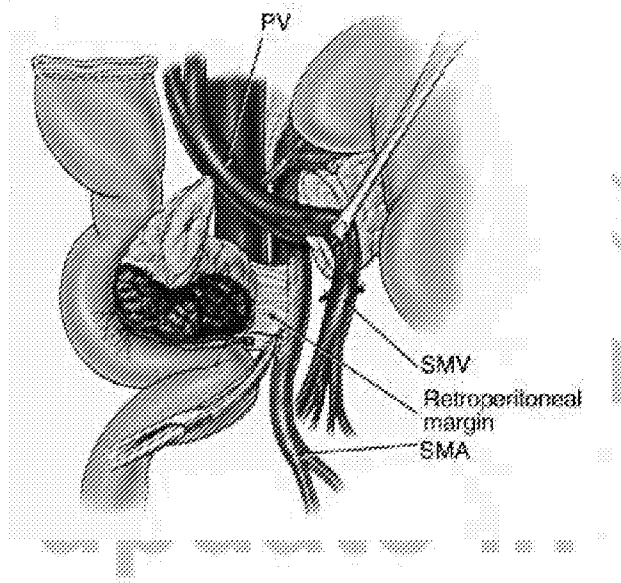


Figure 1. Complete mobilization of the superior mesenteric (SMV) and portal veins, and separation of the specimen from the right lateral border of the superior mesenteric artery (SMA).[554]

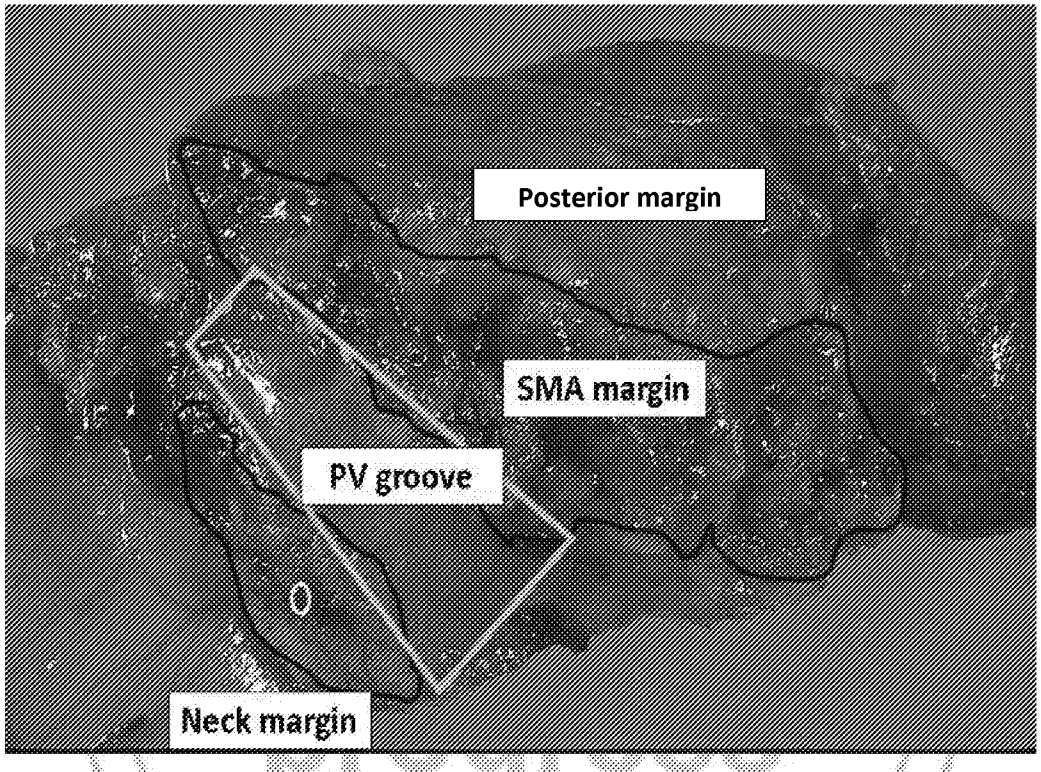
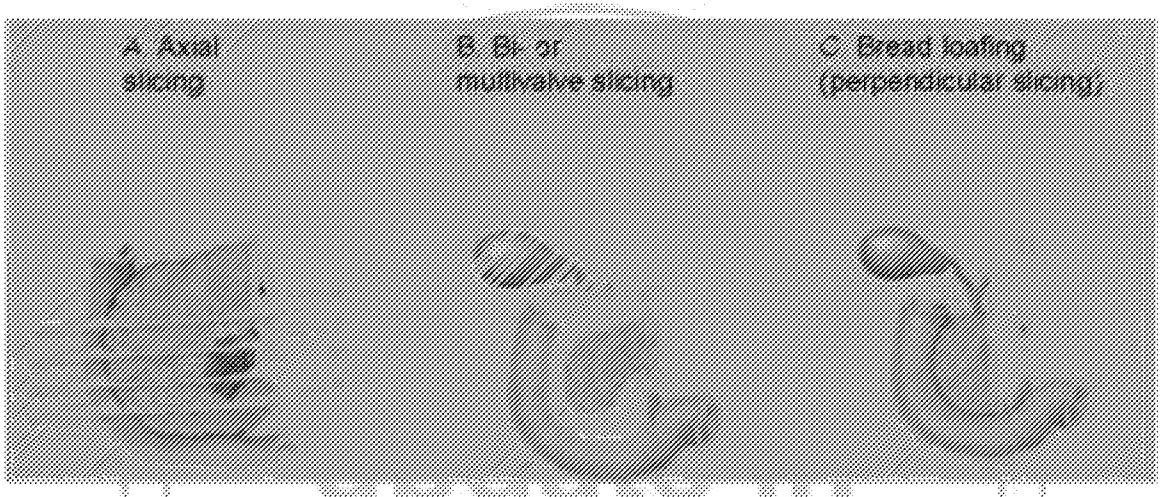


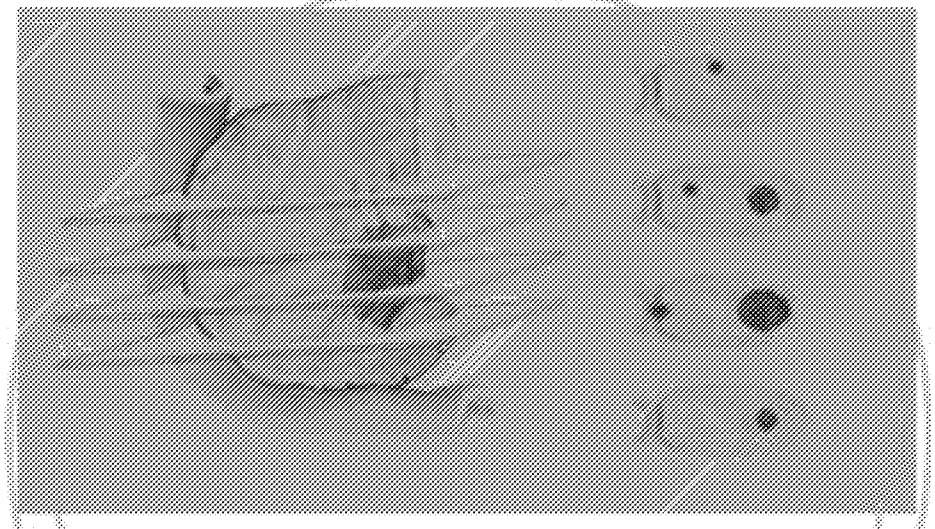
Image courtesy of Dr. N. Volkan Adsay

Figure 2. Whipple specimen with labeled margins.



Courtesy of Mr. Paul Brown, Specialist Medical Illustrator, St James's University Hospital Leeds

Figure 3. Slicing of pancreatoduodenectomy specimens.[459]



Courtesy of Mr. Paul Brown, Specialist Medical Illustrator, St James's University Hospital Leeds

Figure 4. Slicing of the pancreatoduodenectomy specimen in the axial plane to allow circumferential assessment of tumor.[459]

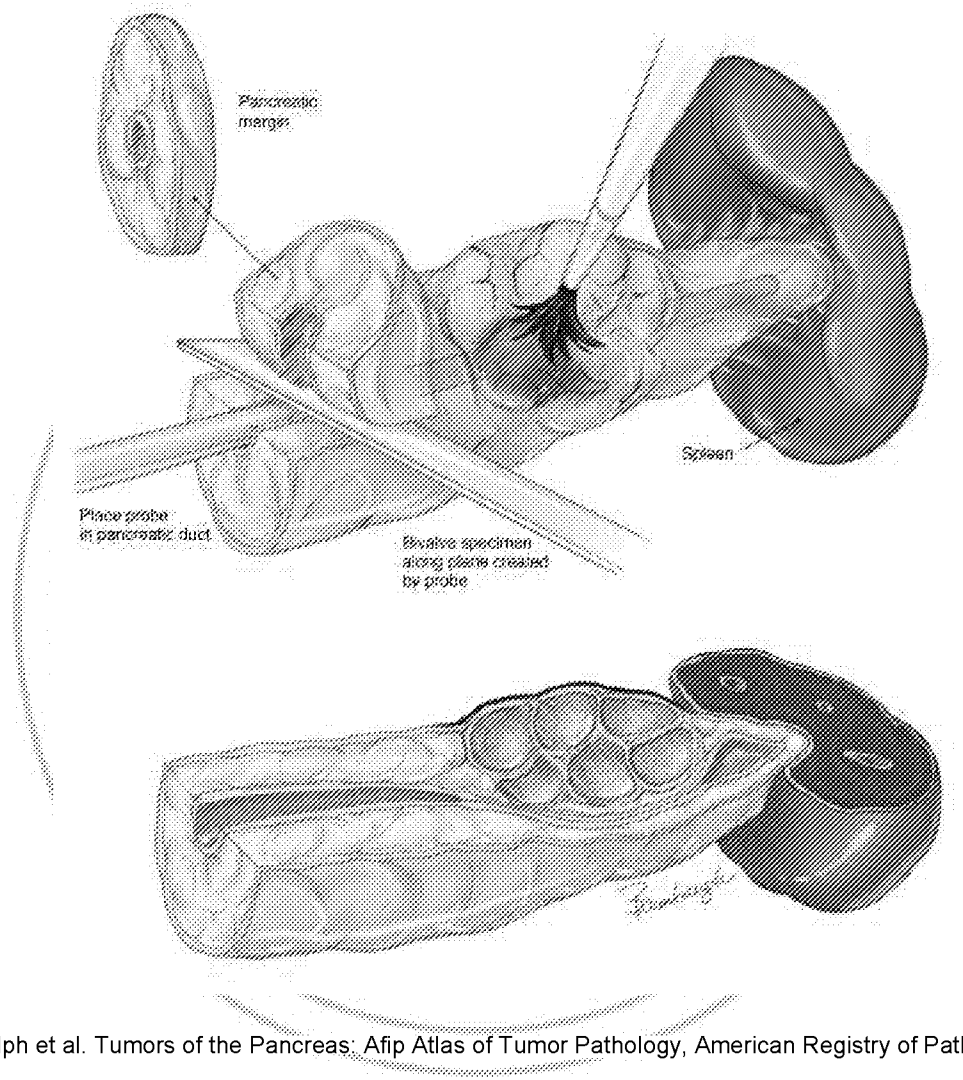


Figure 16-4, from Hruban, Ralph et al. Tumors of the Pancreas: Afip Atlas of Tumor Pathology, American Registry of Pathology, Washington DC 2007

Figure 5. Slicing of the distal pancreatectomy specimen.[473]

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Generic Name: DOXORUBICIN HYDROCHLORIDE
Trade Name: ADRIAMYCIN

Classification: Antineoplastic antibiotic

Actions: Interferes with nucleic acid synthesis by binding with DNA and preventing DNA-directed RNA and DNA transcription. Although most active in the S phase, considered cell-cycle nonspecific since it has some activity during all phases.

Indications: Sarcomas, carcinomas, melanomas, leukemias, lymphomas, neuroblastomas.

Dosage:
Neonatal: Not applicable.
Pediatric: IV: 60–90 mg/m^2 every 3 weeks or 20–30 mg/m^2 3 times every 3 weeks or 30 mg/m^2 every week or 60–90 mg/m^2 by IV drip over 10–96 hours every 3–4 weeks.
Intra-arterially: 25 mg/m^2 every day for 3 days.
Intraperitoneally or into the bladder: 30–60 mg/m^2 in 150 ml of normal saline every month.
Adult: See pediatric dosage.

Preparation: A red powder, available in 10- and 50-mg vials, which can be mixed with sterile water and D_5W or normal saline for IV push administration.

Home Stability: Unopened vials are stable for 2 years if protected from light and stored at room temperature. In solution, doxorubicin is stable for 24 hours at room temperature and 48 hours in the refrigerator. It should be protected from light if not used within 8 hours.

Compatibilities: Not compatible with aminophylline, dexamethasone, keflin, fluorouracil, diazepam, heparin, and hydrocortisone, and the same final filter should not be used with these drugs. Necrotizing colitis has occurred with Ara-C. Barbiturates increase the plasma clearance of doxorubicin. Mercaptopurine increases doxorubicin hepatotoxicity. Doxorubicin decreases the effectiveness of oral digoxin. Compatible with dacarbazine and has been mixed with it in liter solutions and infused over 24 hours for 4 days. However, as with most antineoplastic agents, it is usually advisable not to administer doxorubicin with other admixture solutions.

Administration:
Neonatal: Not applicable.
Pediatric: Administer by slow IV push into the side arm of a free-flowing IV, checking for patency every 2–3 minutes to avoid extravasation of this severe vesicant. This drug should not be given by IV drip through a peripheral IV.
Adult: See pediatric administration.

Contraindications: Allergy to lincomycin, bone marrow depression, poor liver function (dose reductions required for serum bilirubin greater than 1.2), congestive heart failure. Watch for cardiotoxicity in patients with hypertension, coronary artery disease, angina, and previous myocardial infarction. To prevent irreversible cardiotoxicity, cumulative doses should not exceed 550 mg/m^2 with doxorubicin or daunorubicin or 450 mg/m^2 with cyclophosphamide or mitomycin or previous radiation therapy to the chest. Concurrent administration of vitamin E or N-acetylcysteine or weekly or continuous drip administration of doxorubicin may decrease cardiotoxicity. Dose reductions should also be considered if other anthracyclines have been given. Single doses of more than 150 mg should be double-checked.

Side Effects:
Primary:
Hematopoietic: Decreased white blood cells after 7–14 days, with recovery in 1–3 weeks.
Cutaneous: Extensive tissue damage if extravasated; radiation "recall" on previously irradiated areas of the skin, the esophagus, and the lung; complete and often sudden hair loss that is reversible.
Cardiac Toxicity: Pericarditis, myocarditis, EKG changes, hypotension, and usually irreversible cardiomyopathy.
Gastrointestinal: Moderate nausea and vomiting, stomatitis, esophagitis, and diarrhea. Ascites and adhesions occur with intraperitoneal administration.
Nephrotoxicity: Urine will be red for 24–48 hours after administration. Bladder instillations may cause urgency, local irritation, and cystitis.

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FOLFIRI regimen as second-/third-line chemotherapy in patients with advanced pancreatic adenocarcinoma refractory to gemcitabine and platinum salts: A retrospective series of 70 patients.

Subcategory:
Multidisciplinary Treatment

Category:
Cancers of the Pancreas Small Bowel and Hepatobiliary Tract

Meeting:
2011 Gastrointestinal Cancers Symposium

Session Type and Session Title:
General Poster Session B

Abstract Number:
272

Citation:
J Clin Oncol 29: 2011 (suppl 4; abstr 272)

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Abstracts that were granted an exception in accordance with ASCO's Conflict of Interest Policy are designated with a caret symbol (^).

Abstract Disclosures

Abstract:

Background: Gemcitabine-based regimen is a standard of first-line chemotherapy in patients with advanced pancreatic adenocarcinoma (PAC) and 5-FU/oxaliplatin combination is an option for second line (Oettle, 2009). Some data suggest a potential efficacy of 5-FU/irinotecan regimen (FOLFIRI) as first-line treatment (Taïeb, 2007) or in patients with gemcitabine/platinum-refractory advanced PAC (Yoo, 2009; Gebbia, 2010). **Methods:** All consecutive patients with unresectable advanced PAC (01-2005/05-2010) and OMS≤2 received FOLFIRI-1 (irinotecan 180 mg/m^2 D1, n=60) or FOLFIRI-3 regimen (irinotecan 100 mg/m^2 D1/D3 n=10) after failure of gemcitabine- and platinum-based chemotherapies as a systematic policy in two institutions. Following data were analyzed: tumor response, progression free survival (PFS), overall survival rate (OS), and grade 3-4 toxicities. **Results:** Seventy patients were studied. Median age was 60 years

(range: 24-81); 37 (52.9%) were male; 30 (42.9%) were PS 0, 27 were PS 1 and 13 were PS 2. Cancer was locally advanced in 15.7% and metastatic in 84.3% of patients. Before FOLFIRI regimen, patients received 1 line (n=24, 34.3%), 2 lines (n=40, 57.1%) or ≥ 3 lines (n=6, 8.8%) chemotherapy. PFS with previous line was less than 3 months in most patients. Tumor control was achieved in 31 (44.3%) patients (partial response: 5, stable disease: 26). PFS was 17% at 12 months and 3% at 24 months. Median PFS was 23 weeks (range: 2-147). OS was 24% at 12 months and 9% at 24 months. Median OS was 24 weeks (range: 2-147). From the initial diagnosis, 1-year and 2-year survivals were 79% and 32%. Dose adaptation was required in 21 (30.0%) patients. Eighteen (25.7%) patients had grade 3-4 toxicities, mainly hematologic (n=13) or digestive (n=6). Febrile neutropenia occurred in 3 patients without death. **Conclusions:** FOLFIRI regimen after failure of gemcitabine- and platinum-based regimens for advanced PAC in our study had an acceptable toxicity and a surprising efficacy in patients OMS 0-2. These results suggest that FOLFIRI regimen should be further tested as first-line chemotherapy in patients with advanced pancreatic cancer.

Abstracts by C. Neuzillet:

FOLFIRI regimen as second-/third-line chemotherapy in patients with advanced pancreatic adenocarcinoma refractory to gemcitabine and platinum salts: A retrospective series of 70 patients.
Meeting: 2011 ASCO Annual Meeting | Abstract No: 4132 **| First Author: C. Neuzillet**
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FDA Approves Irinotecan Liposome to Treat Pancreatic Cancer

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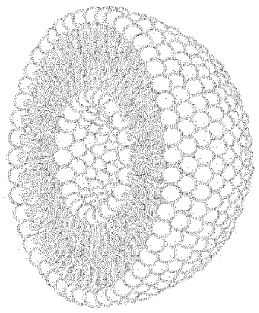
November 24, 2015 by NCI Staff

On October 22, the U.S. Food and Drug Administration (FDA) approved irinotecan liposome (Onivyde®) to be used in combination with fluorouracil and leucovorin to treat patients with metastatic pancreatic cancer whose disease has progressed after gemcitabine-based chemotherapy.

The approval was based on results from a clinical trial of 417 patients with metastatic pancreatic cancer. Patients in the trial were randomly assigned to receive treatment with irinotecan liposome alone, irinotecan liposome in combination with the chemotherapy drugs fluorouracil and leucovorin, or with fluorouracil and leucovorin alone.

Patients treated with all three drugs had a median survival of 6.1 months, compared with 4.2 months for those treated with fluorouracil and leucovorin alone. There was no survival benefit with irinotecan liposome alone compared with fluorouracil and leucovorin alone. Patients treated with all three drugs had a 3.1-month delay in tumor growth compared with 1.5 months for those receiving only fluorouracil and leucovorin.



Cross section of a liposome. Encapsulating irinotecan in a liposome helps the drug stay in the circulation longer, so it is more likely to reach and kill cancer cells.
Credit: Wikimedia Commons

Gemcitabine has been the cornerstone of pancreatic cancer treatment for the past 20 years. A common research approach during much of that time has been to combine gemcitabine with another drug in an attempt to increase its efficacy in previously untreated patients with good performance status, explained Austin G. Duffy, M.D., of the Thoracic and Gastrointestinal Oncology Branch in NCI's Center for Cancer Research.

"But beyond that there has unfortunately been little progress made in terms of subsequent treatment options for patients who have already received a combination of drugs in the first-line setting and whose cancer has advanced," Dr. Duffy noted.

"There are relatively few phase III clinical trials performed in this patient population, the so-called second-line setting," he added. "So any randomized phase III clinical trial, such as this one, that demonstrates a survival benefit in pancreatic cancer is important, both for the obvious reason that it can help individual patients, but also because of the numerous disappointingly negative phase III studies in this disease for drugs that had looked promising in phase II trials."

Despite these results, Dr. Duffy cautioned that it will still be important to see how well patients tolerate this new therapy and its impact on their quality of life.

Positive trial results and the availability of new options for patients with pancreatic cancer are always good news, he said.

"However, nobody will or should be satisfied with these modest gains," he continued. "We need to do better at every stage."

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VOLUME 24 · NUMBER 28 · OCTOBER 1 2006

JOURNAL OF CLINICAL ONCOLOGY

COMMENTS AND CONTROVERSIES

Uridine Diphosphate Glucuronosyltransferase (UGT) 1A1 and Irinotecan: Practical Pharmacogenomics Arrives in Cancer Therapy

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For 50 years the dosing of anticancer drugs has been empirical—small phase I trials predict a tolerable dose, and subsequent studies refine the accuracy of that prediction. Because of population variability, however, a proportion of patients will inevitably experience more severe toxicity at doses selected for general use. Phase III trials routinely report grade 4 myelosuppression rates of up to 80% in certain solid tumors, without a recommendation for a dose adjustment.[1,2] Historically, this risk has been regarded as acceptable, compared with the greater risk of treating the greater part of the population with ineffective doses. Approaches to individualizing therapy have been sought through pharmacokinetic analyses, but recommendations to the community have never been practical.

In recent years, the potential of pharmacogenetic analyses to improve the therapeutic index of cancer therapy in pediatric malignancies has been described.[3,4] For example, thiopurines are subject to variable metabolic disposition through single nucleotide polymorphisms (mutations in a gene sequence that have a prevalence of at least 1%). Clinical studies have shown that identification of the variant population has the potential to ameliorate toxicity while enhancing therapeutic outcome.[5,6] Similar genetic signatures have long been sought in adult solid tumors. In colorectal cancer, several candidate genes have been identified that have the potential to determine risk of toxicity and possibly efficacy of fluoropyrimidines and oxaliplatin.[7-11]

Such approaches have two distinct goals: to minimize toxicity and to maximize the effectiveness of therapy. In 2005, the US Food and Drug Administration (FDA) took two actions that may be perceived as an advance regarding how pharmacogenetic approaches might permit us to reduce the risk of chemotherapy. First, it was determined that a fraction of the population at higher risk for adverse effects associated with the use of standard doses of irinotecan can be identified prospectively. These are patients who, by virtue of a genetic polymorphism, have a lower than normal capacity to metabolize SN-38, the active metabolite of irinotecan. The polymorphism is found in the gene encoding uridine diphosphate glucuronosyltransferase (UGT) 1A1, which facilitates the excretion of SN-38. This risk was emphasized by a warning added to the package insert of irinotecan. The text added to the label states that "individuals who are homozygous for the *UGT1A1*28* allele are at increased risk for neutropenia following initiation of CAMPTOSAR treatment. A reduced initial dose should be considered for patients known to be homozygous for the *UGT1A1*28* allele."[12]

Second, the FDA approved a test to identify these individuals. The genetic test (Invader *UGT1A1* Molecular Assay; Third Wave Technologies Inc, Madison, WI), conducted on genomic DNA isolated from peripheral blood, identifies patients homozygous for the *UGT1A1*28* allele. Such patients clear irinotecan and its metabolites more slowly than the rest of the population, and so have greater exposure to active drug after a standard dose. The FDA-approved label for the test states that "a reduced initial dose [of irinotecan] should be considered for patients known to be homozygous for the *UGT1A1*28* allele."[13] What are practicing oncologists to do with this information?

IRINOTECAN: A KEY DRUG IN COLORECTAL CANCER TREATMENT

The topoisomerase I interactive drug irinotecan was introduced into clinical studies in the early 1990s, and was found to have activity in several malignancies for which limited treatment options existed, most prominently colorectal cancer.[14] Added to fluorouracil (FU), irinotecan resulted in the first major advance in many years in the treatment of colorectal cancer, and the combined administration of these drugs became the standard of care.[15,16] Although studies in the adjuvant treatment of colon cancer have been disappointing,[17] irinotecan remains a valuable agent in managing patients with advanced disease.

The toxicity of irinotecan in combination has been a concern: early studies identified dose-limiting diarrhea and neutropenia as the major adverse effects, and an aggressive preventive regimen of antidiarrheal and infection evolved. Both of these toxicities are of concern, given that severe neutropenia occurs most often in the context of accompanying diarrhea.[18] An analysis of two studies of irinotecan with bolus administration of FU revealed an unacceptable early death rate, attributed largely to the combined occurrence of these two adverse effects.[19] Therefore, it has been recommended that infusional FU be used in the FU/irinotecan regimen, or that doses be reduced, although the impact of the dose reduction on treatment efficacy in

Journal of Clinical Oncology, Vol 24, No 28 (October 1), 2006: pp 4534-4538
DOI: 10.1200/JCO.2006.07.3031

colorectal cancer has not been assessed.[16] With either approach, the variability in toxicity has remained a concern.

Pharmacology of Irinotecan Associates with Variability

The basis of variable toxicity was evident from the initial trials of irinotecan. As with most drugs, those patients with the greatest exposure to the drug were at the highest risk of toxicity.[19] The pharmacology of this class of drugs, however, is complicated. Irinotecan is a prodrug, converted to its active metabolite SN-38 by carboxylesterases, which are distributed ubiquitously in the tissues.[20,21] Furthermore, both irinotecan and SN-38 are lactones that exist in a pH-dependent equilibrium with their ring-opened carboxylate forms; only the lactone is active. Given that plasma pH varies in a narrow range, most inferences regarding exposure to irinotecan or SN-38 are based on total plasma drug measurements. Excretion of the drug and its metabolites primarily is hepatic, with renal excretion playing a minor role.[22] As with many drugs that undergo hepatic disposition, the excretion of SN-38 is facilitated by glucuronidation, a process catalyzed by the *UGT1A* family of phase II enzymes. In addition, SN-38 is subject to oxidation by the cytochrome P450 family.[22] Ratain described the pharmacodynamic importance of glucuronidation by relating glucuronide formation to risk of toxicity.[23,24] A relationship could be shown with both diarrhea and myelosuppression when irinotecan was administered as a single agent. Hence glucuronidation by *UGT1A* enzymes was investigated further as a marker of the variability in irinotecan toxicity.

Differences in Disposition: *UGT1A1*

Like the cytochromes P450 and other enzyme families, the purpose of which is the detoxication of xenobiotics, this family of enzymes achieves broad substrate specificity through variation in the substrate-binding domain. Thus the *UGT1A* family of enzymes is represented in the genome by a series of four invariant exons, the transcribed product of which may be spliced to any one of nine exons representing different substrate-binding domains. The family members are thus designated *UGT1A1*, *1A2*, and so on, in an agreed-on terminology. By virtue of this structural variability, *UGT1A1* is the isoform with the greatest affinity for SN-38, and so is the most important catalyst in its metabolism; recent work also implicates both *UGT1A7* and *UGT1A9* in the process.[25]

UGT1A1 is the enzyme primarily responsible for bilirubin glucuronidation. Lower than normal activity is a feature of Gilbert's syndrome, the genetic basis of which has been elucidated. It has been determined that UGT enzyme levels are regulated primarily through transcriptional control, and that variation in promoter structure influences the rate of transcription. In particular, a series of TA repeats in the proximal promoter vary from five to eight in length: the lower the number of repeats, the more efficient the transcriptional activity of the gene. The commonest alleles are those with six and seven repeats. Gilbert's syndrome is most commonly associated with homozygous presence of the TA_7 allele (which is also classified as *UGT1A1*28*).[26,27] The frequency of alleles of these repeats varies by ethnic and racial origin: in a white population, approximately 50% are [TA_6/TA_6], 40% are [TA_6/TA_7], and 10% are [TA_7/TA_7] genotypes. The proportion of [TA_7/TA_7] genotypes is also approximately 10% in individuals of African origin, but less than half that in Asians.[28] Other polymorphisms in the *UGT1A1* gene have been shown to influence functional activity and to associate with Gilbert's syndrome or drug toxicity. Missense polymorphisms in exon 1 and in the shared exons 2 to 5 have been described. Of particular importance to East Asian populations is a mutation in exon 1 (a G to A transition termed *UGT1A1*6*) with an allele frequency of approximately 12%, which reduces catalytic function by 60% in homozygotes.[29] The impact of this polymorphism on irinotecan toxicity did not emerge in one study,[30] but was strikingly associated with greater toxicity in a population of Korean patients treated with irinotecan and cisplatin for advanced non–small-cell lung cancer.[31]

UGT1A1 Genotype and Irinotecan Toxicity

Iyer et al[32] first demonstrated that irinotecan disposition might be genetically determined, as shown in liver microsomes, and that *UGT1A1* was responsible for irinotecan glucuronidation. Case reports of toxicity in colorectal cancer patients with Gilbert's syndrome and with *UGT1A1*28* homozygosity[33,34] prompted more extensive investigation. A series of publications then demonstrated evidence that this genotype may be an important influence on the toxicity of irinotecan (Table 1). The analyses in patients treated with single-agent irinotecan (on a schedule of administration once every 3 weeks) demonstrate a relationship of the *UGT1A1*28* genotype to toxicity.[35,36] This relationship is more easily discerned with neutropenia than with diarrhea, which was not common with this regimen. Perhaps such a finding is not unexpected, given that grading of neutropenia through a documented blood count may be more objective than that of diarrhea, which is a retrospective recall of stool number. There are three published analyses of the relationship of genotype to toxicity with combinations involving irinotecan. The study by Ando et al[30] merges hematologic and GI toxicity, and showed that the incidence of either is increased in the patients homozygous or heterozygous for the *28 genotype. The presence of additional chemotherapy drugs was an additional significant risk factor, and this is suggested also in the studies of Rouits and Marcuello.[37,38] In sum, the combination studies replicate the findings of the single-agent analyses; there is a clear association of *UGT1A1*28* homozygosity with neutropenia from irinotecan alone or in combination. For diarrhea, there is a trend reaching significance in only one of the studies. Case reports and the combined analysis of Ando et al[30] indicate that the occurrence of both toxicities, known to be a major risk factor for early death in studies of irinotecan plus FU, may be associated with the genotype, although additional characterization of this aspect is needed. Preliminary analyses from a large cooperative group trial (N9741) in which genotyping was performed in 520 patients confirm the association of *28 homozygosity with risk of grade 4 neutropenia for an arm incorporating irinotecan with oxaliplatin ($P = .004$), but not for weekly irinotecan plus FU (IFL; $P = .46$). A higher risk of febrile neutropenia was also associated with *28 homozygosity. No association was found for severity of diarrhea.[39]

Collectively, these observations together provide definitive evidence that the variant *UGT1A1* genotype is associated with toxicity of irinotecan-containing regimens. Equally important, the observations

Table 1. Clinical Studies of *UGT1A1* Genotype Status and Toxicity of Irinotecan-Containing Therapy

Irinotecan Dose and Schedule	Additional Agents	No. of Patients	*UGT1A1*28* Genotype*	*UGT1A1*28* No. of Patients	Neutropenia/Leukopenia Grade 4 No. of Patients	Neutropenia/Leukopenia Grade 4 %	Diarrhea Grade 3/4 No. of Patients	Diarrhea Grade 3/4 %	Reference
Weekly in 65%	Multiple	118	6/6	93		15†			Ando et al[30]
			6/7	18		44†			
			7/7	7		57†			
P					< .001				
300 mg/m² every 3 weeks	None	20	6/6	9	Trend for lower ANC nadir 6/6 *v* 6/7 *v* 7/7		Only in 6/7 and 7/7		Iyer et al[35]
			6/7	7					
			7/7	4					
P					.04		NS		
350 mg/m² every 3 weeks	None	66	6/6	29	0 of 29	0	Only in 6/7 and 7/7		Innocenti et al[36]
			6/7	24	3 of 24	13			
			7/7	6	3 of 6	50			
P					.02		NS		
85 mg/m² weekly (n = 28) or 180 mg/m² every 2 weeks	FU (63% had FOLFIRI)	75	6/6	31	3 of 31	10	4 of 31	13	Rouits et al[37]
			6/7	35	7 of 35	20	7 of 35	20	
			7/7	7	4 of 7	57	2 of 7	29	
P					.001		NS		
180 mg/m² every 2 weeks in 59%	FU or raltitrexed (59% had FOLFIRI)	95			Grade 3 or 4 hematologic toxicity				Marcuello et al[38]
			6/6	40	6 of 40	15	7 of 40	17	
			6/7	45	12 of 45	27	15 of 45	33	
			7/7	10	4 of 10	40	7 of 10	70	
P					NS		.005		

Abbreviations: ANC, absolute neutrophil count; NS, not significant; FU, fluorouracil; FOLFIRI, fluorouracil, leucovorin, and irinotecan.
*6/6 = [TA_6/TA_6] = homozygous for the *UGT1A1*28* allele; 6/7 [TA_6/TA_7] = heterozygous for the *UGT1A1*28* allele; 7/7 = [TA_7/TA_7] = homozygous for the *UGT1A1*28* allele.
†Patients with either toxicity.

prompt several questions that provide directions for future work. What is the basis for the apparent specificity for neutropenia over diarrhea? Does it matter, given that it is the occurrence of both together that is the life-threatening conjunction associated with at least irinotecan/fluororacil/leucovorin (IFL) therapy? Can measurement of *UGT1A1* genotype contribute to safety of irinotecan combinations? If so, how can this best be established? Can consideration of haplotypes (genetic polymorphisms that are likely to be associated and so vary together in the population) improve the specificity of genetic testing in this context? Such a possibility is suggested in the work of Ando et al,[30] in which association of *UGT1A1*27* was correlated with *28, and those with both had a high risk of toxicity. Consideration of genotypic variation in other key genes mediating drug metabolism in the context of colon cancer chemotherapy should also refine the applicability of genotyping to refine dosing strategies.[7,9,11] However, the most immediate concern is what impact these findings and the FDA actions should have on oncology practice today.

PRACTICAL INTERPRETATION OF THE CURRENT DATA

Should Every Patient Receiving Irinotecan for the First Time Be Considered for Testing?

Yes. A number of limitations in the state of current knowledge must, however, be emphasized. First, as listed in Table 1, not all of the patients with the variant genotype experience life-threatening toxicity with standard-dose irinotecan. However, according to studies of small numbers of patients, some 50% do experience life-threatening toxicity, and more complete studies are needed to provide a more accurate assessment of risk. Second, a normal *UGT1A1* genotype does not ensure lack of toxicity, although the risk clearly is less. Innocenti et al[40] have indeed suggested that we may be underdosing those with the normal genotype and that a reassessment of dosing should be conducted. These two concerns have perhaps been best countered by Wang and Wenshilboum,[3] responding to a criticism that not all myelosuppression was explained by an identified source of thiopurine methyltransferase (TPMT) pharmacogenetic variation: "the fact that TPMT pharmacogenetic testing would allow us to understand, anticipate, and avoid this potentially fatal drug reaction in a subset of patients clearly represents a significant clinical advance." Although the level of understanding of irinotecan pharmacogenomics is substantially less mature than that of the thiopurines, a strong argument can be made that until additional studies provide refinement of the model, *UGT1A1* testing should be considered before the first dose of irinotecan in all patients.

Does the Genotyping Predict for All Toxicities of Irinotecan?

No. It is often pointed out that the predominant toxicity with the schedule of irinotecan administered once every 3 weeks is myelosuppression, and that these results are therefore of questionable relevance for a schedule administered once every 2 weeks, in which diarrhea predominates. The largest study fails to show a significant association between the *UGT1A1*28* homozygous genotype and diarrhea in

patients who received what, is arguably the regimen associated with the highest incidence of diarrhea, IFL.[39] Conversely, the greatest risk from irinotecan toxicity is when diarrhea and neutropenia coexist, a scenario that resulted in an unacceptably high rate of early death with the IFL regimen.[19] Thus, it may be asserted that knowing which patients are at risk for myelosuppression, in itself, is a valuable predictor.

What Dose Should Be Used in UGT1A1*28 Homozygotes?

This has not been established. It is acknowledged that no clear dosing strategy for the variant phenotype has been defined. Concerns regarding undertreatment are appropriate and must be addressed prospectively. With current knowledge, it is clear that patients with this phenotype should receive less drug, but how much less? On the basis of a consideration of SN-38 area under the concentration-time curve, Innocenti et al[40] have recommended a 20% dose reduction. Until more data are available, this seems a reasonable approach. The concern regarding underdosing may be further allayed by a dose escalation to full doses in the second course, in the event of little or no toxicity at the reduced level.

What Additional Studies Need to Be Accomplished?

The limitations described, all of which are valid, indicate the need to better characterize the genotype-phenotype relationships, and their implications for specific therapies. Nonetheless, for immediate practical purposes, those limitations seem to us outweighed by the recognition of a subset of patients at greater risk of toxicity from noncurative therapy, and the availability of a test that can identify them. The test is increasingly available in the pathology departments of major institutions, but our experience has been that not all insurance carriers will agree to support its cost. This coverage issue needs to be resolved.

Although safety is a paramount priority in the management of incurable disease, it is not the only priority. Patients and oncologists have legitimate concerns that dose modifications might be required only in a subset of patients with *UGT1A1*28* genotype. The oncology community as a whole should commit the necessary resources to conduct a hypothesis-driven study of patients who are representative of all three genotypes. This trial would extend the investigation of genotype to *UGT1A1* haplotypes, and to genotypes involving other key determinants of toxicity and/or efficacy. It should establish appropriate dose modification strategies for irinotecan-containing regimens in patients of various genotypes. It should also incorporate molecular correlative studies to analyze simultaneously the pharmacogenomics of response in both tumor and normal tissue—a necessary step to advance the realization of individualized therapy.

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Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Authors	Employment	Leadership	Consultant	Stock	Honoraria	Research Funds	Testimony	Other
Peter J. O'Dwyer						Pfizer (C)		
Dollar Amount Codes (A) < $10,000 (B) $10,000-99,999 (C) ≥ $100,000 (N/R) Not Required								

Author Contributions

Conception and design: Peter J. O'Dwyer
Data analysis and interpretation: Peter J. O'Dwyer, Robert B. Catalano
Manuscript writing: Peter J. O'Dwyer, Robert B. Catalano
Final approval of manuscript: Peter J. O'Dwyer

Can *UGT1A1* genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan? An evidence-based review

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Disclaimer: *The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.*

This evidence-based review addresses the question of whether testing for *UGT1A1* mutations in patients with metastatic colorectal cancer treated with irinotecan leads to improvement in outcomes (e.g., irinotecan toxicity, response to treatment, morbidity, and mortality), when compared with no testing. No studies were identified that addressed this question directly. The quality of evidence on the analytic validity of current *UGT1A1* genetic testing methods is adequate (scale: convincing, adequate, inadequate), with available data indicating that both analytic sensitivity and specificity for the common genotypes are high. For clinical validity, the quality of evidence is adequate for studies reporting concentration of the active form of irinotecan (SN-38), presence of severe diarrhea, and presence of severe neutropenia stratified by *UGT1A1* common genotypes. The strongest association for a clinical endpoint is for severe neutropenia. Patients homozygous for the **28* allele are 3.5 times more likely to develop severe neutropenia compared with individuals with the wild genotype (risk ratio 3.51; 95% confidence interval 2.03–6.07). The proposed clinical utility of *UGT1A1* genotyping would be derived from a reduction in drug-related adverse reactions (benefits) while at the same time avoiding declines in tumor response rate and increases in morbidity/mortality (harms). At least three treatment options for reducing this increased risk have been suggested: modification of the irinotecan regime (e.g., reduce initial dose), use of other drugs, and/or pretreatment with colony-stimulating factors. However, we found no prospective studies that examined these options, particularly whether a reduced dose of irinotecan results in a reduced rate of adverse drug events. This is a major gap in knowledge. Although the quality of evidence on clinical utility is inadequate, two of three reviewed studies (and one published since our initial selection of studies for review) found that individuals homozygous for the **28* allele had improved survival. Three reviewed studies found statistically significant higher tumor response rates among individuals homozygous for the **28* allele. We found little or no direct evidence to assess the benefits and harms of modifying irinotecan regimens for patients with colorectal cancer based on their *UGT1A1* genotype; however, results of our preliminary modeling of prevalence, acceptance, and effectiveness indicate that reducing the dose would need to be highly effective to have benefits outweigh harms. An alternative is to increase irinotecan dose among wild-type individuals to improve tumor response with minimal increases in adverse drug events. Given the large number of colorectal cancer cases diagnosed each year, a randomized controlled trial of the effects of irinotecan dose modifications in patients with colorectal cancer based on their *UGT1A1* genotype is feasible and could clarify the tradeoffs between possible reductions in severe neutropenia and improved tumor response and/or survival in patients with various *UGT1A1* genotypes. ***Genet Med* 2009:11(1):21–34.**

Key Words: *colorectal cancer, UGT1A1*, pharmacogenetics, systematic review

INTRODUCTION

Medical disorder and treatment

Colorectal cancer (CRC) is the third leading cause of new cancer in the United States, with about 150,000 new cases per year. More than 55,000 deaths from CRC were expected in 2006. At least 15% of individuals with new CRC cancers (20,000–25,000) might be candidates for irinotecan therapy.[1–3] For the 70–80% of patients who present with "apparently resectable localized disease," optimal treatment is usually considered to be surgery followed by adjuvant therapy for high-risk cases.[3–6] CRC patients with advanced disease at diagnosis may receive first-line systemic chemotherapy or chemotherapy and radiation therapy, either followed by surgery or used palliatively if surgery is not indicated.[5] Fluorouracil continues to be the first choice of drugs for use in chemotherapy and may be used in combination with leucovorin.[4,6,7] However, other combination chemotherapy regimens involving the use of irinotecan and other drugs seem to improve the median survival over fluorouracil and leucovorin and are increasingly being prescribed for first line and sequential therapy for patients with metastatic CRC.[4,8,9]

UGT enzymes and metabolism of irinotecan

Irinotecan is a topoisomerase I inhibitor that interrupts DNA replication in cancer cells, resulting in cell death.[10–12] The irinotecan prodrug is activated by the enzyme carboxylesterase

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Disclosure: The authors declare no conflicts of interest.

Submitted for publication July 28, 2008.

Accepted for publication September 23, 2008.

DOI: 10.1097/GIM.0b013e31818efd77

to the active metabolite SN-38, which is 100–1000 times more cytotoxic than the parent drug.[10] SN-38 is further catalyzed into an inactive glucuronide derivative, SN-38G by several hepatic and extrahepatic UGT enzymes. The major isozyme involved in this catalyzation is UGT1A1, but others (UGT1A 6, 7, 9, and 10) also have some role.[13] A decrease in the level of functional UGT1A1 enzyme reduces a person's ability to metabolize SN-38 to an inactive form, and low UGT1A1 enzyme levels have been associated with a higher risk for adverse reactions caused by relatively high levels of and/or prolonged exposure to the active form of the drug.[6,14] Based on available tests and the proposed clinical scenario, the UGT1A1 enzyme was the focus of the evidence review with the associated key questions contained in Table 1.

Testing for *UGT1A1* variants

The *UGT1A* gene family includes nine protein coding genes and four pseudogenes, and encodes 13 different isoforms of the UGT1A enzyme (UGT1A1 through UGT1A13p). The isoforms result from alternative splicing of promoters and regions encoding substrate binding domains (multiple exon 1 sites) to common exons 2–5 (Fig. 1).[12,15–20] At least 63 *UGT1A1* variants have been described, including single base pair changes, frame shift mutations, insertions, and deletions in the promoter region, five exons and two introns of the gene.[21] Most are associated with absent, reduced, or inactive enzyme; one is associated with an increased enzyme level, and the effects of some are unknown.

This review focuses on the more commonly tested mutations (Table 2). The first is a two-base pair insertion (TA) in the TATA box in the promoter region of the gene.[22] The result of this mutation is that the $(TA)_6TAA$ sequence, found in the promoter of the wild genotype *UGT1A1*1* allele, becomes $(TA)_7TAA$; this variant is designated as *UGT1A1*28*. The $(TA)_5TAA$ (*UGT1A1*36*) and $(TA)_8TAA$ (*UGT1A1*37*) variants are also described (Table 2), but they are less common and less routinely tested. Other mutations include polymorphisms in exon 1, c.211G>A (*UGT1A1*6*), and g.686C>A (*UGT1A1*27*) (Table 2).

Because information on additional functional polymorphisms in the promoter (e.g., −3279T>G; *UGT1A1*60*) and coding regions (e.g., 1456T>G; *UGT1A1*7*) of the enzyme were limited at the time of the initial review, we did not include studies of these polymorphisms in the analysis.[23–26] However, these studies and others published since the initial review,[26–28] have shown that some polymorphisms are relatively common in specific racial/ethnic groups (e.g., Asians) and may influence metabolism of irinotecan.

Table 1 Key questions relating to the analytic framework

1. Does testing for *UGT1A1* mutations in patients with metastatic CRC treated with irinotecan lead to improvement in outcomes (e.g., irinotecan toxicity, response to treatment, morbidity and mortality) compared with no testing? (Overarching question)
2. What is the analytic validity of the test(s) that identify key *UGT1A1* mutations?
3. What is the clinical validity of *UGT1A1* testing?
   a. How well does *UGT1A1* testing predict phenotypic markers (e.g., increased plasma SN-38 levels or decreased enzyme activity) and associated adverse drug reactions (e.g., diarrhea or neutropenia)?
   b. How well does *UGT1A1* testing in patients with metastatic CRC predict morbidity and mortality?
   c. Do other factors (e.g., race/ethnicity, other medications) independently affect clinical validity?
4. What are the benefits and harms (clinical utility) related to *UGT1A1* testing for patients with metastatic CRC treated with irinotecan?
   a. Based on *UGT1A1* test results, what are the management options for patients?
   b. Do these options improve patient outcomes or management decisions by patients or providers?

Clinical scenario

As noted, the *UGT1A1 *28* allele is associated with reduced levels of enzyme. Therefore, individuals with the wild genotype sequence (**1/*1*) who have average levels of the enzyme will metabolize SN-38 more quickly than those who are either heterozygous (**1/*28*) or homozygous (**28/*28*) for this allele. Higher or more prolonged exposure to the active form of irinotecan is thought to explain many of the adverse drug events associated with irinotecan use, including severe neutropenia and severe diarrhea. Thus, if irinotecan dosage can be modified on the basis of patients' *UGT1A1* genotype, some proportion of these adverse events might be avoided. However, a reduction in dosage might also be associated with reduced tumor response and/or increased morbidity.

In 2004, a change to the prescribing information[29,30] in the Camptosar (irinotecan) Injection Package Insert was announced through an Food and Drug Administration (FDA) Center for Drug Evaluation and Research email alert (NDA 20-571/S-024/S-027/S-028), which stated that:

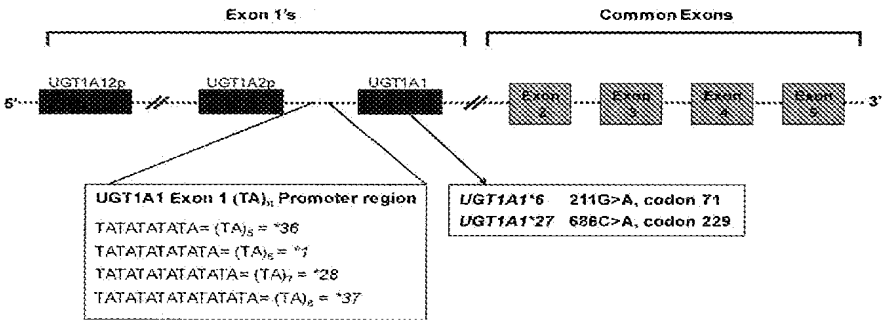

Fig. 1. Schematic of the partial *UGT1A1* gene showing locations of the polymorphisms of interest for this review in the exon 1 promotor region and in exon 1. First exons are alternatively spliced to common exons to produce UGT isoforms. Adapted from *Clin Pharmacol Ther.* 2004;75:495–500 and *Oncology (Williston Park).* 2003;17:52–55.[17,20]

Table 2 *UGT1A1* allele naming conventions, locations, and associated phenotypes

UGT1A1 allele[a]	Variant	Location	Enzyme activity	Associated phenotype
*UGT1A1*1*	$(TA)_6TAA$	Promoter	Normal	Wild type
*UGT1A1*28*	$(TA)_7TAA$	Promoter	Reduced	Gilbert syndrome
*UGT1A1*36*	$(TA)_5TAA$	Promoter	Increased	
*UGT1A1*37*	$(TA)_8TAA$	Promoter	Reduced	Crigler-Najjar, type II
*UGT1A1*6*	c.211G>A; G71R	Exon 1	Reduced	Gilbert syndrome
*UGT1A1*27*	g.686C>A; P229Q	Exon 1	Reduced	Gilbert syndrome

[a]Allele frequencies, stratified by race, are shown in Table 4.

> ". . . a reduction in the starting dose by at least one level should be considered for patients known to be homozygous for the *UGT1A1*28* allele . . . . However, the precise dose reduction in this patient population is not known and subsequent dose modifications should be considered based on individual patient tolerance to treatment."
>
> "Individuals who are homozygous for the *UGT1A1*28* allele are at increased risk for neutropenia following initiation of Camptosar treatment. A reduced initial dose should be considered . . . . Heterozygous patients . . . may be at increased risk for neutropenia; however, clinical results have been variable and such patients have been shown to tolerate normal starting doses."

Subsequently, in August 2005, the Invader® *UGT1A1* Molecular Assay (Third Wave Technologies, Inc., Madison, WI) was cleared by the US FDA Center for Devices and Radiologic Health under 510(k) rules for Drug Metabolizing Enzyme Genotyping Systems.[31,32] The Invader test and other laboratory developed *UGT1A1* tests are currently available from multiple laboratories in the United States and are being marketed to oncologists and pathologists as an aid to clinical decision making.[33,34] In its package insert,[35] Third Wave Technologies, Inc. describes the assay as follows:

> ". . . . . an in vitro diagnostic test for the detection and genotyping of the **1* (TA6) and **28* (TA7) alleles of the UDP glucuronosyltransferase 1A1 (*UGT1A1*) gene in genomic DNA from whole peripheral blood as an aid in the identification of patients with greater risk for decreased UDP-glucuronosyltransferase activity."

METHODS

The purpose of this article is to provide a summary and extend the findings of a more formal evidence report (see Resources section). These evidence reports are to be used by the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group (EWG) to inform the development of formal recommendations for clinical practice.[36] The methods used to identify, review, evaluate, analyze, and summarize the evidence are detailed in Appendix C of the original evidence report (see Resources section). Investigators at Research Triangle Institute (RTI) International were contracted by the National Office of Public Health Genomics at the Centers for Disease Control and Prevention to conduct the initial stage of the review of this evidence, focusing on clinical validity and utility. RTI staff conducted a MEDLINE literature search (through May 2006) for studies addressing the clinical validity and utility of *UGT1A1* genotyping in metastatic CRC patients treated with irinotecan. Based on key questions and discussion with the *UGT1A1* Technical Expert Panel, a list of article inclusion and exclusion criteria was generated. Articles were also identified by search of the references included in selected articles. RTI reviewed abstracts and selected articles found in the search, abstracted data into evidence tables, assessed the quality of individual articles, and prepared a preliminary report. When the RTI report was submitted, members of the Centers for Disease Control and Prevention-sponsored EGAPP initiative team and EGAPP consultants performed searches for and reviewed articles on the analytic validity of *UGT1A1* genotyping and on *UGT1A1* allele/genotype frequencies, and updated the clinical validity and utility searches through December 2006. They also performed additional summarization and statistical analyses, integrated the component sections, and produced a draft evidence report for consideration by the EWG.

With a focus on the application of study data to specific key questions, EGAPP reviewers assessed the quality of evidence for the evaluation components (i.e., analytic and clinical validity, clinical utility) based on standard criteria, including study design and conduct, consistency and generalizability of data, and appropriateness of statistical analyses. Short summaries were written for all individual studies included for assessment of analytic validity, clinical validity, and clinical utility, and included EGAPP and RTI quality ratings (see Resources section). Feedback was sought throughout the review from the Technical Expert Panel, other technical consultants, and the EWG.[36] In addition, a draft of the evidence report was sent to nine expert peer reviewers. The report was revised in response to comments from the reviewers and resubmitted to the EWG along with a summary of comments and their disposition.

The focus of this report is on patients with metastatic CRC treated with irinotecan. The analytic framework is shown in Figure 2, with the numbers indicating the key questions shown in Table 1. These key questions were developed by the EWG and further refined in discussions with a Technical Expert Panel. Key question 1 is the overarching question: "Does testing for *UGT1A1* mutations in patients with metastatic CRC treated with irinotecan lead to improvement in outcomes (e.g., irinotecan toxicity, response to treatment, morbidity and mortality) compared to no testing?" If direct evidence is insufficient to answer key question 1, key questions 2 through 4 are used to elicit intermediate information to address the overarching question through a "chain of evidence." In reviewing the available evidence, we often used questions from the ACCE (Analytic validity, Clinical validity, Clinical utility, and Ethical, Legal and

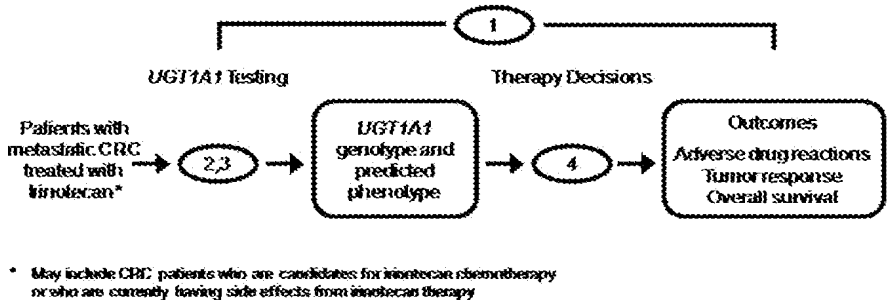

Fig. 2. The analytic framework: testing for *UGT1A1* mutations in patients with metastatic colorectal cancer (CRC) treated with irinotecan (Camptosar). This schematic shows the analytic framework underlying the current review. The numbers indicate the four key questions contained in Table 1.

Social implication) review framework[37] to identify and organize the specific information needed to address the key questions.

RESULTS

Analytic validity

Identification of relevant literature

Through MEDLINE searches, we identified 17 articles that were included in analyses (Appendix C of the original evidence report, see Resources section). Searches of the gray literature (e.g., unpublished reports, web sites, government documents) identified laboratories offering *UGT1A1* testing, as well as a US FDA 510(k) summary and relevant committee reports (see Resources section).

Analytic sensitivity and specificity

Table 3 summarizes the results of four method comparison studies on testing for **28* (TA7) and **1* (TA6) alleles, using sequencing as the referent method.[18,31,38,39] In these studies, genotypes for all of 190 samples homozygous or heterozygous for **28* were correctly identified (estimated analytic sensitivity 100%; 95% confidence interval [CI] 98–100%). The homozygous wild genotype (**1/*1*) was correctly identified in all of 131 samples (estimated analytic specificity 100%; 95% CI 97–100%). A 2007 study (not included in the Table 3 analysis) was very consistent, reporting 100% concordance in 88 samples tested by sequencing and PCR/capillary electrophoresis and the Invader assay (**28/*28*, $N = 13$; **28/*1*, $N = 46$; **1/*1*, $N = 29$).[40] Very little data are available to support estimates of analytic sensitivity for other promoter (**36*, **37*) and exon 1 variants (**6*, **27*).[38]

Test reproducibility and failure rates

Data on the reproducibility of Invader UGT1A1 Molecular Assay results and assay failure rates were reported as part of the FDA 510(k) approval process.[31] Twenty blood samples (six **1/*1* wild genotypes, five **28/*1* heterozygotes, four **28/*28* homozygotes, and five undisclosed genotypes) were each tested five times at three different sites (300 test results). Of the 49 initial "invalid calls" or sample failures, 40 were due to invalid positive or negative control results, and nine to low signal intensity. Failure rates on the first run were 9.3%, 0%, and 7.0% for the three sites. Six samples failed again when retested (6 of 600; 1%, 95% CI 0.4–2.2%). Incorrect results were reported for 11 samples, all from one site, for an overall correct call rate of 98.8% (883/894; 95% CI 97.8–99.4%). Nine of these 11 incorrect results may have been sample mix ups, an example of preanalytic errors that are also expected to occur in clinical practice. If the erroneous results caused by sample mix ups are excluded, the overall correct analytic call rate for the Invader assay was 99.8% (95% CI 99.2–99.9%). In a report of Invader testing for *UGT1A1*1*, **28*, **6*, and **27*, Hasegawa et al.[38] observed failure rates of 6.7% (3 of 45) in tests of**1/*1* homozygotes, 10% (6 of 60) in *UGT1A1*6* testing, and 21.7% (13 of 60) in *UGT1A1*27* testing, but these relatively high-failure

Table 3 Analytic validity of *UGT1A1* testing for genotypes involving the **28* variant

				Analytic		
				Sensitivity[a]		Specificity[a]
Source	*N*	Test method	Referent method	**28/*28*	**28/*1*	**1/*1*
Monaghan[19]	12	Radioactive PCR	Sequencing	4/4	5/5	3/3
Pirulli[40]	40	DHPLC	Sequencing	19/19	8/8	13/13
Hasegawa[39]	60	Invader (RUO)	Sequencing	4/4	11/11	42/42[b]
Invader[32]	212	Invader (IVD)	Sequencing	30/30	109/109	73/73
All	324			57/57	133/133	131/131
Overall estimate (95% confidence interval)					100% (98–100%)	100% (97–100%)

[a]Test result/referent result.
[b]Does not include three sample failures.

Table 4 Consensus *UGT1A1* allele frequencies stratified by race

	Common allele frequencies (95% confidence interval)		Other allele frequencies (95% confidence interval)			
Race	Studies (patients)	**28* (TA7)	**36* (TA5)	**37* (TA8)	**6* (211G>A)	**27* (686C>A)
White	11 (2517)[19,20,58–66,72]	0.334 (0.309–0.361)	0.003 (0.001–0.008)[58,64,65]	0.002 (0.001–0.009)[58,64]	0.005 (0.001–0.03)[60]	0.000[67]
Asian/Asian American[a]	4 (454)[58,64,68,69]	0.139 (0.112–0.171)	0.000 (0.00–0.09)[58,64]	0.000 (0.00–0.09)[58,64]	0.13[b] (0.10–0.17)[67–69]	0.023 (0.014–0.035)[67,69]
African/African American	3 (411)[58,70,71]	0.404 (0.358–0.452)[58,70,71]	0.058 (0.039–0.085)[58,70,71]	0.043 (0.026–0.070)[58,70,71]	0.00[67]	0.00[67]

[a]The estimates in this table were derived from studies that provided genotype frequency data. Two other studies reported only allele (**28, *36, *37*) frequencies in small control groups of healthy Japanese[66] and African Americans/Jamaicans,[66,82] estimates were consistent.
[b]Studies published subsequent to this review reported allele frequencies for the*6 allele among Asian control groups of 0.08 (95% CI 0.06–0.10) and 0.15 (95% CI 0.10–0.21).[28,71]

rates may have been related to low DNA concentrations in the samples tested.

Since we completed this review, a 2007 study compared the failure rates for sequencing, PCR/capillary electrophoresis, and the Invader assay in tests of 119 samples containing **1, *28, *36,* and **37* variants.[40] The study's authors reported first-run failure rates of 5.0% for sequencing and 1.7% for the PCR/capillary electrophoresis method, with all failures resolved by repeat analysis. The Invader assay failed on the first and second runs in 7.6% of samples (9 of 119; three **1/*1*, two **28/*1*, one **28/*28*, and three other).

Limitations

Although most data were collected using the Invader technology, other technologies are being used. In addition, these data mainly focus on the analytic phase of testing and do not include errors in the preanalytic (e.g., sample handling or labeling errors) and postanalytic (e.g., data entry or interpretive/reporting errors) phases.[41,42] A large proportion of the data were reported by early Invader investigators[38,43] or by the manufacturer of the Invader kit as part of its FDA submission.[31] Only two of four studies[31,39] reported that the samples were blinded to those performing the assays, to rule out retesting to get the "right" answer.

Clinical Validity

Identification of relevant literature

Because of the limited literature, the analysis was restricted to nine studies, including several that allowed entry to individuals with tumors other than CRC.[14,44–51] *UGT1A1*1* and **28* account for 98–99% of the *UGT1A1* polymorphisms in the white population and were the focus of most studies identified. Studies on polymorphisms that are more commonly found in other racial/ethnic groups (e.g., *UGT1A1*6* and **27* in Asians) were more limited. In addition, homozygosity for **28* is specified as the primary risk factor in the Camptosar (irinotecan) package insert.[29] Consequently, we chose to limit the review of clinical validity to these common alleles for which testing is broadly available.

UGT1A1 genotypes and SN-38 levels

The metabolism of the prodrug irinotecan and its relationship to *UGT1A1* genotypes has been described earlier. One way to assess the "exposure" to SN-38 is a ratio of the area under the curve (AUC) for SN-38G (the inactive form of irinotecan) to the AUC for SN-38 (the active form). Essentially, this compares the integrated time dose exposure for the inactive form (SN-38G) to that of the active form (SN-38). High values indicate that most exposure is to the inactive form; low values indicate increased exposure to the active form. The results of six published studies[26,45,50,52–54] showed that the AUC ratios were lowest among individuals homozygous for **28* (**28/*28*), intermediate among those heterozygous for **28* (**1/*28*), and highest among those with the wild genotype (**1/*1*). This indicates that the highest relative exposure to the active form of irinotecan, SN-38, occurs among the individuals homozygous for **28* (**28/*28*). The SN-38 to SN-38G AUC ratios should be viewed as an intermediate measure of irinotecan exposure.

A more appropriate measure of exposures would include the irinotecan dose. The biliary index (BI) is the irinotecan AUC times the ratio of the SN-38 to SN38G AUCs. Two studies provided the BI for cancer patients stratified by *UGT1A1* genotype. One studied 71 CRC patients,[50] the other reported on 20 patients with solid tumors, four of whom had CRC.[45,55] Both found a significant and consistent dose response in the BI from the wild type, through the heterozygotes and homozygotes. These data strongly indicate the highest time-weighted exposure to the active form of irinotecan occurs in individuals homozygous for **28* (**28/*28*). There were no apparent differences in these findings between studies in whites and Asians (see Table KQ3.1 in full evidence review, Resources section).

Irinotecan treatment regimens

Studies selected for evaluating clinical validity (and clinical utility) did not use standardized treatment regimens. Table 5 provides a brief description of the treatment regimens used in the studies we evaluated.[14,44–51] In several studies, multiple treatment protocols were evaluated. Because we could not account for the effect of variations in treatment regimens in subsequent analyses, we examined the homogeneity of results to determine whether treatment variations had a significant effect on study results. In other words, if the analysis of a clinical validity measure (e.g., severe neutropenia) was found to be homogeneous within a comparison group (e.g., **28* homozygotes compared with **1/*1* wild genotype), it was assumed that a given treatment regimen did not have a significant impact on that measure.

UGT1A1 genotypes and severe diarrhea

The severity of diarrhea is graded on a subjective scale from 1 (mild) to 4 (severe or life threatening).[56] The overall observed rates of severe diarrhea (Grades 3 and 4) among participants in the six studies selected for analysis[44–48,51] was 24% (95% CI 19–30%). When stratified by *UGT1A1* genotypes, the rates of severe diarrhea were 18% (95% CI 11–28%) among those with the wild genotype, 27% (95% CI 20–36%) among those het-

Table 5 Chemotherapy treatment regimens used in studies selected for analysis

Carlini et al.[51]

- Group 1 (15 patients) received 1000 mg/m^2 Capectabine orally twice daily on days 2–15 of 3-wk cycle with 125 mg/m^2 of irinotecan (90-min IV infusion) on days 1 and 8 of each cycle.
- Group 2 (52 patients) received 900 mg/m^2 Capectabine orally twice daily for the same period with 100 mg/m^2 of irinotecan (90-min IV infusion) on days 1 and 8 of each cycle.

Font et al.[44]

- 70 mg/m^2 of irinotecan (90-min IV infusion) + 25 mg/m^2 docetaxel (30-min IV infusion) on days 1, 8, and 15 followed by a 1-wk rest (28-day cycles).

Innocenti et al.[14]

- 350 mg/m^2 of irinotecan (90-min IV infusion) once every 3 wk.

Iyer et al.[45]

- 300 mg/m^2 of irinotecan (90-min IV infusion) once every 3 wk.

Marcuello et al.[46]

- Regimen A: 350 mg/m^2 of irinotecan (45-min IV infusion) once every 3 wk.
- Regimen B: Regime A + 3 mg/m^2 Tomudex in 15 min IV every cycle.
- Regimen C: 80 mg/m^2 of irinotecan (45-min IV infusion) every wk + 1 dose 2250 mg/m^2 5-FU (48 min continuous infusion) every cycle.
- Regimen D: 180 mg/m^2 of irinotecan (45-min IV infusion) every 2 wk + 5-FU and leucovorin.

Massacesi et al.[47]

- 80 mg/m^2 of irinotecan (30-min IV infusion) on days 1, 8, 15, 22, and 36, 43, 50, and 57 days. 3 mg/m^2 of raltitrexed 2–4 hr later (15-min IV infusion) on days 1, 22, and 45.

Rouits et al.[48]

- IRIFUFOL (28 patients): 85 mg/m^2 of irinotecan (90-min IV infusion) + 1200 mg/m^2 5-FU (7-hr IV infusion) and 100 mg/m^2 bolus L-folinic acid, each wk.
- FOLFIRI (47 patients): 180 mg/m^2 of irinotecan (90-min IV infusion) + 2500 mg/m^2 5-FU (continuous infusion) and 400 mg/m^2 bolus L -folinic acid, biweekly.

Soepenberg et al.[49]

- 70 or 80 mg/m^2 of irinotecan given orally to fasted patients once daily for 5 days.

Toffoli et al.[50]

- Modified FOLFIRI (90% of patients): 180 mg/m^2 of irinotecan (2-hr IV infusion) on day 1 + 400 mg/m^2 of 5-FU bolus followed by 2,400 mg/m^2 of 5-FU (46-hr IV infusion) + 200 mg/m^2 of LV on day 1 every 2 wk.
- FOLFIRI (10% of patients): 180 mg/m^2 of irinotecan (2-hr IV infusion) on day 1 + 400 mg/m^2 bolus of 5-FU followed by 600 mg/m^2 of 5-FU (22-hr IV infusion) on days 1 and 2 + 200 mg/m^2 of LV on days 1 and 2 every 2 wk.

erozygous for **28*, and 27% (95% CI 12–48%) among those homozygous for **28*. The analysis showed the severe diarrhea rates between studies to be homogeneous among participants with the same genotype (Q values of 10.4, 7.1, and 9.5; P values of 0.1, 0.3, and 0.2). Figure 3 shows the corresponding summary risk ratios (RRs) from these studies (with the risk among 154 study participants with the wild genotype serving as the referent category) of 1.40 (95% CI 0.94–2.08) for 155 participants heterozygous for **28*, and 1.63 (95% CI 0.64–4.14) for 41 participants homozygous for **28*. The results were homogeneous within groupings (Q values of 3.0 and 8.5; P values of 0.7 and 0.1, respectively).

UGT1A1 genotypes and severe neutropenia

Neutropenia is a decrease in the number of circulating neutrophils (a type of white blood cell that usually accounts for 50–70% of circulating white blood cells) and can be caused by bone marrow suppression associated with the use of antineoplastic chemotherapy drugs. Because neutrophils help defend against bacterial infections, chronic neutropenia can be life threatening. Neutropenia is graded based on the absolute neutrophil count, or number of neutrophil cells per mm^3 (Grade 1, 1500–1999; Grade 2, 1000–1499; Grade 3, 500–999; and Grade 4, <500 cells/mm^3).[56] The overall observed rate of severe neutropenia (Grades 3 and 4 combined) among participants from the eight studies[14,45–51] selected for analysis was 16% (95% CI 13–19%). When stratified by *UGT1A1* genotypes, the rates were 9.8% (6.8–14%) among those with the wild genotype, 18% (14–23%) among those heterozygous for **28*, and 38% (22–57%) among those homozygous for **28*. The results were homogeneous within genotype (Q values of 4.5, 7.1, and 7.9, and P values of 0.7, 0.4, and 0.2, respectively).

Figure 4 shows the corresponding severe neutropenia RRs from these studies, with the risk among participants with the wild genotype serving as the referent category. The summary RRs were computed using original data and a random effects model. Summary RRs were 1.82 (95% CI 1.16–2.85) for participants heterozygous for **28* (based on samples from 276 heterozygotes and 282 participants with the wild genotype) and 3.51 (95% CI 2.03–6.07) for those homozygous for **28* (based on samples from 57 homozygotes and 263 participants with the wild genotype). The RRs were homogeneous within comparison groups (Q values of 1.2 and 5.2, and P values of 0.9 and 0.5, respectively). Overall, these data provide clear evidence that rates of severe neutropenia differ significantly based on the three major *UGT1A1* genotypes, and that there is a dose response relationship between the number of mutant alleles and rate of severe neutropenia.

Clinical sensitivity and specificity of UGT1A1 genotypes as an indicator of risk for severe neutropenia

Table 6 shows the clinical sensitivity and specificity for severe neutropenia of *UGT1A1* genotyping among participants from the eight studies included in the analysis.[14,45,47–51] A positive *UGT1A1* test is defined as an individual homozygous for the **28* allele (**28/*28*), and the outcome of interest is severe (Grade 3 or 4) neutropenia. We defined clinical sensitivity as the proportion of individuals with severe neutropenia who were homozygous for **28*, and clinical specificity as the proportion of individuals without severe neutropenia who were not homozygous for **28*. We estimated (based on a random effects model) that the tests in these studies had an overall clinical sensitivity of 23% (95% CI 15–34%) and an overall clinical specificity of 92% (95% CI 90–94%).

It is also possible to compute the expected clinical sensitivity and specificity from parameters obtained earlier in this review as shown by the flowchart in Figure 5. The chart shows how a theoretical population of 20,000 white metastatic CRC patients initiating irinotecan therapy is first stratified by *UGT1A1* genotypes on the basis of the consensus estimate of the **28* allele frequency (0.334 from Table 4) and the Hardy-Weinberg prin-

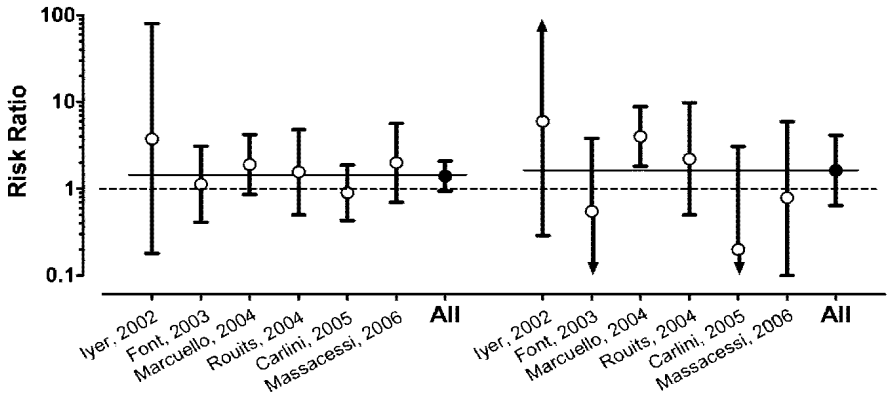

Fig. 3. Risk ratios for severe diarrhea among cancer patients treated with irinotecan by *UGT1A1* genotype from six published studies.[44–48,51] The studies are listed on the *x*-axis, sorted by the risk ratio comparing rates in heterozygotes (**1/*28*) to wild-type individuals (**1/*1*) on the left-hand side. The risk ratios for homozygotes (**28/*28*) versus wild type is the right-hand side. Bars indicate the 95% confidence interval (CI) with the consensus estimate (All) for the two comparison groups. The dotted line indicates a risk ratio of 1.00 (no difference). The two thin solid lines indicate the consensus estimates for the two groups of 1.40 (95% CI 0.94–2.08) and 1.63 (95% CI 0.64–4.14), respectively.

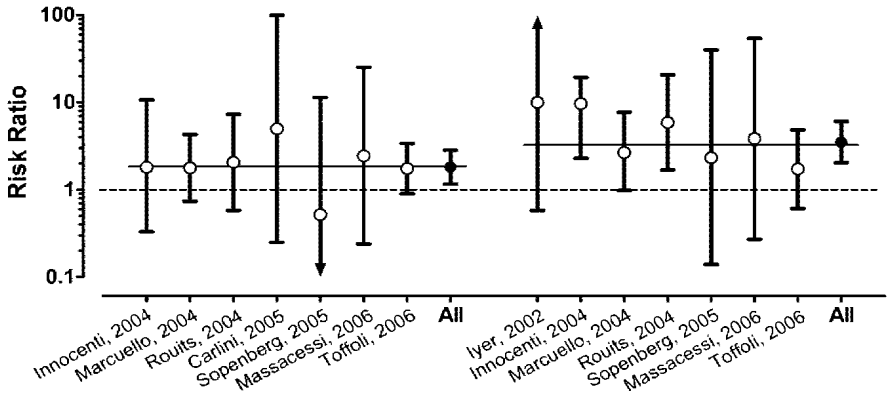

Fig. 4. Risk ratios for severe neutropenia by *UGT1A1* genotype from eight published studies.[14,45–51] The studies are listed on the *x*-axis, stratified by heterozygote individuals (*1/*28) versus wild type (*1/*1) on the left-hand side and homozygote individuals (*28/*28) versus wild type on the right-hand side. Two results (Iyer 2002 for heterozygotes, Carlini 2005 for homozygotes) are not shown as the risk ratio could not be computed due to no observations in one or more groups. The bars indicate the 95% confidence interval (CI) with the consensus estimate (All) for the two comparison groups. The dotted line indicates a risk ratio of 1.00 (no difference). The two thin solid lines indicate the consensus estimates for the two groups of 1.82 (95% CI 1.16–2.85) and 3.51 (95% CI 2.03–6.07), respectively.

ciple. Among the 8871 patients with the wild genotype, the baseline rate of severe neutropenia (9.8%) would result in 869 of them experiencing this adverse drug reaction. Using the RRs of 1.82 and 3.51, the number of adverse reactions in the individuals heterozygous and homozygous for **28* can also be computed. The expected clinical sensitivity and specificity of 24% and 91%, respectively, agree closely with the consensus rates of 23% and 92% computed from published observations (Table 6). The corresponding positive predictive value derived from Figure 5 is 52% (767/1464) and the negative predictive value is 86% (1 − (869 + 1603)/(8871 + 8898)).

Limitations

Nearly all of the clinical validity information was collected from populations consisting of non-Hispanic whites. When studies did include people of other racial/ethnic groups, the results were not stratified by race/ethnicity. Treatment protocols (including irinotecan dosage, method of delivery, and frequency of treatment) varied widely both within- and between-studies, and these variations could affect both the overall rate of adverse events and the RRs for specific adverse events. Although we did not find strong evidence of such an impact, most studies contained too few subjects to be confident. Some studies reported adverse events after the first cycle of treatment, others after the completion of treatment, and still others provided both. Although one study showed that adverse events among patients homozygous for the **28* allele most often occurred in the first cycle of treatment,[50] not enough studies provided clinical outcomes at both times to allow for a meaningful subanalysis by treatment time. Several studies included patients who had cancer at sites other than the colon. However, we could not determine the impact of including these studies, because none of the studies stratified their results by cancer site. Lastly, several studies included patients with less common genotypes. However, because these patients were always included in larger groupings, it was not possible to combine results for patients with these genotypes across studies.

Table 6 Clinical sensitivity and specificity of *UGT1A1* genotyping for severe neutropenia

Study	True positive	False negative	True negative	False positive	Sensitivity (%)	Specificity (%)
Carlini 2005[51]	0	2	59	5	0	92
Innocenti 2004[44]	4	5	48	2	44	96
Iyer 2002[45]	2	0	18	2	100	90
Marcuello 2004[46]	4	18	73	6	18	92
Massacesi 2006[47]	1	3	52	6	25	90
Rouits 2004[48]	4	10	59	3	29	95
Soepenberg 2005[49]	0	1	21	1	0	95
Toffoli 2006[50]	4	33	195	18	11	92
All[a] (95% confidence interval)					23 (15–34)	92 (90–94)

[a]Using a random effects model.

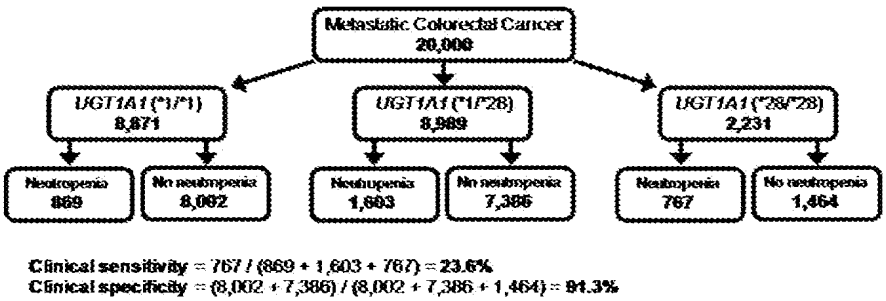

Fig. 5. Flow diagram showing the derivation of clinical sensitivity and specificity of *UGT1A1* genotyping to identify severe neutropenia in a hypothetical cohort of 20,000 white individuals with metastatic colorectal cancer. The clinical sensitivity and specificity are derived using previously reported parameters (e.g., allele frequency, risk ratios), stratified by *UGT1A1* genotype. Overall, the clinical sensitivity is 24% with a specificity of 91% (false positive rate of 9%).

UGT1A1 allele frequencies by racial group

The pharmacokinetics of irinotecan does not seem to differ based on gender or race. However, clinically relevant *UGT1A1* alleles and genotype frequencies do differ by race/ethnicity. Table 4 shows estimated *UGT1A1* *28, *36, *37, *6, and *27 allele frequencies among people of white, Asian, and African descent. Estimates of allele frequencies for the *28 allele are based on 11 studies in white populations,[18,19,57–66] four studies in mixed Asian and Asian American populations,[58,63,67,68] and three studies in African and African American populations.[57,69,70] Fewer studies reported on allele frequencies of the less common alleles (*36 and *37 promoter alleles; *6 and *27 polymorphisms) in whites,[57,59,63,64,66] Asians[28,57,63,66–68,71] and people of African descent.[57,66,69,70] Differences between allele frequencies by racial groups are clear, including statistically higher frequencies of *36 (TA5) in African/African American populations,[57,69,70] and of *6 in Asian populations.[28,66–68]

UGT1A1 genotypes and tumor response

Three studies provided information on tumor response, stratified by *UGT1A1* genotype.[44,50,51] One study[44] found a higher rate of stable or partially responsive tumors among *28 heterozygotes and homozygotes combined than among patients with the wild genotype (RR of 1.6; 95% CI 0.8–3.0). The two other studies[50,51] defined a responsive tumor as "partial or complete response" and provided sufficient data to examine response rates by *UGT1A1* genotype. Summary results from the two other studies[50,51] showed tumor response rates of 41% (95% CI 33–40%) among patients with the wild genotype, 47% (95% CI 33–63%) among *28 heterozygotes, and 70% (95% CI 40–84%) among *28 homozygotes. The results were homogeneous within genotype (Q values of 0.2, 2.2, and 0.6, and P values of 0.6, 0.1, and 0.4, respectively). Figure 6 shows an analysis of the tumor response rate (as defined in the studies) versus *UGT1A1* genotype, with individuals having the wild genotype used as the referent category.[50,51] Overall, the *28 heterozygotes had a nonsignificantly higher response rate (RR, 1.09; 95% CI 0.83–1.43), and *28 homozygotes had a significantly higher response rate (RR, 1.70; 95% CI 1.24–2.33; $P = < 0.001$). The studies were homogeneous within genotype (Q values of 0.4 and 0.8, and P values of 0.6 and 0.8, respectively).

UGT1A1 genotypes and mortality

Two of the three studies providing information on tumor response among patients treated with irinotecan also provided some information about mortality.[44,50] The other study[46] provided information only on survival. It was not possible to combine the information from these three studies in a formal analysis. Instead, the findings are summarized in Table 7. The data from Font et al.[44] are for combined heterozygotes and homozygotes, and all patients had lung cancer. The data from Toffoli et al.[50] compare homozygotes (and heterozygotes) with the wild type, and all patients had CRC. The data from Marcuello et al.[46] include 95 patients with CRC and represent combined heterozygotes and homozygotes. None of the differences were statistically signif-

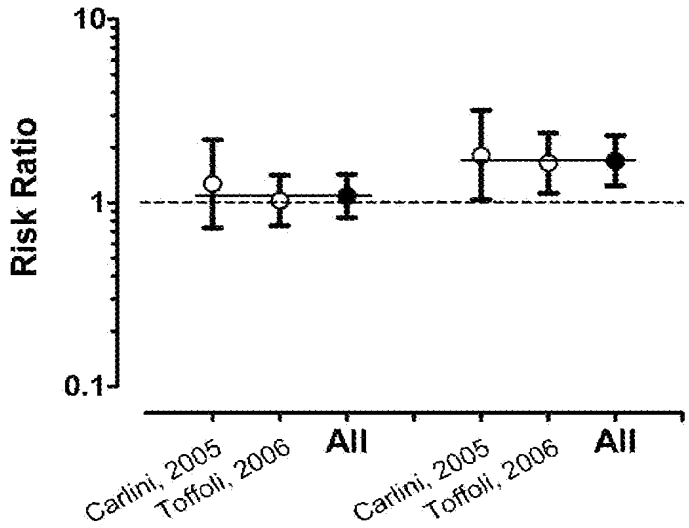

Fig. 6. Risk ratios for tumor response by *UGT1A1* genotype from two published studies.[50,51] The studies are listed on the *x*-axis, with the risk ratios for heterozygote individuals (**1/*28*) versus wild type (**1/*1*) on the left hand side, and the risk ratios for homozygote individuals (**28/*28*) versus wild type on the right hand side. The bars indicate the 95% confidence interval (CI) with the consensus estimate (All) for the two comparison groups. The dotted line indicates a risk ratio of 1.00 (no difference). The two thin solid lines indicate the consensus estimates for the two groups of 1.09 (95% CI 0.83–1.43) and 1.70 (95% CI 1.24–2.33), respectively.

Table 7 Mortality-related outcome data from studies of cancer patients treated with irinotecan, stratified by *UGT1A1* genotype

Study	Outcome measure	Finding
Font et al.[44]	Time to progression	3 mo (*1/*1) vs. 4 mo (other[a])
	Median survival	8 mo (*1/*1) vs. 11 mo (other)
	1 yr survival	21% (*1/*1) vs. 41% (other)
	2 yr survival	14% (*1/*1) vs. 31% (other)
Marcuello et al.[46]	Median survival	32 mo (*1/*1) vs. 24 mo (other[a])
Toffoli et al.[50]	Hazard ratio	0.81 (95% CI 0.45–1.44) (*28/*28 vs. *1/*1)
	Hazard ratio	0.84 (95% CI 0.58–1.21) (*1/*28 vs. *1/*1)
	Median survival	613 days (*1/*1) vs. 686 days (*28/*28)
	Median survival	613 days (*1/*1) vs. 669 days (*1/*28)

[a]Other includes both heterozygotes (**1/*28*) and homozygotes (**28/*28*).

icant. Findings from two studies[44,50] were in the direction of improved survival for **28* homozygotes versus nonwild genotype patients, whereas the third reported a survival advantage for the wild genotype individuals.[46]

Clinical Utility

Options for modifying patient care

There is insufficient information for the less common genotypes to provide clear options for patient management. For CRC patients who have the common **28* polymorphism, the three main options for modifying patient care have been summarized and discussed.[72]

- *Modify the irinotecan regimen*: The Camptosar (irinotecan) package insert provides suggested modified (reduced) dose levels (mg/m^2) for two single-drug regimens of Camptosar (125 mg/m^2 weekly and 350 mg/m^2 every 3 weeks).[29] It states that a reduction by one dose level may be considered for patients 65 years or older, those having low performance status, or those with increased bilirubin levels; reduction in starting dose by at least one level "should be considered for patients known to be homozygous for the *UGT1A1*28* allele." However, the package insert also notes that "the appropriate dose reduction in this patient population is not known."
- *Use other drugs*: Newer drugs (e.g., cetuximab, bevacizumab) can be substituted in a variety of regimens that vary the combination of drugs, as well as the doses, schedules, and duration of infusion for each drug.
- *Treat patients with colony-stimulating factors before the first cycle of chemotherapy to prevent the occurrence of febrile neutropenia:* Such treatments, which cost 2–3000 dollars per dose, are currently recommended by the National Comprehensive Cancer Network for patients with a 20% or greater risk of febrile neutropenia.[73] Although **28* homozygous patients have a 36% risk of severe neutropenia, the proportion associated with fever is unknown. This pretreatment and monitoring of white cell counts might be an acceptable alternate indicator of acceptable dosing.

Additionally, treatment options need to be placed in the context of overall care. The choice of treatment for CRC patients should also reflect the level of risk for various adverse effects that they consider to be acceptable. Thus, the *UGT1A1* test may be useful to identify **28* homozygous patients who may prefer a treatment with low risk of toxicity even if it may not be as effective in fighting their cancer, whereas the testing may not be as useful for those seeking aggressive therapy and willing to accept risk of higher toxicity. Decisions about testing may also be based on the specific planned regimen and dosing. McLeod[74] has proposed that, unless patients receive irinotecan at a dose >150 mg/m^2 (either alone or in combination with a myelotoxic drug) or irinotecan >100 mg/m^2 in combination with another marrow-toxic agent (e.g., oxaliplatin), their increased risk for toxicity is "neither statistically nor clinically significant" and testing may not be warranted.

Will reduction in the irinotecan dose reduce patients' risk of having a severe drug-related adverse event?

Based on the clinical validity and additional information on the pharmacokinetics of irinotecan, it is biologically plausible that a reduced initial dose in **28* homozygous patients could result in a reduction in severe neutropenia. However, no studies (with or without randomization) have genotyped patients before their first treatment, modified starting dosages, and then compared the clinical outcomes (e.g., severe neutropenia, tumor response) based on these modified dosage with outcomes among patients receiving a standard dose. Currently, reducing the irinotecan dosage in subsequent cycles is the standard method of avoiding additional instances of neutropenia. For

example, Toffoli et al.[50] have shown that reducing dosage from 180 mg/m^2 to between 90 and 150 mg/m^2 in all individuals having neutropenia reduced the rate of neutropenia in **28* homozygotes in subsequent cycles. They reported that the odds ratio (OR) for neutropenia among **28* homozygotes patients relative to the wild genotype dropped from 8.6 (95% CI 1.3–57) after the first cycle to 2.0 (95% CI 0.6–7) after the end of therapy on lower doses (2–6 cycles). However, they also found that the point estimates for tumor-related morbidity and mortality were lower among **28* homozygous patients (and to a lesser extent heterozygous patients), possibly due to the effects of "over-dosing." Thus, the reduced drug metabolism rate among these patients in these two groups (i.e., slower inactivation of SN-38) that may cause the increased rate of severe adverse drug events (harm) is possibly also responsible for the apparent increase in tumor response and improved survival (benefit).

Comparing the benefits and harms

The proposed benefit of testing metastatic CRC patients for *UGT1A1* genotype is that the risk for adverse drug-related side effects (e.g., severe neutropenia) among patients found to be homozygous for the **28* genotype (and to a lesser extent for those found to be heterozygous) can be reduced by lowering their initial and/or subsequent doses of irinotecan. The concomitant harm is that reduction in irinotecan dosage many also reduce the effectiveness of chemotherapy in tumor suppression and long-term survival.

To compare these competing effects, we used a model (shown in Table 8) that incorporates estimates of the effect of reducing the initial irinotecan dosage given to **28* homozygous CRC patients on the number of severe neutropenia episodes avoided and on the number of additional CRC tumors nonresponsive to treatment. The numbers are based on the hypothetical population shown in Figure 5, with results shown for projections of the effectiveness of an irinotecan dose reduction from 20% to 100%. Effectiveness of 100% means that the rate of severe neutropenia among **28* homozygous patients receiving the reduced dose will be equivalent to that among patients with the wild genotype receiving the full dose. From the literature, that rate is expected to be about 9.8%. The number needed to test shown in Table 8 indicates the total number of cancer patients that would need to be genotyped (and have reduced dose in all found to be **28* homozygotes) to avoid one case of severe neutropenia in a homozygous patient. Our calculations assume that the reduced dose will cause homozygotes to have the same tumor response rate as individuals with the wild genotype. This may be an oversimplification of the model, as response rates seem to also be dose dependent.

As an example, consider that reducing the irinotecan dose among **28* homozygous individuals is 100% effective in reducing excess neutropenia (Table 8, row 1). How many excess events might be avoided among a hypothetical population of 20,000 whites. A total of 2231 homozygotes (Fig. 5) would occur and with a background rate of 9.8% for severe neutropenia, 219 events would occur if homozygotes had the same rate as wild-type individuals. Because the RR for severe neutropenia among homozygotes is 3.51 (Fig. 4), the actual number would be expected to be 767 or 548 more than the baseline of 218 events. The number needed to test to avoid one individual with severe neutropenia would be 20,000 tests divided by 548 avoided severe neutropenia events or 36 (Table 8, Column 4). The number of nonresponsive CRC tumors among homozygous individuals (**28/*28*) receiving a reduced dose is considered a constant (647) and is computed as follows. The baseline response rate in wild-type individuals is 41%, and the observed response rate for homozygotes is 1.70 times higher, or 69% (Fig. 6). Thus, there were originally 1539 responsive tumors among the homozygotes (0.69 * 2231), but only 892 might still be responsive with a reduced dose (0.40 * 2231). This is a drop of 647 responsive tumors. These 647 additional nonresponsive tumors (harms) can be compared with the avoidance of 548 cases of neutropenia (benefits) and result in an OR of 647:548 or 1.2:1 (Table 8, Column 5).

An alternative approach to comparing the benefits and harms would be to compare number of nonresponsive CRC tumors to the number of deaths resulting from severe neutropenia. An estimated 1 in 110 cases of severe neutropenia might result in death in individuals receiving irinotecan as a first-line treatment,[75–77] and the ratio of nonresponsive tumors to avoided

Table 8 Preliminary estimates of the clinical utility of testing metastatic CRC patients for *UGT1A1* polymorphisms: Benefits and harms among *UGT1A1*28* homozygotes

Effectiveness of irinotecan dose reduction in preventing neutropenia (%)	Projected Total number of neutropenia cases	Cases of neutropenia avoided	Number needed to test to avoid one case of neutropenia	Additional nonresponsive CRC tumors: case of neutropenia avoided
100	219	548	36	1.2:1
90	274	493	41	1.3:1
80	328	439	46	1.5:1
70	383	384	52	1.7:1
60	438	329	61	2.0:1
50	493	274	73	2.4:1
40	548	219	91	2.9:1
30	603	164	122	3.9:1
20	658	109	183	5.9:1
10	713	54	370	12:1

events of severe neutropenia (last column in Table 8) could be converted to this measure by dividing the right-hand side by 110. For example, using the numbers in Row 1, the OR of nonresponsive tumor versus death resulting from severe neutropenia change from 647:494 to 647:(494/110) or about 140:1. According to the very preliminary analysis reported in Table 8, it seems that at high rates of effectiveness (70–100%), each avoided case of neutropenia is associated with one nonresponsive tumor. At lower rates of effectiveness (20–50%), there are likely to be 2–5 times as many nonresponsive tumors as avoided cases of severe neutropenia.

Might individuals with the wild genotype be underdosed?

Given some limited evidence that individuals homozygous for **28* have improved survival[72] (Table 7), it is possible that individuals with the wild genotype are underdosed. Original Phase I studies did not stratify patients by *UGT1A1* genotype and, therefore, higher doses may be well tolerated by wild genotype individuals (**1/*1*). Preliminary data from new Phase I dose-escalation trials with patients stratified by genotype have been recently published (see Resources section).[78–80]

Limitations

In general, the same problems with studies of clinical validity are applicable to clinical utility. The study populations were mainly non-Hispanic whites, treatment regimens varied widely, patients with rare genotypes were grouped with patients with common genotypes, and patients with cancers other than CRC were included in some studies. As a result, the modeling of the benefits (reduction in risk for severe neutropenia) and harms (reduction in treatment effectiveness that may occur among patients homozygous for **28* whose irinotecan treatment dose is reduced) is based on weak evidence that is, in the case of harms, nonsignificant. These limitations underscore the need for caution in interpreting the results and indicate the need for further study.

Recent information

Since the formal literature search, one additional trial[81] has been reported that would have been included in the analysis of clinical validity and clinical utility. Specifically, that study found a higher rate of severe neutropenia among patients homozygous for **28* (RR, 5.4; 95% CI 2.4–12) but no difference among **28* heterozygous patients (RR, 0.8; 95% CI 0.2–2.8). The study reported little or no relationship between patients' *UGT1A1* genotype and risk for severe diarrhea. This study found an improved rate of survival among patients homozygous for **28* ($P = 0.06$).

In another study published since the initial review, Hoskins et al.[82] conducted a meta-analysis that provided more evidence for an association between irinotecan dose and risk of irinotecan-related Grades 3–4 neutropenia. On the basis of commonly used treatment regimens, irinotecan dose levels were stratified into three groups low (<150 mg/m^2), medium (150–250 mg/m^2), and high (>250 mg/m^2). They reported a statistically significant association between genotype and hematologic toxicity at medium and high doses of irinotecan, with ORs of 3.22 for **28* homozygotes versus **28* heterozygotes (95% CI 1.52–6.81, $P = 0.008$), and 27.8 for **28* homozygotes versus wild genotype (95% CI 4.0–195, $P = 0.005$). They did not find these associations at low doses (OR = 1.80; 95% CI 0.37–8.84; $P = 0.41$).

All of these findings are consistent with those reported in the main body of the review and strengthen the findings of the existing evidence review.

DISCUSSION

Quality of evidence

Figure 7 summarizes the quality of evidence for the key questions (Table 1). We rated the quality of evidence as adequate for the analytic validity of the common *UGT1A1* variant **28*, as there are two or more relatively high quality studies providing consistent results. However, the number of challenges do not allow for a confident estimates of the analytic sensitivity and specificity of the tests, even though the point estimates were high. Lastly, the data are restricted mainly to the analytic phase of testing. There are little or no data to estimate the analytic validity of tests for the less common *UGT1A1* variants.

We also rated the quality of evidence for the association of the **28* variant with the active form of irinotecan (SN-38), severe diarrhea and severe neutropenia as adequate, based on a systematic review of lower quality studies for these three outcome measures. Little or no data are available to examine these three outcomes with respect to the less common *UGT1A1* variants. Although it remains plausible, the evidence was inadequate to prospectively examine whether an initial reduction in irinotecan dosage in CRC patients homozygous for **28* was associated with a reduced risk for severe neutropenia. Little or no data are available to allow a direct, prospective comparison of the possible benefits and harms association with dosage

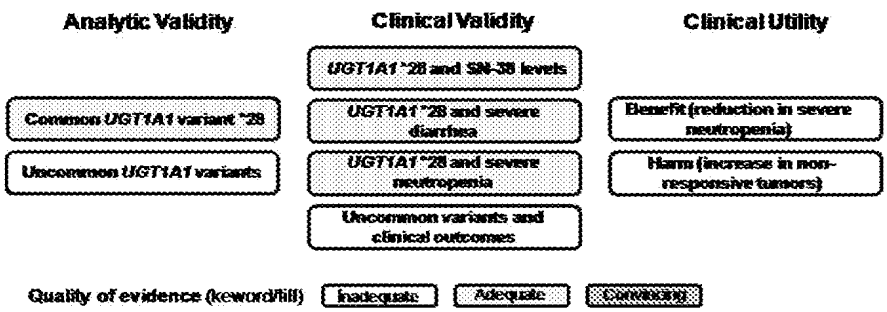

Fig. 7. Graphic display of the quality of evidence for selected components of the current evidence review. For analytic validity, clinical validity, and clinical utility, each of the main components of the evidence review is represented by the text within a box. The quality of evidence is shown by the shading (no shading, inadequate; light shading, adequate; dark shading, convincing).

reduction, but the previous modeling suggests further studies are warranted.

Important gaps in knowledge are as follows:

- There seems to be a clear relationship between *UGT1A1* genotype and severe neutropenia (and some evidence of a relationship with severe diarrhea), but there is no direct or indirect (chain of evidence) evidence to support the clinical utility of modifying an initial and/or subsequent dose of irinotecan in patients with metastatic CRC as a way to change the rate of adverse drug events (e.g., severe neutropenia).
- Even if adverse drug-related events were reduced, this risk reduction may come at the expense of a reduction in tumor responsiveness in **28* homozygotes, leading to an overall net harm.
- The data on the clinical validity of tests for *UGT1A1* variants other than **28* are limited.
- The analytic validity of *UGT1A1* testing in clinical practice is unknown. Laboratories offering such testing may include variants in addition to **28* for which little evidence is available.
- Pre- and postanalytic errors in *UGT1A1* testing have not been reported, but the rate of such errors is likely to be similar to that reported for other genetic tests done in high-complexity laboratories.[41,42] A new external proficiency testing program jointly offered by the American College of Medical Genetics and the College of American Pathologists is likely to provide important evidence about the analytic validity of *UGT1A1* testing in clinical settings.
- There are limited data on *UGT1A1* variants in Hispanic and African American populations.
- If *UGT1A1* testing were recommended for routine use in clinical practice, additional studies would be needed to understand the potential effects of alleles that are rare in whites but more common in other racial/ethnic groups (e.g., **6* in Asians), and testing panels would need to include all variants of clinical significance in the population to be tested.

Research agenda

Analysis of data from the American College of Medical Genetics and College of American Pathologists proficiency testing program will provide needed information about the analytic validity of *UGT1A1* tests offered for clinical use. However, additional studies concerning the clinical validity of tests for the less common *UGT1A1* variants are needed. Given the rarity of these genotypes, these studies will need to include large numbers of subjects receiving treatment. This is feasible, however, because metastatic CRC is relatively common, as is chemotherapy with irinotecan. The most appropriate way to collect the evidence needed to document whether, or how, to modify irinotecan dosage for patients with particular *UGT1A1* genotypes is to mount prospective studies (preferably including randomized trials) comparing outcomes among patients who receive targeted doses of irinotecan versus outcomes among those who receive doses currently recommended for all CRC patients. Such a study should be considered ethical, as it is not known whether the supposed benefits outweigh the possible harms. There are sufficient numbers of subjects for recruitment to be completed in a relatively short period of time.

ACKNOWLEDGMENTS

Funding for this report was provided by National Office of Public Health Genomics, Centers for Disease Control and Prevention. The RTI preliminary evidence review for clinical validity was developed under project number 0208234.036.

The authors acknowledge Nedra Whitehead, PhD; Meera Viswanathan, PhD; and Eric Gillis, MS of RTI International, Research Triangle Park, NC, for their contributions to the full evidence report.

Members of the UGT1A1 Technical Evaluation Panel (TEP). From the EGAPP Working Group: Kathryn A. Phillips, PhD; Joan A. Scott, MS; Steven Teutsch, MD, MPH. Others included EGAPP Consultant Glenn Palomaki, BS; CDC representative Linda A. Bradley, PhD; RTI International representatives Meera Viswanathan, PhD; Nedra Whitehead, PhD; Kathleen N. Lohr, PhD; and RTI consultant Bert O'Neil, MD.

Reviewers of the evidence report for EGAPP included: EGAPP Working Group, D Joe Boone, PhD; Ralph Coates, PhD; Scott Grosse, PhD; Steve Gutman, MD, MBA; Howard McLeod, PharmD; Janelle Hoskins, PhD; Bert O'Neil, MD; Giuseppe Toffoli, MD; Marc Williams, MD; Jim Gudgeon, MS, MBA.

Reviewers of this manuscript for EGAPP included: EGAPP Working Group; Amy Brower, PhD; Janelle Hoskins, PhD; Giuseppe Toffoli, MD.

Resources

Bradley LA, Palomaki GE, Dotson WD, et al. Evidence review: can *UGT1A1* genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan? 2008. Available at: http://www.egappreviews.org.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference MMJ-043PC2	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2013/045495	International filing date *(day/month/year)* 12 June 2013 (12.06.2013)	Priority date *(day/month/year)* 13 June 2012 (13.06.2012)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant MERRIMACK PHARMACEUTICALS, INC.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☒	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☒	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 16 December 2014 (16.12.2014)
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PATENT COOPERATION TREATY

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(PCT Rule 43*bis*.1)

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(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2013/045495	12.06.2013	13.06.2012

International Patent Classification (IPC) or both national classification and IPC
INV. A61K9/00 A61K31/4745 A61K31/513 A61K31/517 A61P35/00

Applicant
MERRIMACK PHARMACEUTICALS, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Fax: +31 70 340 - 3016	see form PCT/ISA/210	Haider, Ursula Telephone No. +31 70 340-8987

Form PCT/ISA/237 (Cover Sheet) (July 2009)

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

      ☐ on paper

      ☐ in electronic form

   b. (time)

      ☐ in the international application as filed

      ☐ together with the international application in electronic form

      ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2-5, 7-9
	No:	Claims	1, 6, 10-27
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	1-27
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

V.1 General remarks

V.1.1 The patentability can be dependent upon the formulation of the claims. The EPO, for example, does not recognise as patentable claims to the use of a compound in medical treatment, but may allow claims to a product, in particular substances or compositions for use in a first or further medical treatment.

Patentability, in particular novelty and inventive step, of claims 1-11, 21-27 has been assessed on the basis of a purpose-limited product claim taking into account the alleged effects of the compound/composition.

V.1.2 Claims 12-20 are product claims referring to liposomal formulations of irinotecan. Said claims contain additional features regarding a dosage regimen and combination partners. The claim being drafted as a product claim, without any medical use, these additional features are not considered as limiting as long as the prior art formulations are "suitable for" the desired way of administration/dosage regimen.

V.1.3 Claims 23, 24 are directed to kits comprising active compounds and instructions for administering. Instructions for use are not considered a technical contribution to the art, and their content is therefore not eligible to confer novelty and/or inventive step to the claims.

V.2 Reference is made to the following documents

D1 "Study of MM-398 Versus 5-Fluorouracil and Leucovorin in Patients With Metastatic Pancreatic Cancer",
11 December 2011 (2011-12-11), pages 1-3, XP055075223
Retrieved from the Internet: URL:http://clinicaltrials.gov/archive/NCT01494506/2011_12_16

D2 Chang-Sung Tsai ET AL: "Nanovector-based therapies in advanced pancreatic cancer",
Journal of Gastrointestinal Oncology, 1 September 2011 (2011-09-01), pages 185-194, XP055075231,
DOI: 10.3978/j.issn.2078-6891.2011.034
Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3397610/pdf/jgo-02-03-185.pdf

D3 J. M. HOSKINS ET AL: "UGT1A1*28 Genotype and Irinotecan-Induced Neutropenia: Dose Matters",
JNCI JOURNAL OF THE NATIONAL CANCER INSTITUTE,
vol. 99, no. 17, 5 September 2007 (2007-09-05), pages 1290-1295,
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DIGESTIVE AND LIVER DISEASE, W.B. SAUNDERS, GB,
vol. 43, no. 11, 1 July 2011 (2011-07-01), pages 912-916, XP028296448,
ISSN: 1590-8658, DOI: 10.1016/J.DLD.2011.07.001

D5 JEFFREY R INFANTE ET AL: "Phase I and pharmacokinetic study of IHL-305 (PEGylated liposomal irinotecan) in patients with advanced solid tumors",
CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE,
vol. 70, no. 5, 2 September 2012 (2012-09-02), pages 699-705,
XP035132528

D6 "Study of MM-398 With or Without 5-Fluorouracil and Leucovorin, Versus 5-Fluorouracil and Leucovorin in Patients With Metastatic Pancreatic Cancer",
9 August 2012 (2012-08-09), pages 1-3, XP055075259,
Retrieved from the Internet: URL:http://clinicaltrials.gov/archive/NCT01494506/2012_08_09

V.3 Novelty (Article 33(2) PCT)

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 1, 6, 10-27 is not new in the sense of Article 33(2) PCT.

V.3.1 **Document D1**, prejudicial to novelty of **claims 1, 10-20, 23, 26, 27**

Document D1 discloses a study of liposomal irinotecan, MM-398, in patients with metastatic exocrine pancreatic cancer, in a dosage of 120 mg/m^2 IV once every 3 weeks. Patients were pre-treated with gemcitabine.

V.3.2 **Document D2**, prejudicial to novelty of **claims 1, 6, 10-27**

Document D2 discloses (the references in parentheses applying to this document) promising results of the use of MM-398 at a dosage of 120 mg/m^2 once every 3 weeks iv for 90 minutes, with or without 5-FU/leucovorin, in two phase I trials involving gemcitabine-refractory pancreatic patients (page 189, right-hand column, paragraph 2).

V.4 Inventive Step (Article 33(3) PCT)

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1-27 does not involve an inventive step in the sense of Article 33(3) PCT.

Claims 1, 6, 10-27 are not novel, thus no opinion on the presence of an inventive step is required.

Closest prior art is document D2 (see above).

Claim 2 defers from the disclosure of D2 in that a dosage adjustment is foreseen for patients homozygous for the UGT1A1*28 allele.

The technical effect due to this difference may be lower toxicity (pages 9-10 of the present description).

The objective technical problem to be solved is therefore to provide a better treatment for a specific patient group, i. e. patients homozygous for the UGT1A1*28 allele.

Inventive step assessment: It is well known in the art that irinotecan-induced toxicity is linked to the dose of irinotecan in these patients (D3, D4), and dose adjustment would appear an obvious measure for the skilled person (see also D4, in particular page 915, right-hand column, paragraph 5).

Claims 3-5, 7-9 differ from the disclosure of D2 in that more details on the dosage regimen for the combination therapy of liposomal irinotecan, 5-FU and leucovorin are given.

No technical effect is apparent for that difference.

The objective technical problem to be solved is therefore find an appropriate dosage regimen for the combination therapy mentioned in D2.

Inventive step assessment: Standard FOLFIRI chemotherapy with non-liposomal irinotecan comprises 180 mg/m^2 irinotecan, 200 mg/m^2 leucovorine and 2400 mg/m^2 5-FU in a cycle period of 2 weeks (see, e. g. D4). D2 mentions 120 mg/m^2 of liposomal irinotecan as the maximum tolerated dose. Dosage finding is part of routine practise for the skilled person. The dosages and modes of administration proposed in

present claims 2-5, 7-9 do not appear to comprise any surprising technical teaching in view of the disclosure of D2 and D4; consequently, the subject-matter of claims 3-5, 7-9 does not involve an inventive step.

Re Item VI

Certain documents cited

The examination has been carried out assuming that the priority of the application is valid. However, attention is drawn to the fact that documents D5 and D6 may become relevant for the subject-matter of the present application during national/regional phase examination if the priority claimed for the present application turns out to be invalid.

Re Item VIII

Certain observations on the international application

VIII.1 Claim 27 lacks clarity as it refers to co-administration in a method of claims 1, 2, wherein said claims do not disclose co-administration (Article 6 PCT).

VIII.2 Claim 19 refers to the pancreatic cancer of claims 12-18, while these claims do not mention the disease (Article 6 PCT).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
MMJ-043PC2		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2013/045495	12/06/2013	13/06/2012
Applicant		
MERRIMACK PHARMACEUTICALS, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/045495

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/00 A61K31/4745 A61K31/513 A61K31/517 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Study of MM-398 Versus 5-Fluorouracil and Leucovorin in Patients With Metastatic Pancreatic Cancer", , 11 December 2011 (2011-12-11), pages 1-3, XP055075223, Retrieved from the Internet: URL:http://clinicaltrials.gov/archive/NCT01494506/2011_12_16 [retrieved on 2013-08-14] the whole document	1,10-20, 23,26,27

-/--

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2013	22/08/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Haider, Ursula

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chang-Sung Tsai ET AL: "Nanovector-based therapies in advanced pancreatic cancer", Journal of Gastrointestinal Oncology, 1 September 2011 (2011-09-01), pages 185-194, XP055075231, DOI: 10.3978/j.issn.2078-6891.2011.034 Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3397610/pdf/jgo-02-03-185.pdf [retrieved on 2013-08-14]	1,6, 10-27
Y	page 189, right-hand column, paragraph 2	2-9
Y	J. M. HOSKINS ET AL: "UGT1A1*28 Genotype and Irinotecan-Induced Neutropenia: Dose Matters", JNCI JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 99, no. 17, 5 September 2007 (2007-09-05), pages 1290-1295, XP055022025, ISSN: 0027-8874, DOI: 10.1093/jnci/djm115 the whole document	2
Y	HEDIA BRIXI-BENMANSOUR ET AL: "Phase II study of first-line FOLFIRI for progressive metastatic well-differentiated pancreatic endocrine carcinoma", DIGESTIVE AND LIVER DISEASE, W.B. SAUNDERS, GB, vol. 43, no. 11, 1 July 2011 (2011-07-01), pages 912-916, XP028296448, ISSN: 1590-8658, DOI: 10.1016/J.DLD.2011.07.001 [retrieved on 2011-07-07] page 915, right-hand column, paragraph 5	2-9
X,P	JEFFREY R INFANTE ET AL: "Phase I and pharmacokinetic study of IHL-305 (PEGylated liposomal irinotecan) in patients with advanced solid tumors", CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 70, no. 5, 2 September 2012 (2012-09-02), pages 699-705, XP035132528, ISSN: 1432-0843, DOI: 10.1007/S00280-012-1960-5 the whole document	1-27

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	"Study of MM-398 With or Without 5-Fluorouracil and Leucovorin, Versus 5-Fluorouracil and Leucovorin in Patients With Metastatic Pancreatic Cancer", , 9 August 2012 (2012-08-09), pages 1-3, XP055075259, Retrieved from the Internet: URL:http://clinicaltrials.gov/archive/NCT01494506/2012_08_09 [retrieved on 2013-08-14] the whole document -----	1-27

1

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2013/045495	12.06.2013	13.06.2012

International Patent Classification (IPC) or both national classification and IPC
INV. A61K9/00 A61K31/4745 A61K31/513 A61K31/517 A61P35/00

Applicant
MERRIMACK PHARMACEUTICALS, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

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If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Fax: +31 70 340 - 3016	see form PCT/ISA/210	Haider, Ursula Telephone No. +31 70 340-8987

Form PCT/ISA/237 (Cover Sheet) (July 2009)

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))

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      ☐ on paper

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4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

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Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2-5, 7-9
	No:	Claims	1, 6, 10-27
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	1-27
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

V.1 General remarks

V.1.1 The patentability can be dependent upon the formulation of the claims. The EPO, for example, does not recognise as patentable claims to the use of a compound in medical treatment, but may allow claims to a product, in particular substances or compositions for use in a first or further medical treatment.

Patentability, in particular novelty and inventive step, of claims 1-11, 21-27 has been assessed on the basis of a purpose-limited product claim taking into account the alleged effects of the compound/composition.

V.1.2 Claims 12-20 are product claims referring to liposomal formulations of irinotecan. Said claims contain additional features regarding a dosage regimen and combination partners. The claim being drafted as a product claim, without any medical use, these additional features are not considered as limiting as long as the prior art formulations are "suitable for" the desired way of administration/dosage regimen.

V.1.3 Claims 23, 24 are directed to kits comprising active compounds and instructions for administering. Instructions for use are not considered a technical contribution to the art, and their content is therefore not eligible to confer novelty and/or inventive step to the claims.

V.2 Reference is made to the following documents

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11 December 2011 (2011-12-11), pages 1-3, XP055075223
Retrieved from the Internet: URL:http://clinicaltrials.gov/archive/NCT01494506/2011_12_16

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Journal of Gastrointestinal Oncology, 1 September 2011 (2011-09-01), pages 185-194, XP055075231,
DOI: 10.3978/j.issn.2078-6891.2011.034
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D3 J. M. HOSKINS ET AL: "UGT1A1*28 Genotype and Irinotecan-Induced Neutropenia: Dose Matters",
JNCI JOURNAL OF THE NATIONAL CANCER INSTITUTE,
vol. 99, no. 17, 5 September 2007 (2007-09-05), pages 1290-1295,
XP055022025

D4 HEDIA BRIXI-BENMANSOUR ET AL: "Phase II study of first-line FOLFIRI for progressive metastatic well-differentiated pancreatic endocrine carcinoma",
DIGESTIVE AND LIVER DISEASE, W.B. SAUNDERS, GB,
vol. 43, no. 11, 1 July 2011 (2011-07-01), pages 912-916, XP028296448,
ISSN: 1590-8658, DOI: 10.1016/J.DLD.2011.07.001

D5 JEFFREY R INFANTE ET AL: "Phase I and pharmacokinetic study of IHL-305 (PEGylated liposomal irinotecan) in patients with advanced solid tumors",
CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE,
vol. 70, no. 5, 2 September 2012 (2012-09-02), pages 699-705,
XP035132528

D6 "Study of MM-398 With or Without 5-Fluorouracil and Leucovorin, Versus 5-Fluorouracil and Leucovorin in Patients With Metastatic Pancreatic Cancer",
9 August 2012 (2012-08-09), pages 1-3, XP055075259,
Retrieved from the Internet: URL:http://clinicaltrials.gov/archive/NCT01494506/2012_08_09

V.3 Novelty (Article 33(2) PCT)

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 1, 6, 10-27 is not new in the sense of Article 33(2) PCT.

V.3.1 **Document D1**, prejudicial to novelty of **claims 1, 10-20, 23, 26, 27**

Document D1 discloses a study of liposomal irinotecan, MM-398, in patients with metastatic exocrine pancreatic cancer, in a dosage of 120 mg/m^2 IV once every 3 weeks. Patients were pre-treated with gemcitabine.

V.3.2 **Document D2**, prejudicial to novelty of **claims 1, 6, 10-27**

Document D2 discloses (the references in parentheses applying to this document) promising results of the use of MM-398 at a dosage of 120 mg/m^2 once every 3 weeks iv for 90 minutes, with or without 5-FU/leucovorin, in two phase I trials involving gemcitabine-refractory pancreatic patients (page 189, right-hand column, paragraph 2).

V.4 Inventive Step (Article 33(3) PCT)

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1-27 does not involve an inventive step in the sense of Article 33(3) PCT.

Claims 1, 6, 10-27 are not novel, thus no opinion on the presence of an inventive step is required.

Closest prior art is document D2 (see above).

Claim 2 defers from the disclosure of D2 in that a dosage adjustment is foreseen for patients homozygous for the UGT1A1*28 allele.

The technical effect due to this difference may be lower toxicity (pages 9-10 of the present description).

The objective technical problem to be solved is therefore to provide a better treatment for a specific patient group, i. e. patients homozygous for the UGT1A1*28 allele.

Inventive step assessment: It is well known in the art that irinotecan-induced toxicity is linked to the dose of irinotecan in these patients (D3, D4), and dose adjustment would appear an obvious measure for the skilled person (see also D4, in particular page 915, right-hand column, paragraph 5).

Claims 3-5, 7-9 differ from the disclosure of D2 in that more details on the dosage regimen for the combination therapy of liposomal irinotecan, 5-FU and leucovorin are given.

No technical effect is apparent for that difference.

The objective technical problem to be solved is therefore find an appropriate dosage regimen for the combination therapy mentioned in D2.

Inventive step assessment: Standard FOLFIRI chemotherapy with non-liposomal irinotecan comprises 180 mg/m^2 irinotecan, 200 mg/m^2 leucovorine and 2400 mg/m^2 5-FU in a cycle period of 2 weeks (see, e. g. D4). D2 mentions 120 mg/m^2 of liposomal irinotecan as the maximum tolerated dose. Dosage finding is part of routine practise for the skilled person. The dosages and modes of administration proposed in

present claims 2-5, 7-9 do not appear to comprise any surprising technical teaching in view of the disclosure of D2 and D4; consequently, the subject-matter of claims 3-5, 7-9 does not involve an inventive step.

Re Item VI

Certain documents cited

The examination has been carried out assuming that the priority of the application is valid. However, attention is drawn to the fact that documents D5 and D6 may become relevant for the subject-matter of the present application during national/regional phase examination if the priority claimed for the present application turns out to be invalid.

Re Item VIII

Certain observations on the international application

VIII.1 Claim 27 lacks clarity as it refers to co-administration in a method of claims 1, 2, wherein said claims do not disclose co-administration (Article 6 PCT).

VIII.2 Claim 19 refers to the pancreatic cancer of claims 12-18, while these claims do not mention the disease (Article 6 PCT).

Cancer Treatment Reviews 35 (2009) 431–436

Contents lists available at ScienceDirect

Cancer Treatment Reviews

journal homepage: www.elsevierhealth.com/journals/ctrv

ANTI-TUMOUR TREATMENT

Pancreatic cancer: Current and future treatment strategies

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ARTICLE INFO

Article history:
Received 24 October 2008
Received in revised form 20 February 2009
Accepted 25 February 2009

Keywords:
Pancreatic cancer
Treatment strategies
Chemotherapy
Targeted therapy

SUMMARY

Pancreatic cancer is a disease with a high mortality rate and short survival, as a result of the high incidence of metastatic disease at diagnosis, the fulminant clinical course and the lack of successful therapeutic strategies. The administration of chemotherapeutic agents for the treatment of advanced disease has failed and currently, research focuses on the understanding of molecular pathways in order to investigate the role of targeted therapy. Trials on adjuvant and neo-adjuvant therapy of pancreatic cancer are also ongoing. This review presents the recent developments with newer chemotherapeutic and molecular-targeted agents, identifying the efforts for individualized treatment strategies.

Introduction

Pancreatic cancer is the fourth most common cause of adult cancer death, accounting for an estimated 37,680 new cases and 34,290 deaths in USA for 2008.[1] The high mortality rate is due to the high incidence of metastatic disease at initial diagnosis, the aggressive clinical course and the failure of systemic therapies. In only 5–25% of the patients presenting with pancreatic cancer will the tumor be operable. The median disease-free survival following complete resection of pancreatic cancer and adjuvant administration of gemcitabine is 13.4 months and 6.9 months for untreated patients. However, the longer disease-free survival has not translated in any advantage in overall survival.[2] In addition, the median survival in locally advanced disease (40% of the patients at diagnosis) is 8–12 months and 3–6 months for those patients presenting with metastatic disease (40–45%).[3] The administration of cytotoxic agents for the treatment of advanced disease has had disappointing results and currently, research focuses on the understanding of molecular pathways in order to evaluate the role of targeted therapy, while trials on combinations of newer chemotherapeutic drugs in metastatic disease and adjuvant therapy of pancreatic cancer are ongoing.

Chemotherapy for metastatic disease

The goal of systemic chemotherapy is to minimize the patients' disease-related symptoms and to prolong survival. 5-Fluorouracil (5-FU) combinations compared with no chemotherapy or best supportive care provided a survival advantage [33 weeks for the treated group compared with 15 weeks in the untreated group ($P < 0.002$)][4] for pancreatic cancer patients, but a meta-analysis demonstrated no survival benefit among 5-FU combinations and 5-FU alone. Data for 5365 patients from 43 randomized controlled trials were included in this meta-analysis. Survival benefit over best supportive care was demonstrated in 5-FU-based chemotherapy in 9 randomized trials. However, trials comparing 5-FU alone vs 5-FU-based combinations did not show any statistical differences, nor did various 5-FU combinations compared among themselves.[5]

Gemcitabine has been the reference regimen since its approval in 1996. It is a prodrug which requires cellular uptake and phosphorylation to active metabolites, which inhibit DNA chain elongation and lead to DNA fragmentation and cell death.[6] Its approval came through a phase III trial, in 126 patients who were randomized either to gemcitabine 1000 mg/m^2 weekly × 7 followed by 1 week of rest, weekly × 3 every 4 weeks thereafter (63 patients), or weekly bolus 5-FU at a dose of 600 mg/m^2. The primary end point was the clinical benefit. Clinical response was experienced in 23.8% of gemcitabine-treated patients compared with 4.8% of 5-FU-treated patients ($P = .0022$). The median overall survival durations were 5.65 and 4.41 months for gemcitabine-treated and 5-FU-treated patients ($P = .0025$) and the 1-year survival rate was 18% and 2% for the gemcitabine and 5-FU group, respectively.[7]

Since its approval, there has been enough effort to develop gemcitabine combinations for pancreatic cancer patients, which has failed to produce a significant overall survival benefit. Specifically, patients with advanced pancreatic cancer were randomly assigned to either GemOx (gemcitabine 1 g/m^2 as a 100 min infusion on day

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doi:10.1016/j.ctrv.2009.02.005

1 and oxaliplatin 100 mg/m^2 as a 2 h infusion on day 2 every 2 weeks) or gemcitabine (Gem) 1 g/m^2 as a weekly 30 min infusion). Three hundred twenty-six patients were enrolled; 313 were eligible, and 157 and 156 were allocated to the GemOx and Gem arms, respectively. GemOx was superior to Gem in terms of response rate (26.8% vs 17.3%, respectively; $P = .04$), progression-free survival (5.8 vs 3.7 months, respectively; $P = .04$), and clinical benefit (38.2% vs 26.9%, respectively; $P = .03$). Median overall survival for GemOx and Gem was 9.0 and 7.1 months, respectively ($P = .13$). GemOx was well tolerated overall, although a higher incidence of grade 3 and 4 toxicity per patient was observed for platelets (14.0% for GemOx vs 3.2% for Gem), vomiting (8.9% for GemOx vs 3.2% for Gem), and neurosensory symptoms (19.1% for GemOx vs 0% for Gem).[8] However, a pooled analysis of two randomized studies comparing gemcitabine alone with combinations with oxaliplatin or cisplatin suggested that patients with PS 0 had better survival outcomes (8.3 months vs 6.7 months, $P = 0.031$). Therefore, patients with PS 0 may benefit from combinations with platinum analogues.[9]

Another drug, capecitabine, had shown activity in combination with gemcitabine in phase II trials in chemotherapy-naive patients with locally advanced or metastatic pancreatic cancer. However, two phase III trials showed conflicting results. One trial randomized 319 patients to receive GemCap (oral capecitabine 650 mg/m^2 twice daily on days 1–14 plus Gem 1 g/m^2 by 30 min infusion on days 1 and 8 every 3 weeks) or Gem (1 g/m^2 by 30 min infusion weekly for 7 weeks, followed by 1-week break, and then weekly for 3 weeks every 4 weeks). Median overall survival time which was the primary end point was 8.4 and 7.2 months in the GemCap and Gem arms, respectively ($P = .234$). Post hoc analysis in patients with good Karnofsky performance status (score of 90–100) showed a significant prolongation of median overall survival time in the GemCap arm compared with the Gem arm (10.1 vs 7.4 months, respectively; $P = .014$). The overall frequency of grade 3 or 4 adverse events was similar in each arm. Neutropenia was the most frequent grade 3 or 4 adverse event in both arms.[10] Another phase III trial, by Cunningham et al. randomized 533 patients to receive gemcitabine 1 g/m^2 weekly for 7 weeks of an 8-week cycle, or capecitabine 1660 mg/m^2 for 3 weeks of a 4 week cycle plus weekly gemcitabine 1 g/m^2. The trial demonstrated a statistically significant difference in overall survival time (7.4 months vs 6 months, $P = 0014$) for the combination arm. This result might be attributed to a prolonged capecitabine administration (1660 mg/m^2 daily for 21 days every 4 weeks).[11]

UFT is a combination of tegafur (a prodrug of 5-fluorouracil) and uracil that is orally administered. The administration of UFT for several weeks may simulate the effects of a continuous infusion of 5-fluorouracil. The combination chemotherapy of gemcitabine with UFT in metastatic pancreatic cancer is well tolerated for most patients but with modest response rates and clinical benefit, as shown in phase II trials. However, a randomized phase III study should be conducted in order to further test the efficacy of the regimen.[12]

An oral fluoropyrimidine (S-1) has also been developed in order to potentiate the antitumor activity of 5-FU and reduce gastrointestinal toxicity. In a phase II study, the combination of 40 mg/m^2 orally twice daily for days 1–14 with gemcitabine 1 g/m^2 (days 1 and 8), repeated every 3 weeks proved to be effective, with acceptable toxicity. S-1 at a dose of 80 mg/m^2 for days 1–14 has been also evaluated in combination with irinotecan 100 mg/m^2 on days 1 and 15, every 28 days. The reported response rate was 44%, the time to progression was 4.9 months, and the median survival was 11.3 months.[13]

Trials have also investigated the role of three or four-drug regimens in metastatic pancreatic cancer. In a randomized multicenter phase III trial, 52 patients were randomly assigned to 40 mg/m^2 cisplatin and 40 mg/m^2 epirubicin both given on day 1, 600 mg/m^2 gemcitabine given intravenously over 1 h on days 1 and 8, and 200 mg/m^2 5-FU a day given by continuous infusion on days 1–28 of a 4 week cycle (PEFG regimen), and 47 were assigned to 1 g/m^2 gemcitabine given intravenously over 30 min once a week for 7 of 8 consecutive weeks in cycle 1 and for 3 of 4 weeks thereafter. The primary endpoint was 4 month progression-free survival. More patients receiving PEFG than gemcitabine alone were alive without progressive disease at 4 months (60% vs 28%; hazard ratio [HR] 0.46). One-year overall survival in the PEFG group was 38.5% and in the gemcitabine group was 21.3% (HR 0.68). More patients assigned to PEFG showed disease response than did those assigned to gemcitabine (38.5% vs 8.5%; odds ratio 6.60, $P = 0.0008$). More patients in the PEFG group had grade 3–4 neutropenia and thrombocytopenia than in the gemcitabine group ($P < 0.0001$).[14] Additionally, a phase II–III trial (ACCORD 11) compared FOLFIRINOX with gemcitabine alone. Chemotherapy-naïve patients, aged 18–75 years, with histologically or cytologically confirmed measurable metastatic pancreatic cancer were randomized to receive gemcitabine (G) (1 g/m^2 IV weekly × 7 for 8 weeks then weekly × 3 out of 4 weeks) or (oxaliplatin 85 mg/m^2 d1 + irinotecan 180 mg/m^2 d1 plus leucovorin 400 mg/m^2 d1 followed by 5-FU 400 mg/m^2 bolus d1 and 2400 mg/m^2 46 h continuous infusion biweekly). The primary endpoint was the response rate. Main grade 3–4 toxicities (FOLFIRINOX arm vs G) were grade 3 neutropenia (32%/17.5%), grade 4 neutropenia (19.5%/0%), grade 3–4 thrombocytopenia (12%/0%), grade 3 vomiting (17%/2.5%), grade 3 hepatic dysfunction (elevated transaminases) (0%/15%) and grade 3–4 fatigue (27%/15%). Confirmed partial responses (FOLFIRINOX/G) were 38.7% (12/31) and 11.7% (4/34) according to the investigators and median duration of response was 6.3 and 4.6 months, respectively. Partial response and stable disease were documented for 21/31 evaluable patients in FOLFIRINOX arm and expert review confirmed 13 partial responses (41.9%) and 6 stable diseases (19.3%).[15] Lastly, capecitabine (750 mg/m^2 orally twice daily for 14 days), gemcitabine (750 mg/m^2) and docetaxel (30 mg/m^2 on days 4 and 11) administered every 21 days was evaluated in 35 patients with encouraging results concerning overall survival (11.2 months) and 2-year survival rate (20%).[16]

On the other hand, efforts have been made to define the optimal infusion rate of gemcitabine in order to achieve its maximum efficacy. Preclinical studies have shown that prolonged gemcitabine infusion at a fixed dose rate (FDR) of 10 mg/m^2 per minute compared with the standard administration of 1 g/m^2 in 30 min resulted in higher intracellular levels of gemcitabine triphosphate, the active metabolite of gemcitabine. However, it has not been proved yet that FDR infusion offers a survival advantage in patients with pancreatic cancer, while some studies have shown increased hematologic toxicity.[17]

Targeted therapy

Recently, research is focusing on the understanding of molecular pathways and the investigation of molecular factors for the treatment of pancreatic cancer, since there are no significant clinical advances with chemotherapeutic agents.

The vascular endothelial growth factor (VEGF) is overexpressed in pancreatic adenocarcinoma and has been shown that its inhibition has a negative impact on tumor growth and metastasis. Bevacizumab (Avastin) is a recombinant humanized anti-VEGF monoclonal antibody which was initially studied in a phase II trial, in 52 patients with metastatic pancreatic cancer, in combination with gemcitabine 1 g/m^2 intravenously over 30 min on days 1, 8, and 15 every 28 days. Bevacizumab, 10 mg/kg, was administered after gemcitabine on days 1 and 15. Eleven patients (21%) had con-

firmed partial responses, and 24 (46%) had stable disease. The 6 month survival rate was 77% and the 1-year survival rate was 29%. Median survival was 8.8 months; median progression-free survival was 5.4 months.[18] The results of the study prompted CALGB to conduct a phase III trial (80303) comparing the combination of gemcitabine at a dose of 1 g/m^2 plus bevacizumab at a dose of 10 mg/kg on days 1 and 15 of a 28 day cycle vs gemcitabine alone in 602 advanced pancreatic cancer patients. The preliminary analysis showed that the combination was not found to improve survival (5.7 months for the combination arm vs 6 months for the gemcitabine arm). However, patients with PS score 0 had longer survival (8 months) compared with other patients with PS score >1 (4.8 months).[19] The combination of chemotherapy with bevacizumab was also examined in a phase II trial where gemcitabine–oxaliplatin (GemOx) plus bevacizumab were administered in 82 advanced pancreatic cancer patients. The results of the study were a 6 month survival of 65.0%, a median survival of 8.1 months and a median time to progression of 5.7 months.[20] In addition, the AVITA study, a phase III, randomized, double-blind, placebo-controlled trial which compared gemcitabine (1 g/m^2 by 30 min infusion weekly for 7 weeks, followed by a 1-week break, and then weekly for 3 weeks every 4 weeks) plus erlotinib 100 mg/day with or without bevacizumab every 2 weeks (5 mg/kg) showed improved progression-free survival (4.6 months vs 3.6 months, $P = 0.0002$), but not overall survival (7.1 months vs 6 months, $P = 0.2087$), compared to gemcitabine plus erlotinib.[21]

Also, EGFR activation through the phosphorylation of the intracellular domain of its tyrosine kinase leads to a signaling cascade, which has been targeted for anticancer drug development. EGFR's overexpression is a negative prognostic factor and contributes to short survival of pancreatic cancer patients. Erlotinib, is an oral tyrosine kinase inhibitor which interrupts EGRF signaling pathway and has been approved as treatment for locally advanced and metastatic pancreatic cancer patients. Specifically, Moore et al conducted a randomized, double-blind, international, phase III trial where 569 patients were randomly assigned 1:1 to receive standard gemcitabine plus erlotinib (100 or 150 mg/day orally) or gemcitabine plus placebo. The primary end point was overall survival. Overall survival based on an intent-to-treat analysis was significantly prolonged on the erlotinib/gemcitabine arm with a hazard ratio (HR) of 0.82 (median 6.24 months vs 5.91 months, $P = .038$). One-year survival was also greater with erlotinib plus gemcitabine (23% vs 17%; $P = .023$). Progression-free survival was significantly longer with erlotinib plus gemcitabine with an estimated HR of 0.77 (3.75 months vs 3.55 months, $P = .004$). Objective response rates were not significantly different between the two arms, although more patients on erlotinib had disease stabilization. There was a higher incidence of some adverse events with erlotinib plus gemcitabine, but most were grade 1 or 2. Of the 282 patients who received erlotinib, 79 had no rash, 102 had grade 1 rash, and 101 had a grade 2 or higher skin rash. Patients younger than 65 ($P = .01$) and those with a good PS ($P = .03$) had a higher likelihood of developing rash. The presence of a rash was associated with a higher likelihood of achieving disease control ($P = .05$) after controlling other prognostic factors. Although the survival benefit with the addition of erlotinib was modest, the occurrence of skin rash was associated with a significant and clinically meaningful difference in survival ($P = .037$; HR, 0.74). The median survival rates for patients with grade 0, 1, and 2 rash were 5.3, 5.8, and 10.5 months; the 1-year survival rates were 16%, 9%, and 43%, respectively ($P = .001$). Moreover, 162 pancreatic cancer biopsies were analyzed immunohistochemically for EGFR staining. Response to treatment with the combination of erlotinib and gemcitabine was not associated to EGFR expression and was superior in both EGFR positive and negative group of patients.[22]

The TARGET trial was a phase I study of a chemotherapy doublet (gemcitabine plus capecitabine), combined with a biologic doublet (bevacizumab plus erlotinib) in patients with advanced pancreatic adenocarcinoma. Patients with advanced disease were treated at 4 cohorts of escalating capecitabine doses (days 1–21): 910 mg/m^2, 1160 mg/m^2, 1400 mg/m^2, and 1660 mg/m^2. The maximum tolerated dose was 1660 mg/m^2. The doses of co-administered gemcitabine (1 mg/m^2 days 1, 8, and 15), bevacizumab (5 mg/kg days 1 and 15), and erlotinib (100 mg/day) every 28 days were constant. Among 14 evaluable patients, there were 5 confirmed partial responses (36%) and in 9 patients (64%) a 50% decrease in CA 19-9 was demonstrated.[23]

SWOG S0205 was a phase III trial where 735 eligible patients (PS $\geqslant$ 2, only 13% of patients had a PS = 2) were randomized to weekly gemcitabine or gemcitabine with cetuximab at a loading dose of 400 mg/m^2 on week 1 and 250 mg/m^2 for the following weeks, didn't demonstrate any difference in terms of survival (6 months vs 6.5 months in the cetuximab arm, $P = .14$). The progression-free survival was in favour of the combination (3 months vs 3.5 months in the cetuximab arm, $P = .058$). The response rate was 7% in each arm. Ninety patients presented with at least one grade IV toxicity, 14% in the combination arm and 11% in the monotherapy arm.[24] Also, in a multicenter, randomized phase II trial, 84 patients with advanced pancreatic cancer were randomly assigned to either 250 mg/m^2 of weekly cetuximab, after a loading dose of 400 mg/m^2, plus 1 g/m^2 gemcitabine and 35 mg/m^2 cisplatin on days 1 and 8 of a 21 day cycle or to the same chemotherapeutic regimen without cetuximab. No significant differences were noted between the groups both for objective response (17.5% vs 12.2% in the cetuximab and non-cetuximab group, respectively, $P = 0.549$), and disease control (3.5% higher in the non-cetuximab group, $P = 0.504$). No significant differences between the groups were also noted in median progression-free survival (3.4 vs 4.2 months, $P = 0.847$) and median overall survival (7.5 vs 7.8 months, $P = 0.739$).[25]

However, the addition of cetuximab to gemcitabine–oxaliplatin combination in a phase II trial showed 38% response rate and a 6 month survival rate 54%.[26] These results are awaited to be further confirmed in phase III trials. Another randomized phase II trial also examined the toxicity and efficacy of weekly irinotecan plus docetaxel with or without cetuximab. Grade 3–4 toxicities were significant in both arms, with 4% death rate in the cetuximab arm, compared with 2% in the non-cetuximab arm and shorter median survival time in the cetuximab arm (5.3 months vs 6.5 months).[27]

Finally, sorafenib, a small molecule inhibiting VEGFR2 and Raf-1, was evaluated in a phase II trial, at a dose of 400 mg orally twice daily, in combination with gemcitabine. Sorafenib proved to be well tolerated but ineffective in metastatic pancreatic cancer, offering a median survival of 4 months and a 6 month survival rate of 23%.[28]

Also, another tyrosine kinase inhibitor of the VEGF and PDGF receptor, sunitinib, is being evaluated as second-line therapy in a phase II study by the CALGB.

Second-line therapy

CONKO 003 trial reported that metastatic pancreatic patients with confirmed disease progression on gemcitabine as first-line treatment, had significant survival benefit with oxaliplatin (85 mg/m^2 on days 8 and 22) plus 5-FU (2 g/m^2 over 24 h) and folinic acid (200 mg/m^2 on days 1–8 and 15–22) (OFF regimen) every 42 days, over combination treatment of 5-FU plus folinic acid (FF regimen). The initial design of the study was OFF vs BSC (best supportive care) but the BSC arm was early replaced by the FF regimen due to lack of acceptance of BSC as a control arm. The progression-

free survival was significantly different ($P = 0.012$) and the median survival time from initiation of second-line therapy was 20 weeks vs 13 weeks for the OFF and FF arms, respectively ($P = 0.014$).[29]

Chemoradiation in locally advanced disease

A phase III trial by Chauffert et al. which included 119 patients with locally advanced unresectable pancreatic cancer compared induction chemoradiation and systemic chemotherapy with chemotherapy alone. The patients were randomized to receive 60 Gy (2 Gy per fraction) with concomitant 5-FU (300 mg/m^2/day, days 1–5 for 6 weeks) and cisplatin (20 mg/m^2/day, days 1–5, during weeks 1 and 5) or induction gemcitabine (1 g/m^2 weekly for 7 weeks). Both groups were given maintenance gemcitabine (1 g/m^2 weekly) until progression or toxicity. The results of the trial were in favour of the gemcitabine group, as chemoradiation schedule was more toxic and less effective. The median survival of the chemoradiation group was 8.6 months compared with 13 months in the gemcitabine group. One-year survival was 32% and 53%, respectively, and grade 3–4 toxicity was higher in both induction and maintenance courses of the chemoradiation group.[30]

Moreover, E4201, a randomized phase III study examined the administration of gemcitabine in combination with radiation therapy vs gemcitabine alone in patients with localized, unresectable pancreatic cancer. The study was closed early because of slow accrual. Median overall survival was 11 months for the combination vs 9.2 months for the gemcitabine arm ($P = 0.034$) and also, 24 month-survival was 12% and 4% for the two groups, respectively. However, the combination of gemcitabine with radiation therapy was more myelosuppressive and was also associated with considerable gastrointestinal toxicity and fatigue.[31]

New agents

Gemcitabine is considered to be the most active agent in the treatment of metastatic pancreatic cancer. Although most studies have used gemcitabine combinations with rather disappointing results, studies have also begun to evaluate the role of new agents in the treatment of metastatic pancreatic cancer. In addition, advances in the treatment of metastatic pancreatic cancer might be achieved by investigating strategies of matching each individual's cancer with the most effective available drugs. A novel micellar formulation of paclitaxel in a low molecular weight biodegradable synthetic polymer has been developed. The substitution of cremophor EL by bioabsorbable polymer results in higher maximally tolerated dose and lower toxicity. In a phase II study, 56 chemotherapy-naive pancreatic cancer patients were treated with 3 h infusion of the new formulation of paclitaxel at a dose of 300–435 mg/m^2 every 21 days. The overall response rate was 6.7%, the median time to progression was 3 months and the median overall survival was 6.2 months. The most common grade 3 toxicities were neutropenia (18%), fatigue (18%), infection (13%) and peripheral sensory neuropathy (11%). These results suggest that the new formulation of paclitaxel was well tolerated and resulted in progression-free survival similar to that seen historically with gemcitabine.[32]

Telomerase is expressed in 85–90% of pancreatic cancer and immunogenic telomerase peptides have been characterised. A phase I/II study was conducted to investigate the safety, tolerability, and immunogenecity of telomerase peptide vaccination. Survival of the patients was also recorded. Forty-eight patients with non-resectable pancreatic cancer received intradermal injections of the telomerase peptide GV1001 at three dose levels, in combination with granulocyte–macrophage colony-stimulating factor. The treatment period was 10 weeks. Monthly booster vaccinations were offered as follow-up treatment. The vaccine was well tolerated and 1-year survival for the evaluable patients in the intermediate dose group was 25%. These data indicate that induction of an immune response is correlated with prolonged survival, and the vaccine may offer a new treatment option for pancreatic cancer patients, encouraging further clinical studies.[33]

Also, the identification of new targets will hopefully provide with promising strategies for individualized treatment. Such a new target is S100P, which has been found to be overexpressed in pancreatic, lung and breast cancer. The overexpression leads to tumor growth and metastasis and high levels of S100P has shown resistance to cytotoxic drugs in vitro and gemcitabine in vivo. Cromolyn binds S100P and increases chemosensitivity of gemcitabine in experimental models.[34]

Additionally, preclinical testing has shown that patients with BRCA-2 germline mutations are sensitive to mitomycin-C and this is being tested in pancreatic cancer patients (7% are associated with BRCA-2 mutations) at Johns Hopkins in a phase II trial.[35]

Finally, studies have shown that the overexpression of a gemcitabine transporter in pancreatic cancer cells (hENT-1, human equilibrative nucleoside transporter 1) is associated with longer overall survival in patients treated with gemcitabine.[36]

Adjuvant therapy

The majority of patients diagnosed with pancreatic cancer present at an advanced stage that precludes cure. In patients with localized disease, surgical resection is the only potentially curative therapy. Both local and systemic recurrences are common after a pancreaticoduodenectomy or total pancreatectomy. This pattern of failure suggests that both systemic and local adjuvant therapy may have a positive impact on survival. In an attempt to improve outcome, the efficacy of adjuvant chemoradiotherapy and chemotherapy has been evaluated in several trials. However, the role of adjuvant treatment in pancreatic cancer is an area of conflict. The first study showing survival benefit with adjuvant chemoradiotherapy with bolus 5-FU (20 months vs 11 months, 5-year survival 18% vs 8%) was GITSG. However, the study was criticized for the small number of patients, the early closing and the low doses of radiation therapy.[37] In a retrospective study, 5-FU-based chemotherapy and radiation was evaluated in 616 (observation only 345, adjuvant chemoradiation 271) pancreatic cancer patients at Johns Hopkins Hospital. Overall median survival was 17.9 months. Groups were similar with respect to tumor size, nodal status, and margin status, but the chemoradiation group was younger ($P < .001$), and less likely to present with a severe comorbid disease ($P = .001$). Patients with carcinomas larger than 3 cm ($P = .001$), grade 3 and 4 ($P < .001$), margin-positive resection ($P = .001$), and complications after surgery ($P = .017$) had poor long-term survival. Patients receiving chemoradiation experienced an improved median (21.2 vs 14.4 months; $P < .001$), 2-year (43.9% vs 31.9%), and 5-year (20.1% vs 15.4%) survival compared with no chemoradiation. After controlling for high-risk features, chemoradiation was still associated with improved survival (relative risk).[38]

The European Organization of Research and Treatment of Cancer (EORTC) compared 5-FU (25 mg/kg/day continuous infusion for 5 days every 4 weeks) with concurrent radiotherapy using a split course (40 Gy) with observation only in patients with resected pancreatic and periampullary cancer. Klinkenbijl et al. were able to show a trend toward benefit in terms of median survival (24.5 months vs 19.0 months; $P = 0.208$). The subgroup analysis looking only at pancreatic cancer patients showed a trend toward benefit in median survival (17.1 months vs 12.6 months; $P = 0.099$). This study was also criticized for suboptimal dose of radiotherapy and split courses. Lower radiother-

apy dose and split courses may have allowed cancer repopulation between courses, thereby, under-estimating the benefit of chemoradiotherapy.[39]

ESPAC-1 (European Study Group for Pancreatic Cancer) was the largest randomized study investigating the role of chemoradiotherapy with 5-FU administration. Patients were randomized to receive chemoradiotherapy alone (20 Gy over a two-week period plus 5-FU), 75 patients chemotherapy alone (5-FU), 72 patients chemoradiotherapy and chemotherapy, and 69 patients observation only. The estimated 5-year survival rate was 10% among patients assigned to receive chemoradiotherapy and 20% among patients who did not receive chemoradiotherapy ($P = 0.05$). The 5-year survival rate was 21% among patients who received chemotherapy and 8% among patients who did not receive chemotherapy ($P = 0.009$). The benefit of chemotherapy persisted after adjustment for major prognostic factors.[40] However, this latter finding is in contrast to earlier studies of adjuvant chemotherapy with 5-FU combinations from Norway and Japan that did not suggest a prolonged beneficial effect of 5-FU on survival. Thus, the results for adjuvant regimens based on systemic 5-FU with or without external radiotherapy are conflicting.

RTOG 9704 evaluated the benefit of adding gemcitabine to chemoradiotherapy with 5-FU. In this Intergroup trial involving RTOG, ECOG and SWOG, 442 eligible patients post gross total resection of pancreatic adenocarcinoma (pathological stage T 1–4, N 0–1, M0) were randomized to receive pre- and post- 5-FU chemoradiotherapy vs pre- and post-chemoradiotherapy with gemcitabine. 5-FU was administered as a continuous infusion (CI) at a dose of 250 mg/m^2/day. Gemcitabine was administered at a dose of 1000 mg/m^2 IV weekly. Both were given over 3 weeks pre- and 12 weeks post-chemoradiotherapy. The dose of chemoradiotherapy was 50.4 Gy, 1.8 Gy/fraction/day, with 250 mg/m^2/day 5FU during radiation for all patients. Patients were stratified by nodal status (uninvolved vs involved), primary tumor diameter (<3 cm vs >3 cm) and surgical margins (negative vs positive vs unknown). Survival was the primary end point. Patients with pancreatic head tumors ($n = 380$) experienced significantly improved survival, with median and 3-year survival of 18.8 months and 31% for the gemcitabine arm vs 16.7 months and 21% for the 5-FU arm, respectively ($P = 0.047$). When analysis was inclusive of patients with body/tail tumors ($n = 442$) no significant difference in survival was found ($P = 0.20$). No significant difference in non-hematologic grade >3 toxicity was seen. The grade 4 hematologic toxicity rate was 14% in the gemcitabine arm and 2% in the 5-FU arm ($P < 0.0001$) with no difference in febrile neutropenia/infection.[41]

Also, CONKO-001 study randomized 368 patients to receive gemcitabine 1 g/m^2 on days 1, 8, 15 every 4 weeks, for 6 months, as adjuvant treatment after complete resection vs observation. During a median follow-up of 53 months, 133 patients (74%) in the gemcitabine group and 161 patients (92%) in the observation group developed recurrent disease. Median disease-free survival was 13.4 months for the gemcitabine group and 6.9 months for the observation group ($P < .001$). Median overall survival was also in favour for the gemcitabine group (22.8 months vs 20.2 months, $P < 0.005$), while 1-year overall survival was 72% for the gemcitabine arm and 72.5% for the observation arm, 3-year overall survival 36.5% vs 19.5% and 5-year overall survival 21% vs 9.0% for the gemcitabine and observation group, respectively.[42] The RTOG 9704 and CONKO-001 results support the administration of adjuvant chemotherapy with gemcitabine, but the role of radiation therapy is still controversial.

ESPAC-3 is a study which will give an answer regarding chemotherapy with gemcitabine vs 5-FU in completely resected disease. For the moment, adjuvant 5-FU and gemcitabine are both applied in current practice and the addition of gemcitabine to chemoradiotherapy with 5-FU in head pancreatic cancer is justified.

Neo-adjuvant therapy

The rationale of neo-adjuvant therapy is based on the improvement of local control and survival by downsizing the tumor and subsequently achieving R0 resection. Regarding the neo-adjuvant setting, Evans et al. assessed the outcome of 86 patients with pancreatic adenocarcinoma and potentially resectable disease who received chemoradiation with 7 weekly infusions of gemcitabine (400 mg/m^2) plus radiation therapy (30 Gy in 10 fractions over 2 weeks). The patients underwent restaging after the completion of therapy and 74% of the patients had a successful pancreaticoduodenectomy. Median survival was 34 months for the patients who underwent surgery and 7 months for the unresected patients ($P < 0.01$) and the 5-year survival was 36% and 0% for the two groups, respectively.[43]

Varadhachary et al. conducted a phase II trial in 90 patients with stage I/II disease who underwent chemotherapy with gemcitabine and cisplatin in addition to chemoradiation and pancreaticoduodenectomy. Chemotherapy was given every 2 weeks for 4 doses (gemcitabine 750 mg/m^2 – cisplatin 30 mg/m^2) and chemoradiation consisted of 4 weekly infusions of gemcitabine (400 mg/m^2) plus radiation delivered for 5 days per week. Sixty-six percent of the patients underwent pancreaticoduodenectomy and had a median survival of 31 months compared with 10.5 months of the unresected patients ($P < 0.01$).[44] The encouraging survival observed in the above trials supports the continued investigation of gemcitabine-based preoperative therapy in resectable disease.

Future directions

Several clinical trials are open and are awaited to give information on the treatment of pancreatic disease. A phase II study of erlotinib plus everolimus (RAD 001) in patients with previously treated advanced disease and a phase I trial of radiotherapy with concurrent bevacizumab, erlotinib and capecitabine for locally advanced pancreatic cancer are ongoing. Regarding resectable disease, a phase II randomized study of preoperative chemotherapy with gemcitabine plus erlotinib with or without radiation therapy and a phase II trial of pancreaticoduodenectomy plus postoperative cisplatin, interferon alpha 2b and 5FU combined with radiation treatment are still involved in patient recruitment.

Conclusion

Pancreatic cancer remains a major therapeutic challenge with the majority of patients having advanced disease at the time of diagnosis and consequently a dismal prognosis. The addition of a molecular-targeting agent, such as erlotinib, to standard gemcitabine in the metastatic setting is an effective treatment prolonging median overall survival by 10 days compared with gemcitabine alone, as reported in a phase III trial by Moore et al. On the other hand, most chemotherapy doublets with gemcitabine have not produced better survival outcomes. However, combinations with platinum analogues or capecitabine, especially in good performance status patients, achieved a prolongation in survival.

In addition, the combination of oxaliplatin-5FU-folinic acid should be considered as a standard second-line treatment for gemcitabine refractory patients.

In the adjuvant setting, current studies have supported the significant role of gemcitabine, either as monotherapy or in combination with chemoradiotherapy with 5-FU, while ESPAC-3 is an ongoing trial expected to give answers on adjuvant chemotherapy. In the meanwhile, the administration of both 5-FU and gemcitabine are accepted adjuvant treatment strategies. With regard to

neo-adjuvant therapy, there are no data to support it outside of clinical trials.

Lastly, drug development focuses on the identification of new targets which will, hopefully, be the basis of individualized strategies for the treatment of advanced disease, with a significant clinical impact, as well.

Conflict of interest statement

None declare.

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review

Annals of Oncology 00: 1–8, 2013
doi:10.1093/annonc/mdt166

Second-line treatment in advanced pancreatic cancer: a comprehensive analysis of published clinical trials

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Received 22 January 2013; revised 20 March 2013; accepted 21 March 2013

Background: There is currently no standard of care for the second-line treatment of advanced pancreatic cancer. The aim of this analysis was to compare the different therapeutic approaches in this setting.
Methods: We carried out a systematic analysis of second-line studies in advanced pancreatic cancer that have progressed on or following gemcitabine and published or presented from 2000 to 2012.
Results: Forty-four clinical trials (*t*) were identified; of which 34 met the inclusion criteria treating an aggregate total of 1503 patients (*n*). Patients who received treatments (*t*: 33; *n*: 1269) had a median overall survival (OS) of 6 months compared with 2.8 months for patients who received best supportive care only (*t*: 2; *n*: 234) ($P = 0.013$). The gemcitabine and platinum-based combination (t: 5; *n*: 154) provided a median progression-free survival and OS of 4 and 6 months compared with 1.6 and 5.3 for the rest of the regimens (*t*: 29; *n*: 1349) ($P = 0.059$ and 0.10, respectively) and 2.9 and 5.7 for the combination of 5-fluorouracil and platinum agents (*t*: 12; *n*: 450) ($P = 0.60$ and 0.22, respectively).
Conclusion(s): Although not conclusive, these data showed that the advantage of second-line chemotherapy in pancreatic cancer is very limited and there is a need for more studies.
Key words: analysis, cancer, pancreatic, review, second-line, treatment

introduction

Pancreatic cancer has an estimated 5-year survival rate of 5%–6% and the majority of patients present with unresectable disease [1, 2]. For the past 10–15 years, gemcitabine has been considered the front-line chemotherapy in both locally advanced and metastatic disease due to its positive effect on quality of life and—to a lesser extent—overall survival [3]. While gemcitabine-based combinations have not been shown to be unequivocally more effective compared with gemcitabine alone, several analyses have suggested benefit in defined subpopulations such as patients with good performance status (PS) and metastatic disease [4–6]. Recently, FOLFIRINOX has emerged as an alternative to gemcitabine in the first-line setting after demonstrating superior survival outcome (median OS 11.1 versus 6.8 months, $P < 0.001$) [7]. However, this regimen is not suitable for patients with poor performance status (PS) and for these patients gemcitabine-based therapy will remain a favorable first-line option [7, 8]. In the second-line setting, there is no consensus on the optimal treatment. This is due, in part, to the paucity of trials in this patient population. In addition, only ≤50% of patients who fail first-line treatment are still physically fit enough to be offered second-line treatment [4, 7]. It has also not been unequivocally established that chemotherapy provides better efficacy compared with best supportive care (BSC), since studies that tried to address this question were underpowered and poorly designed [9, 10]. To further address these questions, we carried out a comprehensive analysis of the second-line trials in locally advanced or metastatic pancreatic cancer.

methods

The primary objectives of this study were to determine whether treatment provides any superior effect over BSC and to determine the regimen that provides the best outcome. Secondary objectives were to compare the outcome of platinum-based compounds in combination with either gemcitabine or 5-fluorouracil (5-FU) and to determine the trend of treatment outcomes over time. We identified the data for this analysis by performing a PubMed search using the term 'second-line therapy AND advanced pancreatic cancer'. In addition, we reviewed the references of the relevant articles and the abstracts presented in ASCO, GI ASCO, ESMO, ECCO, and WCGC. Searches were limited to human studies published in English from 2000 to 2012. Exclusion criteria

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were trials that used chemotherapy other than gemcitabine in the first-line setting, novel investigational or targeted agents other than erlotinib in the second-line setting. Targeted agents were excluded since they represent a class of drugs with different mechanisms of action. However, since erlotinib is the only targeted agent that showed a survival benefit in the first-line setting [11], trials that used erlotinb were included. The following details were extracted: study start and completion dates, number of patients, second-line regimen, and outcomes, including the percentage of responders or the response rate (RR), the median progression-free survival (PFS), and overall survival (OS). In the trials that included more than one arm, each arm was analyzed separately.

statistical analysis

For each trial or arm in the analyses, using results as presented in the relevant publications, the percentage who responded (RR), the median PFS and the median OS were obtained and used as the primary data being analyzed. In an exploratory manner, we compared the distributions of those three outcome variables (RR, PFS, and OS) according to the following categorical variables with the Wilcoxon rank sum test: BSC versus all others, 5-FU plus platinum agents versus all others (but excluding BSC), gemcitabine plus platinum agents versus all others (but excluding BSC), taxane-based regimens versus all others (but excluding BSC), erlotinib-based regimens versus all others (but excluding BSC), and gemcitabine plus platinum agents versus 5-FU plus platinum agents. Exact tests were used as appropriate. All reported P-values are two tailed. In view of the number of tests carried out, we considered $P < 0.005$ as statistically significant, while $0.005 < P < 0.05$ indicated a strong statistical trend.

results

The results of the search identified 38 published trials and 6 abstracts presented at scientific meetings. These 44 trials (t) comprised of 53 arms (a) and treated an aggregate total of 2384 patients (n). Out of these 44 trials, 7 used targeted therapy other than erlotinib [12–18], 2 used novel investigational chemotherapy [19, 20], and the efficacy data were not reported in one trial [7]. Therefore, only 34 trials met the inclusion criteria [9, 10, 21–52] comprising of 38 arms and treating an aggregate total of 1503 patients. The search results are summarized in Figure 1 and supplementary Table S1, available at *Annals of Oncology* online .

BSC versus treatments

In order to determine whether second-line treatment has any impact on outcome, we reviewed the clinical trials that included a BSC arm in their designs. Two phase III trials compared BSC to 'active' treatments [9, 10]. The first study by the German CONKO-study group was a phase III trial that randomized patients in a 1 : 1 ratio to BSC or OFF (oxaliplatin, folinic acid, and 5-FU) [10]. A total of 165 patients were required to demonstrate a statistical difference in survival. However, only 46 patients were accrued and this trial was terminated early. Patients on the OFF arm (n: 23) had median OS of 4.82 months compared with 2.30 months in the BSC arm (n: 23) ($P = 0.008$). In the second study by Jacobs et al., the physician's best choice (BC) including BSC (n: 211) was compared with rubitecan, an oral topoisomerase I inhibitor that showed promising activity in previous studies (n: 198) [9]. The majority of patients on the BC arm (89%) received alternative chemotherapy leaving only 11% of patients (n: 23) to receive BSC only. In addition, 49% of patients on the BC arm crossed over to the rubitecan arm at time of progression. This trial reported no significant difference in median OS between BC and rubitecan (3.3 versus 3.8 months, $P = 0.62$). Patients who crossed over to the rubitecan arm had a longer median survival compared with patients who did not (5.2 versus 2 months, $P < 0.0001$). In our analysis, we compared the outcomes of BSC in these two trials (a: 2; n: 234) to the outcomes of all treatments administered in the remaining 36 analyzed arms (a: 36; n: 1269). We found a trend toward an improved OS with treatments compared with BSC only

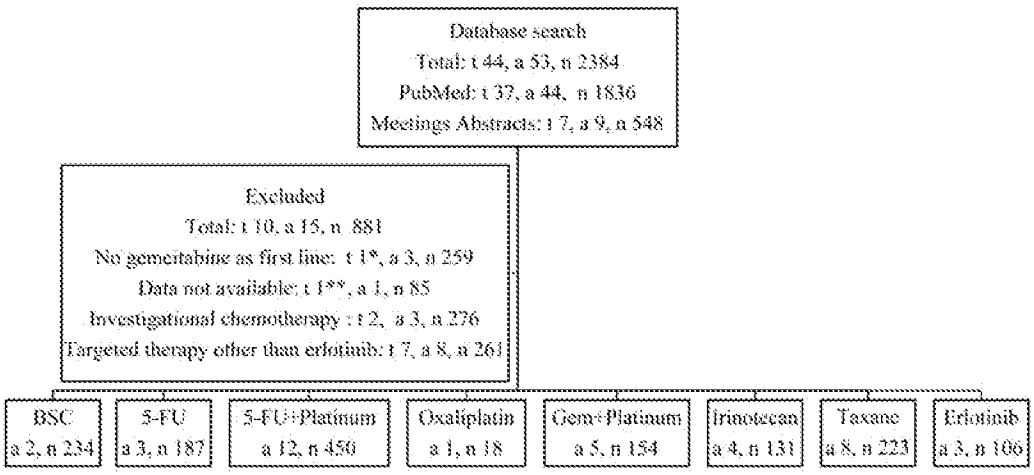

Figure 1. Study selection. t, number of trials; a, number of arms; n, number of treated patients; BSC, best supportive care; 5-FU, 5-fluorouracil; Gem, gemcitabine. In the trial by Conroy et al., patients were randomized to two arms FOLFIRINOX or gemcitabine then received a second-line of gemcitabine if they progressed on FOLFIRINOX* or 5-FU-based regimen if they progressed on gemcitabine**.

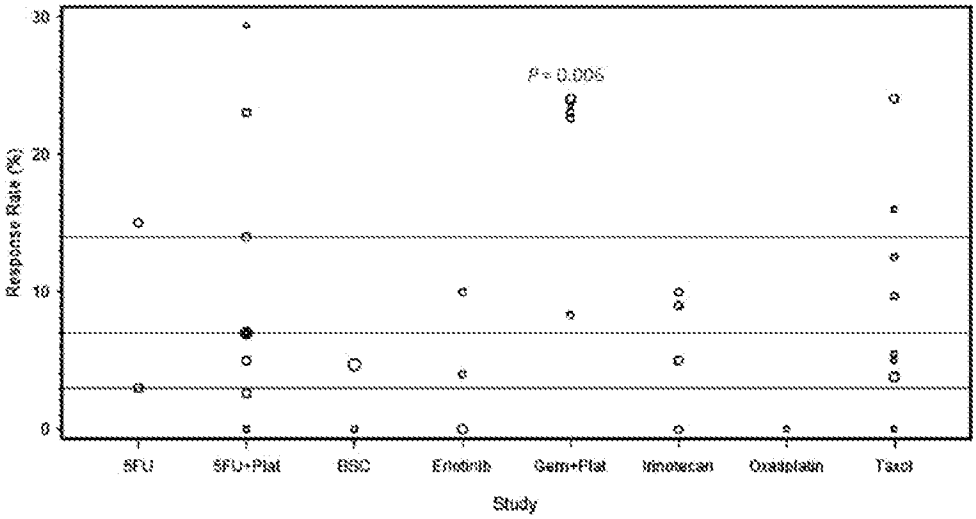

Figure 2. The response rate (RR) of each of the analyzed studies presented as dot plots. The three horizontal lines in each figure represent the quartiles of all the data combined. Circle size is proportional to the number of patients on each trial. The combination of gemcitabine (Gem) and platinum agents (Plat) provided a trend toward an improved RR ($P = 0.006$) compared with the other regimens.

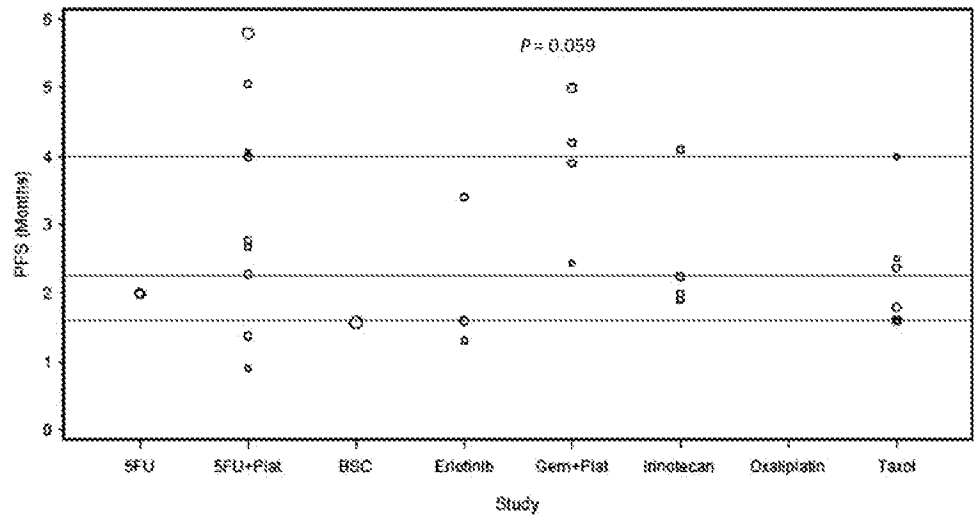

Figure 3. The median progression-free survival (PFS) of each of the analyzed studies presented as dot plots. The three horizontal lines in each figure represent the quartiles of all the data combined. Circle size is proportional to the number of patients on each trial. The combination of gemcitabine (Gem) and platinum agents (Plat) provided a trend toward an improved PFS ($P = 0.059$) compared with the other regimens.

($P = 0.013$). However, there was no statistical difference in RR or PFS ($P = 0.20$ and 0.26, respectively) (Figures 2–4).

5-FU in combination with platinum agents versus other treatments

The combination of platinum agents and 5-FU has shown activity in several GI malignancies including esophageal, gastric, and colorectal cancers [53, 54]. We examined the activity of this combination in the second-line setting in pancreatic cancer. Twelve trials evaluated the efficacy of 5-FU in combination with either oxaliplatin or cisplatin, treating a total of 450 patients. Oxaliplatin was combined with either 5-FU—in 8 trials (*n:* 279) [10, 21, 22, 26–28, 31, 33]—or capecitabine—in 2 trials (*n*: 54) [29, 34]. Two trials used cisplatin in combination with either 5-FU (*n:* 100) [32] or S-1 (*n:* 17) [30]. The median number of treated patients per trial was 30 with a range of (15–100). Of these 12 trials, the CONKO-003 trial was the only phase III randomized study comparing OFF (oxaliplatin, folinic acid, and 5-FU) to FF (folinic acid and 5-FU) [22]. The CONKO-003 trial showed a survival benefit of adding oxaliplatin to 5-FU (5.89 versus 3.09 months, $P = 0.01$). In our analysis, the combination of 5-FU and platinum agents provided a median RR of 7% with a range of (0–29.4). The median PFS and OS were 2.9 and 5.7 months with a range of (0.9–5.8) and (1.3–10.7), respectively. The combination of 5-FU and platinum agents (*a*: 12, *n:* 450) did not show superior outcomes compared with the rest of the treatments (*a*: 26, *n:* 1053) in terms of RR, PFS or OS ($P = 0.50$, 0.27, 0.76, respectively) (Figures 2–4).

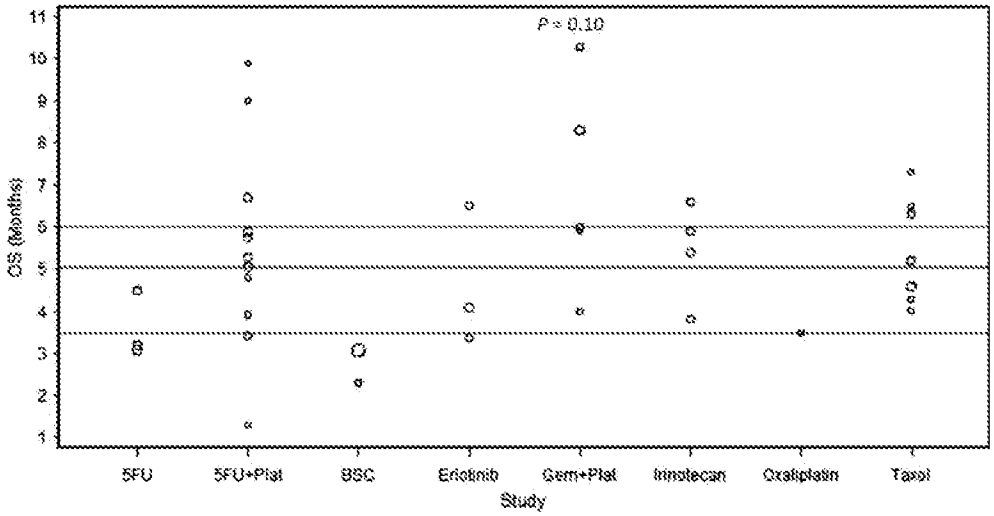

Figure 4. The median overall survival (OS) of each of the analyzed studies presented as dot plots. The three horizontal lines in each figure represent the quartiles of all the data combined. Circle size is proportional to the number of patients on each trial. The combination of gemcitabine (Gem) and platinum agents (Plat) did not provide a significant improvement in OS ($P = 0.10$) compared with the rest of the regimens.

gemcitabine in combination with platinum agents versus other treatments

Combined analyses have suggested a potential survival benefit from adding platinum agents to gemcitabine compared with gemcitabine alone in the first-line setting in advanced pancreatic cancer [4–6]. We sought to determine the efficacy of this combination in the second-line setting. Five trials investigated the effect of adding platinum agents to gemcitabine after disease progression on gemcitabine, treating a total of 154 patients. Gemcitabine was combined with oxaliplatin in 2 trials (*n:* 50) [45, 49]; while the remaining three trials investigated the combination of gemcitabine with liposomal cisplatin (*n:* 24) [46], cisplatin plus 5-FU and epirubicin (*n:* 46) [47], or cisplatin plus 5-FU and irinotecan (*n:* 34) [48]. Gemcitabine was administered as a fixed dose rate (FDR) of 10 mg/m^2/min in four trials [45, 47–49] and as a standard infusion rate over 30-min in one trial [46] (supplementary Table S2, available at *Annals of Oncology* online). The median number of treated patients per trial was 33 with a range of (17–46). The RR ranged from 8.3 to 24% with a median of 23%. The median PFS and OS were 4 and 6 months with a range of (2.4–5) and (4–10.3), respectively. When compared with other treatments (*a*: 33, *n:* 1349) the combination of gemcitabine and platinum agents (*a*: 5, *n:* 154) provided a trend toward an improved RR and PFS ($P = 0.006$ and 0.059, respectively) with no significant improvement in OS ($P = 0.10$). When compared with 5-FU in combination with platinum agents (*a*: 12, *n:* 450), the combination of gemcitabine and platinum agents (*a*: 5, *n:* 154) showed a strong trend toward an improved RR ($P = 0.03$) with no difference in PFS or OS ($P = 0.60$, 0.22, respectively) (Figures 2–4).

taxane-based regimens versus other treatments

Taxane-based chemotherapy is considered the standard of care in many malignancies including breast and lung cancers [55, 56]. We analyzed the activity of this treatment in the second-line setting in pancreatic cancer. Seven trials used taxane-based regimens treating a total of 223 patients. Of these seven trials, only one treated patients on two arms, irinotecan plus raltitrexed (*n*: 19) versus raltitrexed alone (*n*: 19) [38]. Taxane was used as a single agent in four trials (*n:* 108) [38–40, 44] and in combination with either capecitabine—in two trials (*n:* 55) [41, 43]—irinotecan (*n:* 19) [38] or oxaliplatin (*n:* 41) [42]—in 2 trials. The median number of treated patients per trial was 21 with a range of (18–52). The RR ranged from 0 to 24% with a median of 8.7%. The median PFS and OS were 2 and 5.2 months with a range of (1.6–4) and (4.3–7.3), respectively. Our analysis showed no superior outcomes for taxane-based therapy (*a*: 8, *n:* 223) in comparison with other regimens (*a*: 30, *n:* 1280) in terms of RR, PFS, or OS ($P = 0.81$, 0.33, 0.59, respectively) (Figures 2–4).

erlotinib versus other treatments

Erlotinib is the only targeted agent that showed a survival benefit when combined with gemcitabine in the first-line setting [11]. In an attempt to identify the activity of this agent in the second line, we analyzed the three trials that used erlotinib in this setting and treated a total of 106 patients. One trial used erlotinib as a single agent (*n:* 50) [51], while two trials used erlotinib in combination with capecitabine (*n:* 30) [50] or bevacizumab (*n:* 26) [52]. The median number of treated patients per trial was 30 with a range of (26–50). The RR ranged from 0 to 10% with a median of 4%. The median PFS and OS were 1.6 and 4.1 months with a range of (1.4–3.4) and (3.7–6.5), respectively. Our analysis demonstrated that erlotinib-based regimens (*a*: 3, *n:* 106) failed to show any statistical significant improvement in RR, PFS, or OS when compared with the other regimens (*a*: 35, *n:* 1397) ($P = 0.39$, 0.21, 0.52, respectively) (Figures 2–4).

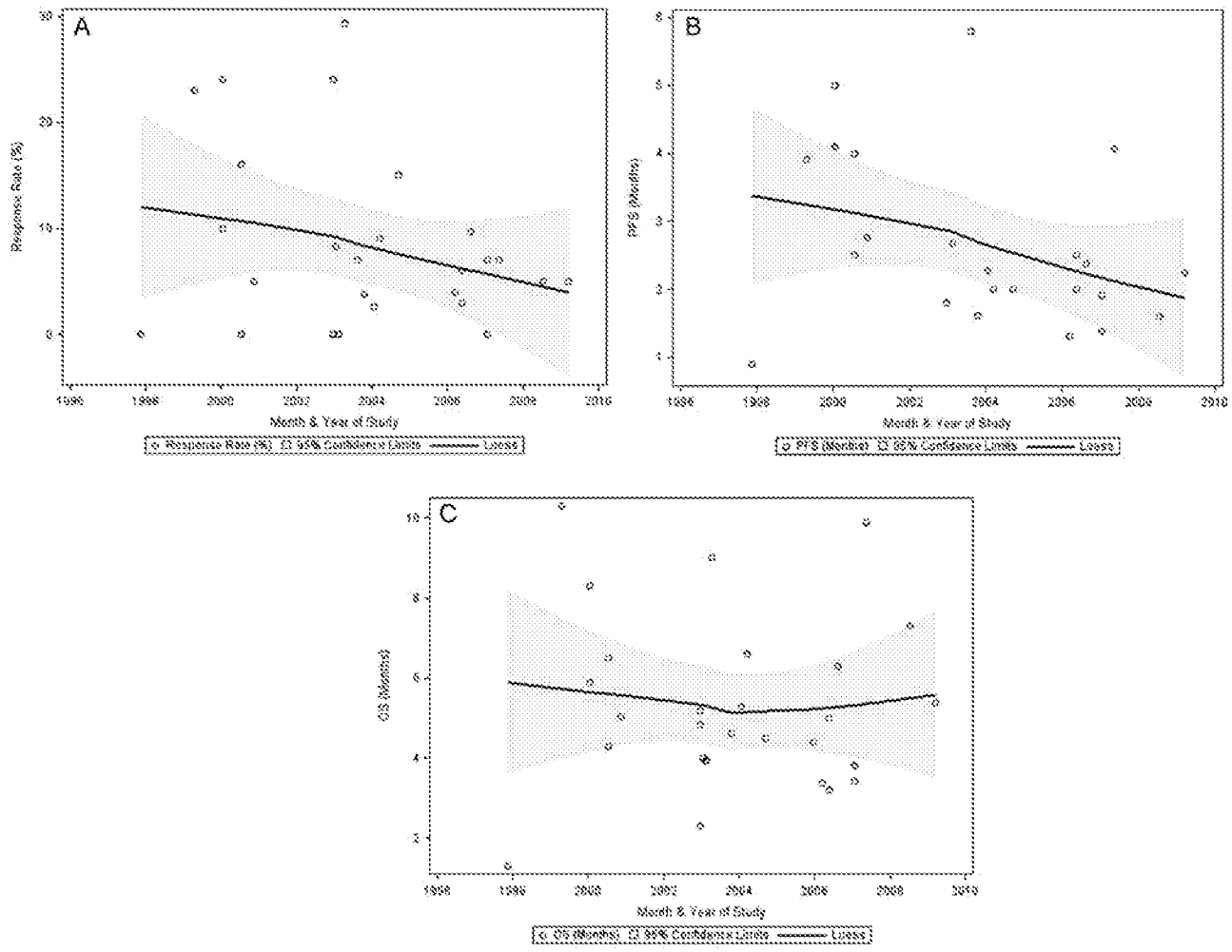

Figure 5. The treatment outcome trends over time plotted against the month and the year of the studies including (A) the response rate (RR), (B) the progression-free survival (PFS), and (C) the overall survival (OS).

treatment effect trend over time

Given the lack of progress in pancreatic cancer treatment, we analyzed the outcome trends over time in the second-line setting. We plotted the RR, PFS, and OS of each of the analyzed regimens over the last 13 years as shown in Figure 5. The earliest starting date of the studies was November 1997 and the latest ending date was August 2010. The median RR was 8.3%. The median PFS and OS were 2.9 and 6 months, respectively. Unexpectedly, there was a negative trend for RR and PFS over time while there was no change in OS.

discussion

There is currently no standard of care for locally advanced or metastatic pancreatic cancer that has progressed following either FOLFIRINOX [7] or gemcitabine-based regimen [6, 11]. While there are potential options, there is no proven benefit for any regimen and treatment choice is generally an extrapolation from front-line studies. This comprehensive analysis indicates a benefit of treatment, mainly with the combination of gemcitabine and platinum agents, in patients who have progressed on gemcitabine in the first-line setting.

Given the modest impact of chemotherapy in pancreatic cancer, the first question is whether there is a proven benefit associated with any therapy compared with BSC. In contrast to other GI malignancies such as colorectal and gastric cancers where the evidence of chemotherapy benefit over BSC in the second-line setting is established [57, 58], such evidence is lacking in pancreatic cancer. The German CONKO Group trial was stopped early due to insufficient accrual [10]. Likewise in the study by Jacobs et al., only 11% of the patients on the BC arm received BSC only (*n:* 23) with almost 50% crossover rate to the treatment arm [9]. In our analysis, the treatments provided a trend toward an improved OS compared with BSC only (median OS of 6 versus 2.8 months, $P = 0.013$). However, these results are limited by the small patient samples on the BSC arms and the lack of quality-of-life assessment on both of these trials. Indeed, randomizing patients to BSC will remain a challenge given this patient population's poor prognosis.

Owing to the improvement in OS provided by the addition of oxaliplatin to 5-FU (*n:* 76) compared with 5-FU (*n:* 84) in the CONKO 3 study (5.89 versus 3.09 months, $P = 0.010$), this regimen has been widely used in the second-line setting [22]. In the CONKO 3 study, patients on the combination arm

received more cycles of chemotherapy and had lower pain level assessment, which could be attributed to a better disease control. As expected, patients with good PS derived the most survival benefit. Although our analysis demonstrated no statistical significant improvement in outcomes of the 5-FU and platinum agents combination (*a*: 12; *n:* 450) compared with the rest of the regimens (*a*: 26, *n:* 1053), it did show a similar efficacy compared with gemcitabine and platinum agents combination (*a*: 5; *n:* 154) in terms of PFS and OS. Of note, these analyzed regimens used different platinum agents, 5-FU doses, and schedules.

Indeed gemcitabine remains the first-line treatment option for patients who are not candidates for FOLFIRINOX. However, the majority of patients develop resistance to gemcitabine in a short period of time suggesting a pre-existence of resistant cell subpopulations or stromal alterations [59, 60]. The combination of gemcitabine and platinum agents (*a*: 5, *n:* 154) was the only regimen that provided superior outcomes compared with the rest of the regimens (*a*: 33, *n:* 1349) in terms of RR and PFS ($P = 0.006$ and 0.059, respectively). However, the improvement in RR and PFS did not translate into a survival benefit ($P = 0.10$). This may have been influenced by subsequent treatments, the method of gemcitabine administration (FDR of 10 mg/m^2/min versus 30-min infusion standard rate), and the amount of cycles the patients were able to receive based on the regimen's tolerability (supplementary Table S2, available at *Annals of Oncology* online).

Despite many efforts to improve the outcomes of the second-line treatments in advanced pancreatic cancer, these outcomes remain dismal. We demonstrated a worsening trend over the last decade in RR (median 8.3%) and PFS (median 2.9 months) with no change in OS (median 6 months) (Figure 5). One possibility to explain these trends is the incorporation of the RECIST criteria 'Response Evaluation Criteria in Solid Tumors (RECIST)' in the assessment of tumor response and time to progression in trials conducted after the year of 2000, resulting in a strict standardized evaluation of outcomes [61]. Noteworthy, neither PFS nor RR was found to be validated surrogate of OS in pancreatic cancer. It has been established that performance status and disease stage, locally advanced versus metastatic, have a major impact on outcome over any treatment effect in pancreatic cancer [62]. However, here we found no evidence for correlation between any of these variables and PFS or OS (data not shown).

To our knowledge, this is the first analysis to compare systematically the efficacy of the most widely used regimens in the second-line setting in pancreatic cancer. Our analysis is limited by the small sample size, the lack of randomization, the heterogeneity of the patients' characteristics and regimens, and the exploratory nature of our statistical design. In addition, our data should be interpreted carefully due to the large selection bias since only $\leq$50% of patients who received first-line treatment qualified for a second line.

Furthermore, these second-line regimens have been used in patients who were not gemcitabine-naïve. This practice is likely to change since FOLFIRINOX became the standard first line in patients with good performance status. As a result, gemcitabine would become, by default, the standard second-line agent. Whether gemcitabine is the appropriate choice and whether it should be used as a single agent or in combination with other agents after FOLFIRINOX failure remains to be determined.

Novel approaches in pancreatic cancer treatment are desperately needed. There have been some advances in the recent years in the molecular and biological understanding of this disease. These advances include the discovery of the important role of the stroma in the drug delivery to the cancer cells [63], the diverse genetic alteration especially in metastatic disease [64], and the impact of stem cells on disease resistance to chemo and radiation therapy [65]. These discoveries may provide the future landscape of pancreatic cancer treatment.

In conclusion, our data support the use of chemotherapy over best supportive care in the second-line setting in pancreatic cancer. The combination of platinum agents with either gemcitabine or 5-FU is preferred in comparison with other regimens. However, the survival benefit provided by these combinations is limited and should be interpreted with caution given the selection bias in this patient population. There is a clear need for well-designed, randomized, and adequately powered clinical trials in the second-line setting after FOLFIRNOX failure. Indeed, future efforts must focus on individual therapy strategies including identifying genetic mutations and new biomarkers predictive of response, in addition to studying the molecular biology of these chemotherapy agents (i.e. ERCC-1, methylation of the MLH1 gene, RRM1). Nevertheless, exploiting recent understanding of the pancreatic tumor and stroma microenvironments in order to improve the therapeutic outcome in this disease is needed.

funding

This work was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

disclosure

The authors have declared no conflicts of interest.

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Pharmacokinetic Interrelationships of Irinotecan (CPT-11) and Its Three Major Plasma Metabolites in Patients Enrolled in Phase I/II Trials[1]

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ABSTRACT

Irinotecan (CPT-11) is an analogue of 20(*S*)-camptothecin with promising activity against several tumor types. In patients, CPT-11 is metabolized to 7-ethyl-10-hydroxycamptothecin (SN-38) and to the β-glucuronide of SN-38. Recently, we identified an additional metabolite of CPT-11, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin (APC; L. P. Rivory *et al.*, Cancer Res., *56:* 3689–3694, 1996). The aim of this study was to investigate the interrelationships of all four compounds to identify factors that might be responsible for the large interpatient variability in CPT-11 and SN-38 kinetics. The plasma kinetics of CPT-11, SN-38, the β-glucuronide of SN-38, and APC were studied in 19 patients for a total of 33 cycles (115–600 mg/m^2). Although the area under the concentration curves (AUCs) of all compounds studied increased with dose, there was considerable variability. Ratios of the AUCs of the appropriate compounds were used as estimates of the major routes of metabolism (conversion of CPT-11 to SN-38, metabolism of CPT-11 to APC, and glucuronidation of SN-38). Each ratio varied more than 10-fold across the patient population, and the apparent extent of conversion of CPT-11 to SN-38 was highest at the 115 mg/m^2 dose level. Interestingly, AUC_{SN-38} was greater in patients with both high AUC_{CPT-11} and AUC_{APC}. We conclude that the variability of the pharmacokinetics of CPT-11 and SN-38 is likely to be due to extensive interpatient differences in the pathways implicated in the metabolism of CPT-11.

INTRODUCTION

CPT-11[3] (Fig. 1) is a semisynthetic derivative of 20(*S*)-camptothecin (1) developed in Europe (Campto) for use in colorectal adenocarcinoma that is not responsive to standard 5-fluorouracil-based chemotherapy (2). The camptothecin family of compounds are cytotoxic agents that inhibit the nuclear enzyme topo I (3). topo I plays an important role in overcoming some of the topological problems that arise during the replication and transcription of DNA, principally through the reduction of DNA supercoiling associated with strand separation. Relaxation of DNA by topo I proceeds through transient nicked DNA-enzyme complexes, many of which are stabilized by camptothecins, thereby preventing DNA religation and release of enzyme. These complexes result in the arrest of replication forks and the formation of permanent double-stranded breaks during the S phase of the cell cycle (3). SN-38, a metabolite of CPT-11 produced *in vivo* by carboxylesterases, has an activity *in vitro* that is 100-1000-fold superior to that of CPT-11 itself (4). In patients, SN-38 is glucuronidated to SN-38G, which is present in significant concentrations in plasma, bile, and urine (5, 6). Recently, it has been shown that tardive diarrhea, which is an important toxicity of CPT-11 in most studies, may be associated with higher values of a biliary index calculated from the product of the AUCs of CPT-11 and SN-38 divided by that of SN-38G (7). Therefore, not only is the metabolism and disposition of CPT-11 likely to be important for the activity of the drug, but it would also seem that kinetic factors could be strong correlates of its toxicity. Recently, we reported the identity of the second polar metabolite, which we observed in the plasma of patients treated with CPT-11 (8). This metabolite, APC (Fig. 1), is the product of a ring-opening oxidation of the terminal piperidine ring of CPT-11. In this paper, we present the plasma pharma-

Received 1/2/97; revised 4/23/97; accepted 4/23/97.

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[1] Supported in part by grants from the Association pour la Recherche sur le Cancer (France) and the Groupement des Entreprises Françaises dans la Lutte Contre le Cancer. L. P. R. is the recipient of a National Health and Medical Research Council (Australia)/Institut National de la Santé et de la Recherche Médicale (France) Exchange Fellowship. This investigation was completed thanks to a travel bursary awarded to L. P. R. by the Société Française du Cancer.

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[3] The abbreviations used are: CPT-11, irinotecan; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, β-glucuronide of SN-38; APC, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin; AUC, area under the concentration curve; AUMC, area under the momem curve; topo I, topoisomerase I; $t_{1/2\beta}$, terminal half-life; REC, relative extent of conversion; REM, relative extent of metabolism; REG, relative extent of glucuronidation; Vd_{SS}, volume of distribution at steady state; *CL*, total body clearance; MRT, mean residence time; C_{max}, maximum plasma concentration.

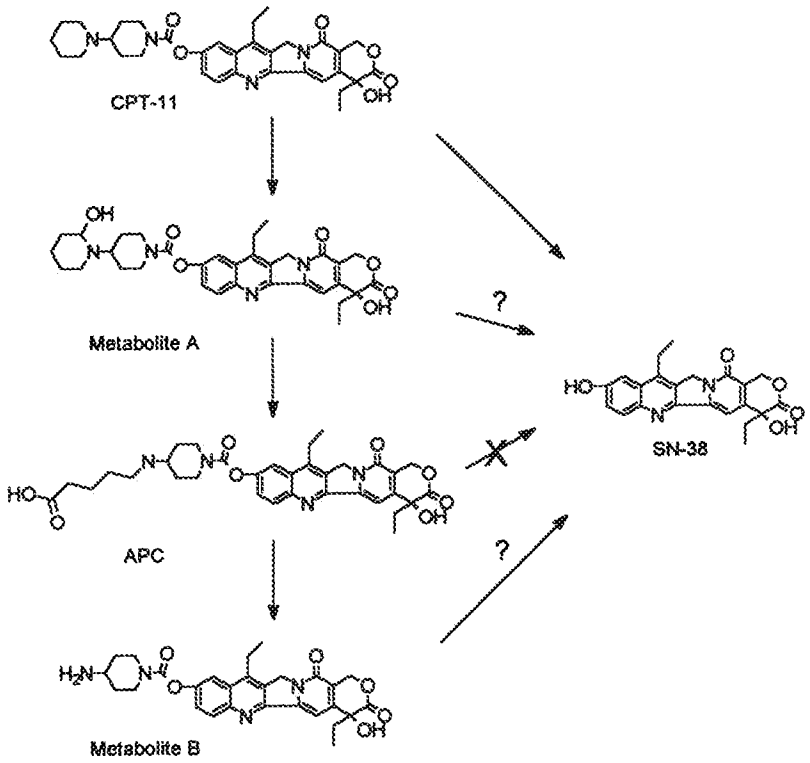

Fig. 1 The chemical structures and pathways implicated in the metabolism of CPT-11. There is some evidence (8) to suggest that production of APC occurs via a monohydroxylated metabolite of CPT-11 (Metabolite A) and that APC may be further metabolized (Metabolite B). Metabolites A and B, which remain to be formally identified and characterized, may participate in the production of SN-38. APC is itself not appreciably transformed to SN-38 by human liver carboxylesterase or liver microsomes (15). Although not shown above, SN-38 is also further glucuronidated.

Table 1 Characteristics of patients studied

	Dose of CPT-11 (mg/m^2)				
	115	300	350	500	600
No.	5	1	8	3	2
Cycles	13	1	9	6	4
Mean age (yrs)	54	61	50	56	56

cokinetics of CPT-11, SN-38, SN-38G, and APC in patients treated with CPT-11 and examine their interrelationships.

MATERIALS AND METHODS

Patients and Pharmacokinetic Studies. A total of 19 patients enrolled in Phase I and II trials in France was studied during treatment with CPT-11 for colorectal adenocarcinoma ($n = 9$), malignancy of the cervix ($n = 3$), metastatic adenocarcinoma of unknown primary origin ($n = 3$), and other cancers ($n = 4$). The patients, whose characteristics are shown in Table 1, had WHO/Eastern Oncology Cooperative Group performance scores of 0–2. They represented either newly recruited patients (Bordeaux) or those for which plasma samples had been previously collected (other centers). CPT-11 was administered i.v. over 30 or 90 min every 3 weeks at doses ranging from 115–600 mg/m^2, except for two patients who received CPT-11 at 115 mg/m^2 as part of a once a week for 3 weeks protocol. CPT-11 lactone (20 mg/ml) was provided by Rhone-Poulenc Rorer SA (Antony, France) and diluted further into 250 ml of sterile 0.9% NaCl. Pharmacokinetic evaluation was performed for a total of 33 cycles. Heparinized blood samples were collected before the commencement of drug infusion, at 15 min in the case of the 30-min infusion protocol and at 30 and 60 min for the 90-min perfusion, at the end of the infusion and then at 5, 10, 15, 30, 45, and 60 min and 2, 4, 8, 12, and 24 h. In some cases, samples were also collected at 48 and 72 h. Plasma was obtained after centrifugation and stored at −20°C until analysis.

Drug Analysis. The plasma concentrations of CPT-11, SN-38, SN-38G, and APC were quantitated by high-performance liquid chromatography after the acidification of deproteinated plasma as described previously (9). Therefore, these compounds were quantitated as total concentrations (lactone + carboxylate). Authentic standards of CPT-11, SN-38, and APC were kindly supplied by Rhône-Poulenc Rorer. The concentrations of SN-38G were estimated using the calibration curve of SN-38, and the relative factor of fluorescence of 0.63 was determined under identical conditions (9).

Pharmacokinetic Analysis. The AUCs of the compounds of interest were calculated using the trapezoidal method and extrapolated to infinity using the terminal rate constant estimated from a regression of the linear semi-log concentration *versus* time profile at later time points. The *CL* of CPT-11 was estimated from the dose divided by the AUC. The AUMC was estimated for CPT-11 also using the trapezoidal rule and extrapolated to infinity. The AUMC was then used to calculate the MRT and the Vd_{ss} of CPT-11 corrected for the influence of the duration of the infusion (τ) using:

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}} - \frac{\tau}{2} \text{ and } Vd_{ss} = \frac{\text{Dose} \times \text{MRT}}{\text{AUC}}.$$

Table 2 The effect of dose on pharmacokinetic parameters of CPT-11 (mean ± SD, $n = 33$)

Dose rate (mg/m^2)	AUC (μM/h)	C_{max} (μM)	CL (liters/h/m^2)	Vd_{ss} (liters/m^2)	$t_{1/2z}$ (h)	MRT (h)
115	9.3 ± 2.3	2.8 ± 1.0	19.3 ± 4.4[a]	121 ± 24	6.6 ± 1.4	6.5 ± 1.6
300–350	42.7 ± 12.6	13.3 ± 6.0	12.9 ± 3.9[a]	76 ± 19	6.1 ± 2.2	6.2 ± 2.2
500	47.7 ± 11.4	9.5 ± 3.4	16.3 ± 4.3	119 ± 48	6.8 ± 2.4	7.8 ± 2.0
600	68.3 ± 13.0	17.3 ± 6.7	13.4 ± 2.7	81 ± 16	4.7 ± 0.2	6.1 ± 0.2

[a] Significantly different, $P < 0.05$ (Kruskal-Wallis test followed by Dunn's multiple comparisons).

The terminal half-life of elimination ($t_{1/2z}$) was estimated as 0.693 divided by the terminal rate constant. The REC of CPT-11 to SN-38, the REM of CPT-11 to APC, and the REG of SN-38 were estimated as:

$$\frac{AUC_{SN\text{-}38}}{AUC_{CPT\text{-}11}}, \frac{AUC_{APC}}{AUC_{CPT\text{-}11}}, \text{ and } \frac{AUC_{SN\text{-}38G}}{AUC_{SN\text{-}38}},$$

respectively.

It should be noted that these are not direct measures of these conversions (see "Discussion") but represent useful pharmacokinetic estimates for the analysis of the dose dependence of metabolic pathways.

Statistical Analysis. Pharmacokinetic parameters (CL, Vd_{SS}, MRT, $t_{1/2Z}$, REC, REM, and REG) were analyzed as a function of the CPT-11 dose level using the Kruskal-Wallis one-way analysis of ranks followed by the Dunn's method for identifying significantly different groups. Correlations between the $t_{1/2Z}$s of related species were carried out with the Spearman rank-order test, as were correlations between AUCs. Multiple regression analysis of the AUCs of CPT-11 and APC as independent variables and SN-38 as the dependent variable was carried out with a partial F test after testing for normality and homoscedasticity (SigmaStat; Jandel Scientific, Corte Madera, CA). Statistical significance was considered to be reached when $P < 0.05$ with a two-tailed distribution. Data are presented as mean ± SD except where indicated otherwise.

RESULTS

CPT-11 Kinetics. Total CPT-11 concentrations decreased rapidly after the end of the infusion period. There was a distinguishable shoulder in these concentration profiles, sometimes even a second maximum. This behavior, which is likely to be due to either a latent increase in CPT-11 carboxylate concentrations (10) or enterohepatic recycling (11), precluded the use of conventional multiexponential kinetic analysis. The $t_{1/2Z}$ of elimination was 6.3 ± 1.8 h, and there was no apparent relationship with the dose level (Table 2). $AUC_{CPT\text{-}11}$, on the other hand, rose in a dose-dependent fashion (Table 2 and Fig. 2). The CL averaged 16.1 ± 4.8 liters/h/m^2 and was highest at the 115 mg/m^2 dose level (Table 2). The MRT of CPT-11 was 6.6 ± 1.8 h, and the Vd_{SS} was 102 ± 34 liters/m^2. Neither of these parameters seemed to be influenced significantly by the CPT-11 dose level (Table 2).

SN-38 Kinetics. The C_{max} of SN-38 occurred at varying times according to two major groups of patients, those in which peak concentrations coincided with the end of the infusion and those with whom SN-38 concentrations rose steadily, achieving a plateau phase with a maximum between 2 and 4 h postinfusion

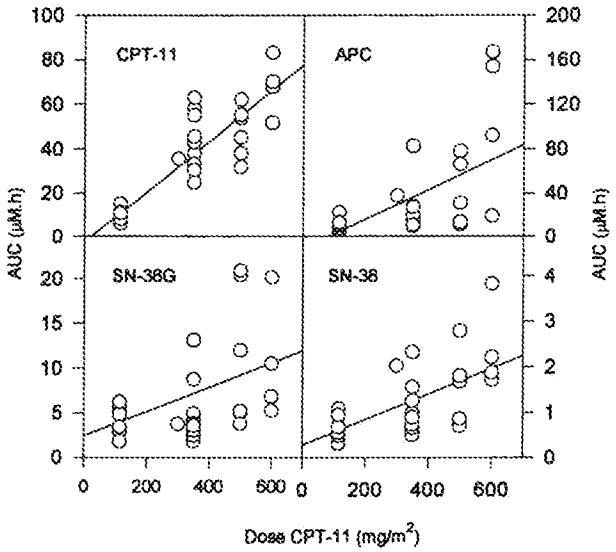

Fig. 2 The relationship between the AUCs of each of the compounds of interest as a function of the dose rate of CPT-11.

(compare Fig. 3*A* to 3*B*). The first situation was present in 14 of 33 cycles, with the peak concentration of SN-38 being manifest at or within the first 15 min postinfusion, although a second peak occurred later in many cases. This variability is reflected in the overall time of peak SN-38 concentration, which was 0.74 ± 0.86 h postinfusion. $AUC_{SN\text{-}38}$ increased with CPT-11 dose rate (Table 2; Fig. 2). The $t_{1/2Z}$ of elimination was 13.3 ± 7.9 h and was not dependent on the dose of CPT-11.

APC Kinetics. APC concentrations peaked consistently at approximately 2 h after the end of the infusion (2.0 ± 0.8 h; Fig. 3, *A* and *B*). In some patients, the concentrations of APC exceeded those of CPT-11, particularly at later times (Fig. 3*B*). The maximal concentrations, which ranged from 0.3 to 18.4 μM across the range of CPT-11 doses, increased with CPT-11 dose rate, as did AUC_{APC} (Table 3, Fig. 2), although both were subject to important interindividual variation.

SN-38G Kinetics. Plasma concentrations of SN-38G rose during the infusion, usually in parallel to those of SN-38 and reached peak concentrations at variable times (1.2 ± 0.6 h). In some of the patients in whom the SN-38 concentration peaked soon after the infusion, the maximum glucuronide concentrations corresponded to the second (later) maximum of SN-38. $AUC_{SN\text{-}38G}$ increased with the dose rate of CPT-11 (Fig. 2), although, as for APC, there was considerable variation.

Table 3 Effect of dose of CPT-11 on pharmacokinetic parameters of metabolites (mean with range in parentheses, $n = 33$)

Metabolite	Parameter	CPT-11 dose rate (mg/m^2)			
		115	300–350	500	600
APC	AUC (μM/h)	11 (4.5–23)	26 (7.0–83)	36 (11–78)	108 (19–167)
	C_{max} (μM)	1.0 (0.30–2.0)	2.7 (0.80–10)	2.4 (0.80–4.2)	11 (1.9–18)
	$t_{1/2z}$ (h)	7.6 (5.0–11)	6.7 (3.9–12)	8.2 (4.7–16)	4.9 (3.4–5.9)
	REM	2.2 (0.65–3.9)	1.1 (0.40–3.2)	1.2 (0.44–2.2)	2.6 (0.65–3.9)
SN-38G	AUC (μM/h)	4.7 (1.9–6.3)	5.0 (1.9–13)	11 (3.8–21)	11 (5.3–20)
	C_{max} (μM)	0.48 (0.22–0.76)	0.57 (0.17–2.3)	0.86 (0.33–1.4)	0.99 (0.49–1.8)
	$t_{1/2z}$ (h)	13 (5.7–29)	12 (4.8–26)	13 (5.6–23)	5.6 (3.7–10)
	REG	7.6 (3.2–11)	5.6 (0.80–14)	8.0 (3.1–14)	4.2 (2.8–5.3)
SN-38	AUC (μM/h)	0.66 (0.31–1.1)	1.2 (0.51–2.3)	1.4 (0.70–2.8)	2.4 (1.7–3.8)
	C_{max} (μM)	0.08 (0.03–0.17)	0.12 (0.04–0.25)	0.15 (0.07–0.22)	0.30 (0.23–0.4)
	$t_{1/2z}$ (h)	13 (7.2–29)	17 (3.8–33)	11 (5.3–21)	7.1 (5.4–11)
	REC	0.07 (0.04–0.11)	0.03 (0.01–0.06)[a]	0.03 (0.02–0.05)[a]	0.04 (0.03–0.05)

[a] Significantly different from the 115 mg/m^2 level, $P < 0.05$ (Kruskal-Wallis test followed by Dunn's multiple comparison).

Interrelationships of Metabolites. The average REC of CPT-11 to SN-38 ranged from 0.009 to 0.11 (0.047 ± 0.029). Although the AUC_{SN-38} was significantly correlated with AUC_{CPT-11} ($r = 0.68$; $P < 0.001$), the REC was significantly higher for the 115 mg/m^2 dose rate than it was for the 300–350 and 500 mg/m^2 dose levels (Table 3).

There was no dose dependence of the extent of metabolism of CPT-11 to APC as estimated from the REM, which ranged from 0.23 to 2.73 (0.98 ± 0.64). AUC_{APC} was correlated to AUC_{CPT-11} ($r = 0.72$; $P < 0.001$). The elimination phases of CPT-11 and APC were consistently parallel (for example, see Fig. 3). Indeed, the terminal half-lives of these compounds (7.1 ± 2.6 and 6.3 ± 1.8 h, APC and CPT-11, respectively) were strongly correlated ($r = 0.87$, $P < 0.001$).

There was no significant dose-dependence of the REG of SN-38 which ranged from 0.8 to 14.7 (6.7 ± 3.4). The elimination phases of SN-38 and SN-38G were also consistently parallel and the terminal half-lives of these compounds (13.3 ± 7.9 and 12.1 ± 6.2 h, SN-38 and SN-38G, respectively) were significantly correlated ($r = 0.67$, $P < 0.001$).

The interrelationship between SN-38 and CPT-11 and APC was probed further following the observation that patients with high AUC_{SN-38} usually had also high AUC_{APC} (Fig. 4). Multiple linear regression with AUC_{CPT-11} and AUC_{APC} as independent variables and AUC_{SN-38} as the dependent variable suggested that AUC_{APC} was a stronger determinant of AUC_{SN-38} than AUC_{CPT-11} but that both were significantly implicated. However, significant heteroscedasticity and the likely multicolinearity between the independent variables render this analysis prone to bias.

DISCUSSION

This is the first pharmacokinetic study of CPT-11 to incorporate quantitation of all three major plasma metabolites of CPT-11: SN-38, SN-38G and APC. We performed this study to examine the interrelationships of the plasma kinetics of these metabolites and the dose of CPT-11 to understand better the disposition of CPT-11 in patients.

The *CL* of CPT-11 was highest at the lowest dose rate (115 mg/m^2). Apart from the results obtained by Negoro *et al.* (12), this observation is at odds with most of the literature concerning CPT-11 pharmacokinetics, although some other studies have reported higher clearances at the lower end of CPT-11 dose ranges (11). The higher *CL* at 115 mg/m^2 CPT-11 was associated with a greater relative extent of the conversion of CPT-11 to SN-38 (REC). Although a causative link could be proposed between these two observations, this is unlikely given that only a small fraction of the dose appears converted to SN-38 (6), indicating that conversion to SN-38 is a minor route of elimination for CPT-11. Also, the apparent K_m of this biotransformation reaction is approximately 60 μM for human liver carboxylesterase (13), which is significantly greater than the range of concentrations encountered in the study. Finally, it must be stressed that even in a simplistic pharmacokinetic model such as the one shown in Fig. 5, REC can be shown to represent the ratio of the rate constant of the formation of SN-38 to that of its elimination and, therefore, not be dependent solely on the formation of SN-38. That is, REC will also be dependent on the effect of dose on the elimination and metabolism of CPT-11 via other pathways (such as formation of APC), not to mention the saturation of SN-38 glucuronidation or enterohepatic recycling. In any case, the relatively small number of patients studied suggests that care should be taken when interpreting these results.

We reported previously the parallel nature of the elimination kinetics of SN-38 and SN-38G (5). The present study confirms these results although the coefficient of determination (r^2) of the correlation of the terminal half-lives of these two compounds was modest. This may be in part due to the difficulty in identifying correctly the terminal phase of eliminination because the plasma concentrations of SN-38 rose and fell at the late time points in some instances, usually in parallel with those of SN-38G (Fig. 3). The REG was variable between patients and this would be expected to influence the disposition of SN-38.

The concentration time profiles of SN-38 were, in some cases, very different from one patient to the next with some exhibiting delayed peak concentrations with plateau-like behavior whilst in some patients, the peak SN-38 concentration oc-

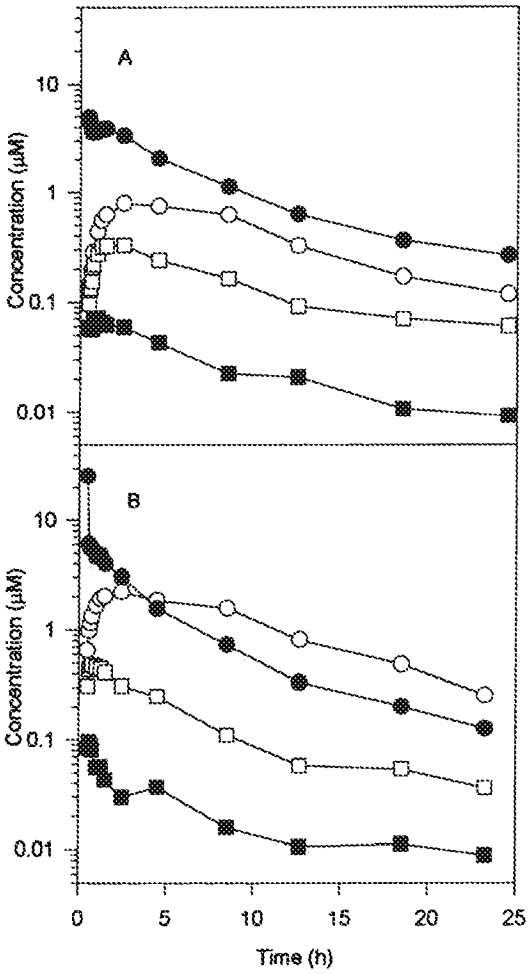

Fig. 3 Plasma concentrations of CPT-11 (●), APC (○), SN-38G (□) and SN-38 (■) in a patient receiving 500 mg/m^2 of CPT-11 (*A*), and in a patient receiving 350 mg/m^2 of CPT-11 (*B*).

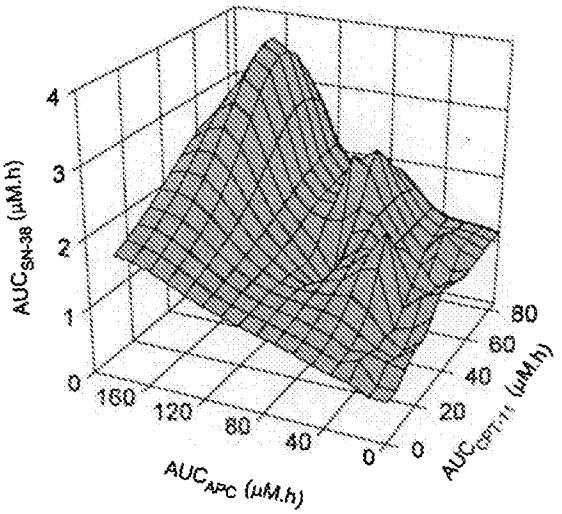

Fig. 4 The interpolated surface for the relationship between the AUCs of CPT-11, APC, and SN-38.

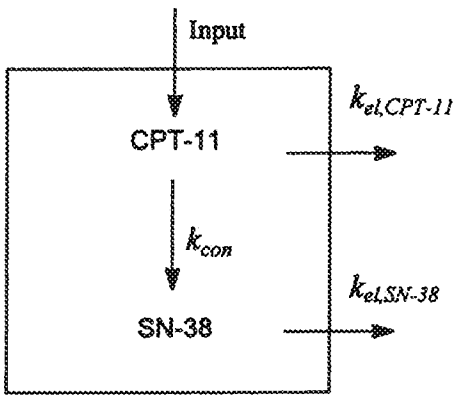

Fig. 5 A simplistic one compartment model for the transformation of CPT-11 to SN-38. Following a bolus input of CPT-11, the REC of CPT-11 to SN-38 (AUC_{SN-38}/AUC_{CPT-11}) can be shown to be equal to:

$$k_{con}/k_{el,SN-38},$$

where k_{con} is the rate constant of conversion of CPT-11 to SN-38 and $k_{el,CPT-11}$ and $k_{el,SN-38}$ are the rate constants of elimination of CPT-11 and SN-38, respectively. Elimination in this case may be comprised of other metabolic conversions (*e.g.*, formation of APC in the case of CPT-11) in addition to renal and other clearances.

curred closely following the end of the infusion. This heterogeneity in kinetic behavior, which we also observed previously in a smaller study, may affect the therapeutic outcome of treatment because it has been demonstrated that protracted exposure to CPT-11 and other camptothecins is accompanied by enhanced anticancer activity in mice bearing human xenografts (14). The reason for this heterogeneity is as yet unknown.

Although AUC_{APC} was correlated to CPT-11 dose there was considerable variability in the extent of formation of APC and, in some patients, APC concentrations were superior to those of CPT-11 several hours following the end of the infusion. Because APC differs from CPT-11 only in the distal piperidine ring, APC is a also a potential prodrug of SN-38. However, APC is not significantly converted to SN-38 by either human liver microsomes or purified human liver carboxylesterase in comparison to CPT-11 (8). CPT-11 itself is a relatively poor substrate in these systems (8, 13) and it is, therefore, unlikely that direct transformation of APC to SN-38 occurs significantly *in vivo*. Nevertheless, we observed that patients with high AUC_{SN-38} usually had both high AUC_{CPT-11} and high AUC_{APC} when the data were analyzed graphically (see Fig. 4). Although this is at first surprising, it is possible that either a precursor or a metabolite of APC is extensively hydrolysed to SN-38 *in vivo* (Fig. 1). Indeed, other metabolites of CPT-11 which are probable intermediates of the oxidative route of metabolism of CPT-11 have been observed in plasma, albeit at much lower concentrations than APC (8). APC may, therefore, be an indicator of the importance of this overall route of metabolism rather than a direct precursor of SN-38.

In conclusion, our current study demonstrates that the kinetics of the two newly described metabolites of CPT-11,

namely APC and the glucuronide of SN-38 are both subject to considerable interpatient variability. The interrelationships between the kinetics of these metabolites and CPT-11 and SN-38 are complex. Also, it is apparent from our results (8) and those of others (6) that the pathways of the metabolism of CPT-11 are still not completely defined.

ACKNOWLEDGMENTS

We are indebted to Dr. F. Bonichon for assistance with the statistical aspects of the study and to C. Garcia for many of the high-performance liquid chromatography analyses.

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Phase I and Pharmacokinetic Trial of Weekly CPT-11

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Purpose: We conducted a phase I and pharmacokinetic trial of CPT-11 (irinotecan) to characterize the maximum-tolerated dose (MTD), toxicities, pharmacokinetic profile, and antitumor effects in patients with refractory solid malignancies.

Patients and Methods: We treated 32 patients with CPT-11 administered as a 90-minute intravenous infusion every week for 4 consecutive weeks followed by a 2-week rest period. Dose levels ranged from 50 to 180 mg/m^2/wk. We determined concentrations of the lactone (active) and total (lactone plus carboxylate) forms of CPT-11 and its metabolite, SN-38, in the plasma and urine of selected patients during and after drug infusion.

Results: Grade 4 diarrhea was the dose-limiting toxicity (DLT) at the 180-mg/m^2/wk dose level. Other toxicities attributed to CPT-11 included dehydration, nausea, vomiting, and asthenia. Hematologic toxicity was mild in most patients. The terminal plasma half-life for CPT-11 (total) was 7.9 ± 2.8 hours, for CPT-11 (lactone) 6.3 ± 2.2 hours, for SN-38 (total) 13.0 ± 5.8 hours, and for SN-38 (lactone) 11.5 ± 3.8 hours. We observed significant correlations between drug dose and peak plasma concentration ($C_p$max) and between drug dose and area under the concentration curve (AUC) for CPT-11, but not for SN-38.

Conclusion: The MTD for CPT-11 in this patient population was 150 mg/m^2/wk when administered on a weekly-times-four schedule repeated every 6 weeks. At dose levels greater than 150 mg/m^2/wk, diarrhea is dose-limiting.

J Clin Oncol 11:2194-2204. © 1993 by American Society of Clinical Oncology.

IN THE LATE 1950s, Wani et al[1] first observed antitumor activity in the extract of bark from the Camptotheca acuminata (Nyssaceae) tree. In 1966, those same investigators identified the alkaloid camptothecin as the active component of the extract. Clinical trials of camptothecin sodium (NSC 100880) were performed in the late 1960s and early 1970s and, while antitumor activity was observed, those trials were halted due to severe and unpredictable hemorrhagic cystitis and myelosuppression.[2-4] In the early 1980s, investigators at Yakult Honsha Co, Ltd in Tokyo, Japan synthesized CPT-11, a water-soluble camptothecin analog with a high degree of antitumor activity.[5-7] Phase I clinical trials of CPT-11 in Japan established that the toxicities associated with CPT-11, primarily nausea, vomiting, diarrhea, and myelosuppression, were more predictable and clinically manageable than those reported for camptothecin sodium.[8,9] Phase II clinical trials in Japan subsequently reported significant clinical activity for CPT-11 in patients with small-cell and non–small-cell lung cancer, colorectal cancer, gastric cancer, squamous cell carcinoma of the cervix, ovarian cancer, and lymphoma.[10-13]

Camptothecins are selective topoisomerase-I inhibitors.[14,15] Topoisomerase-I is a nuclear enzyme that is responsible for the release of torsional strain on DNA that occurs during replication and transcription. By interfering with the religation of parent single-strand DNA following passage of the newly synthesized DNA or RNA, camptothecins produce a lethal accumulation of single-strand DNA breaks in the cell. This novel mechanism of action, coupled with the observation that, for at least some tumors, higher levels of topoisomerase-I occur in tumor cells than in normal tissue, has fueled interest in the clinical development of this family of compounds.[16-18]

Preclinical studies suggest that CPT-11 may primarily behave as a prodrug in vivo and that the majority of antitumor activity may be attributable to its more active metabolite, SN-38. In vitro, SN-38 is 250- to 1,000-fold more potent than CPT-11 in the inhibition of topoisomerase-I activity.[19] A reversible, pH-dependent hydrolysis converts the closed lactone E ring of both CPT-11 and SN-38 to the open, carboxylate form of each compound (Fig 1). Only the closed ring (lactone) forms of CPT-11 and SN-38 are effective topoisomerase-I inhibitors; the carboxylate forms are inactive.[20] In vivo, there is a dynamic equilibrium, with an acidic pH driving the equilibrium to the closed ring form and a basic pH shifting the equilibrium to the inactive, open-ring form.

This report summarizes the phase I clinical and pharmacokinetic study of CPT-11 administered once a week for 4 consecutive weeks followed by a 2-week rest period.

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Submitted February 23, 1993; accepted July 19, 1993.

Supported by G.H. Besselaar Associates, acting on behalf of Yakult Honsha Co, Ltd, and Daiichi Pharmaceutical Co, Ltd, Tokyo, Japan.

Presented in part at the Annual Meeting of the American Society of Clinical Oncology, San Diego, CA, May 1992.

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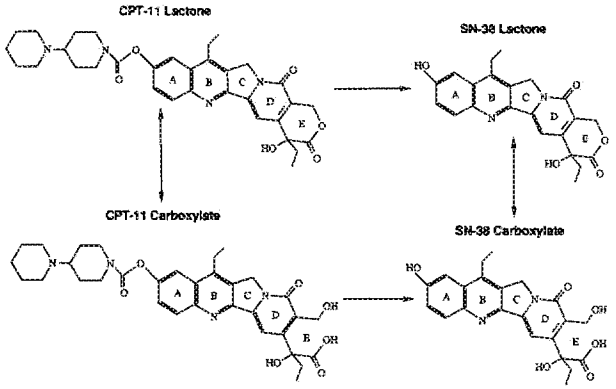

Fig 1. Structures of CPT-11 and SN-38 in the lactone and carboxylate forms. CPT-11 is converted to SN-38 by carboxylesterase. Acidic pH favors the lactone forms and basic pH favors the carboxylate forms.

The goals of this trial were (1) to establish the maximum-tolerated dose (MTD) for CPT-11 administered in this fashion; (2) to determine the qualitative and quantitative toxicities of CPT-11 for this schedule; (3) to obtain a detailed pharmacokinetic profile of CPT-11 and its active metabolite, SN-38; and (4) to collect information about the antitumor effects of CPT-11 in patients with solid tumors.

PATIENTS AND METHODS

Eligibility

All patients entered onto this trial had histologically proven solid malignancies for which no curative therapy was available. Eligibility criteria included the following: (1) age ≥ 18 years; (2) measurable or assessable tumor; (3) predicted life expectancy of ≥ 12 weeks; (4) Southwest Oncology Group (SWOG) performance status of 0, 1, or 2; (5) no surgery, radiation, or chemotherapy within 4 weeks before entry (6 weeks for previous treatment with nitrosoureas or mitomycin); (6) adequate baseline organ function, defined as WBC count ≥ 3,500/μL, platelet count ≥ 100,000/μL, hemoglobin ≥ 9.5g/dL, total bilirubin ≤ 2.0 mg/dL, AST ≤ 3.0-fold upper limit of normal, prothrombin time within normal limits, serum creatinine ≤ 2.0 mg/dL or creatinine clearance ≥ 60 mL/min, serum electrolytes within 10% of normal, and serum glucose ≤ 200 mg/dL; (7) negative skin test for hypersensitivity to CPT-11; (8) negative pregnancy test (for women of childbearing potential) and agreement to use contraception while on study; (9) no concurrent use of commercial or investigational antineoplastic therapy; (10) no acute atrial fibrillation or myocardial infarction in the previous 6 months; and (11) signed informed consent. Before initiation of this trial, institutional review board–approval was obtained at each of the participating centers.

Dosage and Drug Administration

Drug dosage. Data from phase I and early phase II clinical trials conducted in Japan of CPT-11 suggested that a dose of 100 mg/m^2 was well tolerated when administered on a weekly basis. Our trial began with a dosage level of 50 mg/m^2, or approximately half of the MTD identified in Japan. Dose levels of 50, 80, 100, 125, 150, and 180 mg/m^2 were explored in this trial.

CPT-11 preparation. CPT-11 (irinotecan) as the hydrochloride was provided by Daiichi Pharmaceutical Co, Ltd (Tokyo, Japan) in 2-mL or 5-mL vials at a concentration of 20 mg/mL containing 45 mg D-sorbitol and 0.9 mg lactic acid, and adjusted to a pH of 3.5 to 4.5 with sodium hydroxide. Drug was diluted and mixed in 500 mL of dextrose (5%) in water (pH 4.3; range, 3.5 to 6.5) and administered intravenously over 90 minutes.

CPT-11 administration. The first dose of CPT-11 was administered in the hospital, where the patients were observed for a minimum of 24 hours following treatment. If no significant adverse events were observed, subsequent treatments were administered in the outpatient setting. CPT-11 was administered once a week for 4 consecutive weeks, followed by a 2-week rest period. This 6-week period constituted one cycle or course of treatment. A minimum of three patients were entered at each dose level. Using the common toxicity criteria of the National Cancer Institute (NCI), dose-limiting toxicity (DLT) was defined as any of the following events occurring during cycle no. 1 of CPT-11 treatment: (1) grade 3 or greater nonhematologic toxicity (aside from nausea and vomiting), (2) grade 4 vomiting despite therapy with serotonin antagonists, or (3) grade 4 neutropenia persisting for longer than 5 days or grade 4 neutropenia associated with fever. If DLT was observed in one of the first three patients entered at any dose level, an additional three patients were enrolled at that dose level. DLT occurring in two or more patients treated at the same dose level was used as the clinical end point for this study. The MTD was defined as the highest dose level at which no more than one of six patients experienced DLT. In other words, the MTD was identified as one dose level below that at which DLT was observed in two or more patients.

Pretreatment and Follow-Up Studies

A complete history, physical examination, and determination of performance status was obtained on each patient at baseline. Physical examinations and complete review of systems were performed on a weekly basis. The following laboratory studies were obtained at baseline and repeated weekly: complete blood cell count with leukocyte differential, prothrombin and partial thromboplastin times, serum electrolytes, blood urea nitrogen (BUN), serum creatinine, lactic dehydrogenase (LDH), alkaline phosphatase, total bilirubin, AST, ALT, serum calcium, inorganic phosphorus, albumin, total protein, uric acid, urinalysis, and fecal occult blood test. Chest x-rays and resting 12-lead ECGs were obtained at baseline and before each course of therapy. For those patients with measurable disease, tumor measurements were obtained at baseline and recorded in two dimensions. Tumor reassessment was performed after every other cycle of therapy using the same technique as was used for baseline tumor measurement. A complete response required total disappearance of all measurable and assessable cancer for at least 4 weeks and no increase in cancer-associated symptomatology or decrease in performance status. A partial response required at least a 50% reduction in tumor areas as determined by the sum of the products of the greatest length and the maximum perpendicular width of all measurable lesions with no progression in any existing lesion or appearance of any new lesion. All partial responses had to be confirmed by repeat evaluation at least 4 weeks apart. Progressive disease was defined as a greater than 25% increase in tumor area as measured by the aforementioned

method. Stable disease was defined as insufficient change in the tumor to qualify for either a response or progressive disease.

Dose Modification

Initially, doses of CPT-11 scheduled for weeks 2, 3, or 4 were omitted if the patient experienced any grade 3 or greater toxicity. Treatment during that cycle was reinstituted only if all interim toxicities had resolved to grade 2 or less. A new cycle of treatment could only be initiated if the patient met all initial eligibility criteria. Since Grade 3 toxicities were transient and generally well tolerated, the protocol was subsequently amended to allow dose reduction, rather than dose omission, during weeks 2, 3, or 4 of treatment (Table 1). Once a dose was reduced, the patient received that reduced dose for all of the remaining weeks of that treatment course, as well as for the next treatment course. The dose of CPT-11 could be increased one dose level for the subsequent course if that patient experienced no grade 3 or greater toxicity during treatment at the reduced dose.

Dose-Intensity

Since patients could begin a cycle at one dose level and receive subsequent weeks of therapy at a lower dose, we recorded the actual drug dose delivered to each patient during each of the first 4 weeks of cycle no. 1. We then calculated the arithmetic mean dose of CPT-11 for this first cycle of therapy in milligrams per square meter per week. The actual amount of CPT-11 administered was then analyzed with respect to planned drug dose during the first cycle of therapy.

Blood Sampling and Urine Collection

Blood samples were obtained during the first cycle of therapy from at least two patients treated at each dose level. Just before CPT-11 administration on day 0, an indwelling heparin-lock intravenous catheter was placed in the arm contralateral to the drug infusion line. Blood samples were collected at the following time points: 0 minutes (pretreatment blank), 15 and 45 minutes into infusion, end of infusion (EOI), 5, 10, 20, 30, 45, 60, and 90 minutes, and 2, 3, 4, 6, 8, 12, 24, 48, and 72 hours postinfusion. Heparinized blood samples were immediately centrifuged and the plasma extracted with cold methanol and subjected to high-performance liquid chromatography (HPLC) as will be described later. Urine samples were collected at baseline and every 6 hours for 12 hours, then every 12 hours up to 48 hours and stored at −20°C until analysis. Urine samples were centrifuged for 10 minutes at 2,000 rpm to separate any insoluble materials and filtered through a 0.45-μm filter.

HPLC Analysis

A modified reverse-phase HPLC assay developed by Kaneda and Yokokura[21] was used to analyze CPT-11 and SN-38 in plasma, urine, and bile. One-half milliliter of fresh plasma or bile was added to prepared cold (−20°C) methanol (2 mL) and the tube was vortexed for 12 seconds. The mixture was centrifuged at 2,500 rpm for 5 minutes and 10 to 80 μL of extract was immediately subjected to HPLC analysis on two separate HPLC systems. The extraction efficiency for both CPT-11 and SN-38 from plasma was 98%. Analytical conditions for CPT-11 consisted of a C_{18} column (μBondapack, 10 μ, 30 cm × 3.9 mm) preceded by a μBondapack C_{18} precolumn Guard-pack (Waters Associates, Milford, MA). The mobile phase was a mixture of 65% methanol:35% sodium phosphate buffer with 3 mmol/L heptanesulfonic acid (pH 4.0) pumped at a flow rate of 0.8 mL/min (model 510, Waters Associates). Detection was monitored by fluorescence (model 470, Waters Associates) with an excitation set at 375 nm and the emission wavelength set at 430 nm. Analytic conditions for SN-38 consisted of a Novapak C_{18} column (4 μm, 15 cm × 3.9 mm, Waters Associates) with an acetonitrile:water (1:3) mobile phase (pH 6.14) pumped at a flow rate of 1 mL/min. Detection was monitored by fluorescence with excitation set at 375 nm and emission wavelength set at 566 nm. Retention times for CPT-11 and SN-38 under their respective analytic conditions were 6.3 and 4.3 minutes, respectively. Chromatograms and peak height areas were stored and analyzed on a Waters Maxima Workstation. The amount of CPT-11 and SN-38 in each duplicate sample was calculated by comparison of the peak areas with that of the standard curve analyzed on the same day. Standard curves constructed in blank donor plasma were linear (R^2 = .99) for both CPT-11 (2 to 2,000 ng/mL) and SN-38 (2 to 200 ng/mL). Following quantitation of the lactone forms of CPT-11 and SN-38, the plasma extracts were acidified with 2% hydrochloric acid and analyzed for total CPT-11 and SN-38 by the preceding HPLC methods. The chromatographic conditions for quantitation of CPT-11 and SN-38 in the urine were identical to that of plasma and bile, with the exception that the urine samples were diluted (1:5 to 1:300), acidified, and analyzed for total CPT-11 or SN-38 only.

Table 1. Dose Modification

Toxicity Grade	Dose Level Reduction
Leukopenia/neutropenia	
0-1	None
2	1 dose level
3	2 dose levels
4	Omit dose
Neutropenic fever	Omit dose
Diarrhea and other nonhematologic toxicities	
0-1	None
2	1 dose level
3	2 dose levels
4	Omit dose

Pharmacokinetic Analysis

The pharmacokinetic parameters were calculated using model-independent methods.[22] The terminal rate constant (k) was determined by log-linear regression analysis of the terminal phase of the plasma concentration-time curves. The terminal plasma half-lives ($t_{1/2}$s) were calculated by the equation: $t_{1/2} = 0.693/k$. The area under the plasma concentration-time curve (AUC) was calculated by the linear trapezoidal rule up to the last measurable data points with extrapolation to infinity. Clearance was calculated by dividing the total dose of CPT-11 received by the AUC.

RESULTS

Thirty-two patients received 118+ courses of treatment. Patient characteristics are listed in Table 2. The majority of patients (28 of 32) were either asymptomatic or only mildly symptomatic (ie, SWOG performance status of 0 to 1) at the time of enrollment onto this study. Twenty-nine of 32 had received prior chemotherapy (17 had re-

Table 2. Patient Characteristics

No. entered and assessable	32
Age, years	
Median	55
Range	19-78
Performance status	
0	13
1	15
2	4
No. with prior chemotherapy only	17
No. with prior radiotherapy only	2
No with prior chemotherapy and radiotherapy	12
No. with no prior chemotherapy or radiotherapy	1
No. of prior chemotherapy regimens	
Median	2
Range	0-7
No. of courses of CPT-11	
Median	2
Range	1-9+
Total no. of courses of CPT-11 administered	118+
Tumor types	
Colorectal	22
Uterine cervix	2
Breast	2
Kidney	1
Liver	1
Non–small-cell lung	1
Ovary	1
Prostate	1
Stomach	1

ceived prior chemotherapy only, while an additional 12 patients had received prior chemotherapy plus radiation). The median number of prior chemotherapy regimens for all 32 patients was two (range, zero to seven). Six dose levels were evaluated: 50, 80, 100, 125, 150, and 180 mg/m^2/wk. All patients were assessable for toxicity and response. The median number of courses of CPT-11 administered per patient was two (range, one to nine+). A large proportion of patients on this trial had metastatic colorectal cancer (22 of 32, 68.8%), but patients with a variety of other tumor types were also included in the trial. Twenty-one patients (65.6%) were considered heavily pretreated (ie, had received three or more prior chemotherapy regimens and/or had prior abdominopelvic irradiation) and 11 patients (34.4%) were considered lightly pretreated (ie, had received two or fewer prior chemotherapy regimens and had not received prior abdominopelvic irradiation).

Hematologic Toxicity

Hematologic toxicities encountered during cycle no. 1 were relatively mild. The most common hematologic toxicity, granulocytopenia, occurred during the third to fourth week of treatment and is listed in Table 3. Clinically significant granulocytopenia (ie, absolute granulocyte count $< 500/\mu L$, NCI grade 4) was rare and occurred in only one of the 32 patients (3.1%) during cycle no. 1. When all treatment cycles were analyzed, granulocytopenia did not become more frequent or severe with continued treatment; only three of 118 (2.5%) treatment cycles resulted in grade 4 granulocytopenia. All three episodes occurred in patients in the heavily pretreated category and who received doses of CPT-11 $\geq$ 150 mg/m^2. No episodes of neutropenic fever occurred on this study. There was no significant relationship between dose of CPT-11 and severity of leukopenia or granulocytopenia. Grade 4 anemia was observed in only one course of treatment (0.8%) and was not felt to be due to CPT-11 treatment. There were no episodes of grade 3 or 4 thrombocytopenia.

Table 3. Neutropenia: Cycle No. 1

Dose Level (mg/m^2)	No. of Patients	NCI Toxicity Grade				
		0	1	2	3	4
50	4	3	1	0	0	0
80	4	2	0	2	0	0
100	6	1	4	1	0	0
125	6	3	0	2	1	0
150	6	3	1	1	0	1
180	6	2	1	1	2	0

Nonhematologic Toxicity

Table 4 lists the nonhematologic toxicities that occurred during cycle no. 1. Grade 4 diarrhea was the DLT for CPT-11 in this trial. It occurred during the first cycle of treatment at the 180-mg/m^2 dose level in four of six patients (two of four heavily pretreated and two of two minimally pretreated). Onset followed the second or third week of treatment and resulted in dehydration that required hospitalization for parenteral fluid and electrolyte replacement. Once grade 4 diarrhea occurred, it typically lasted 5 to 7 days. Paregoric, loperamide (Imodium, Janssen Pharmaceuticals, Piscataway, NJ), diphenoxylate-

Table 4. Nonhematologic Toxicities: Cycle No. 1 (NCI Grade $\geq$ 3)

Dose Level (mg/m^2)	No. of Patients	Nausea/ Vomiting	Diarrhea	Dehydration	Asthenia	Ileus
50	4	0	0	0	0	0
80	4	0	0	0	0	0
100	6	1	0	1	0	0
125	6	0	1	0	0	1
150	6	0	1	1	1	0
180	6	2	4	2	2	0

atropine (Lomotil; GD Searle & Co, Chicago, IL), bismuth, octreotide, atropine, and scopolamine were all ineffective in reducing its severity or duration. Stool cultures, stains, and examination for fecal leukocytes were negative in these patients. Grade 2 to 3 diarrhea was observed at lower CPT-11 dose levels and responded well to loperamide and/or diphenoxylate-atropine. Grade 3 ileus occurred in one patient with metastatic prostate cancer and was felt to be related to intake of narcotic analgesics, rather than CPT-11.

Prophylactic antiemetics were not routinely used before the initial dose of CPT-11. Grade 3 nausea and vomiting was observed in one of six patients treated at the 100-mg/m^2 dose level, and two of six patients treated at the 180-mg/m^2 level. These episodes were of short duration and responded rapidly to standard antiemetic therapy. Prophylactic antiemetics administered before subsequent doses of CPT-11 were successful in preventing further episodes of grade 3 nausea and vomiting in all but one patient.

Grade 3 weakness and asthenia, defined as generalized weakness resulting in impairment of performance status, was infrequent but appeared to be dose-related. It occurred in one of six patients treated at the 150-mg/m^2 dose level and in two of six patients treated at the 180-mg/m^2 dose level during the first cycle of treatment.

Other toxicities were noted to occur after the first course of therapy. Grade 3 transaminase elevation was observed in two patients treated at the 50-mg/m^2 dose level and in one patient treated at the 125-mg/m^2 level. In one patient, this occurred shortly after initiation of parenteral hyperalimentation and was not considered to be a CPT-11–induced toxicity. Another patient with an underlying hepatoma and slightly elevated transaminase levels at baseline experienced a transient increase in AST and ALT levels during cycle no. 4 of treatment. It was not clear whether the increase was due to drug, tumor, or a combination of both. Hepatitis serologies were negative. Treatment was withheld for 3 weeks and transaminases gradually returned to baseline. This patient was able to receive subsequent treatment with CPT-11 without a recurrence of the increase in AST or ALT. Another patient who developed grade 3 transaminase increases was found to have infectious mononucleosis following cycle no. 6 of CPT-11 at the 125-mg/m^2 level. Symptoms resolved spontaneously over 4 weeks and the patient was able to resume therapy at the same dose of CPT-11 without any further episodes of transaminase elevation.

One patient with colon cancer metastatic to the liver and lungs experienced grade 4 diarrhea at the 180-mg/m^2 level and received a dose reduction in cycle no. 2 to the 125-mg/m^2 level. The patient required hospitalization on day 3 of cycle no. 2 due to a second episode of grade 4 diarrhea. Increased interstitial lung markings were noted on admission chest x-ray and arterial blood gases showed mild hypoxemia (pAO_2 = 77 mm Hg) while on nasal oxygen at 2 L/min. Sputum cultures were negative, but a blood culture from the third hospital day was positive for Klebsiella pnuemoniae. Although there was no leukocytosis at the time, there was a left shift on differential. Appropriate antibiotics were administered, but the patient remained dyspneic and died on hospital day 16. Permission for bronchoscopy or autopsy was denied. Possible causes of this patient's death include progression of preexisting interstitial pulmonary metastases from colon cancer, noncardiogenic pulmonary edema (adult respiratory distress syndrome), bacterial pneumonia and sepsis, or pulmonary toxicity from CPT-11.

Determination of Dose-Intensity

Following the initial 26 cycles of therapy, the protocol was amended to include dose-modification criteria that would allow dose reduction, rather than omission of CPT-11 during weeks 2, 3, and 4 of each cycle based on interim toxicities (Table 1). Given that a patient who started at one dose level could actually receive CPT-11 at lower dose levels during subsequent weeks of the cycle, we analyzed the actual dose delivered (in milligrams per square meter per week) during the first 4 weeks of cycle no. 1 in relation to the dose level at which therapy was initiated. These results are depicted graphically in Fig 2. A maximal CPT-11 dose-intensity of 134.4 mg/m^2/wk was achieved at the 150-mg/m^2 dose level. Further dose escalation increased the frequency of toxicity without increasing delivered dose-intensity of CPT-11.

Responses

Partial responses occurred in two patients with recurrent colorectal cancer treated on this phase I study. A 41-year-old man with Dukes C colon cancer had progressive disease after 6 months of adjuvant fluorouracil (5-FU) and levamisole and disease progression following two cycles of another phase I agent. Following two cycles of CPT-11 at the 80-mg/m^2 dose level, the sum of the products of the perpendicular dimensions of his measurable tumor shrank from 42.0 cm^2 to 8.3 cm^2 (80.2% reduction). Therapy continued and this response lasted for 8 months. The second patient to respond was a 30-year-old woman with Dukes B colon cancer who had received adjuvant local irradiation with 5-FU sensitization following surgical re-

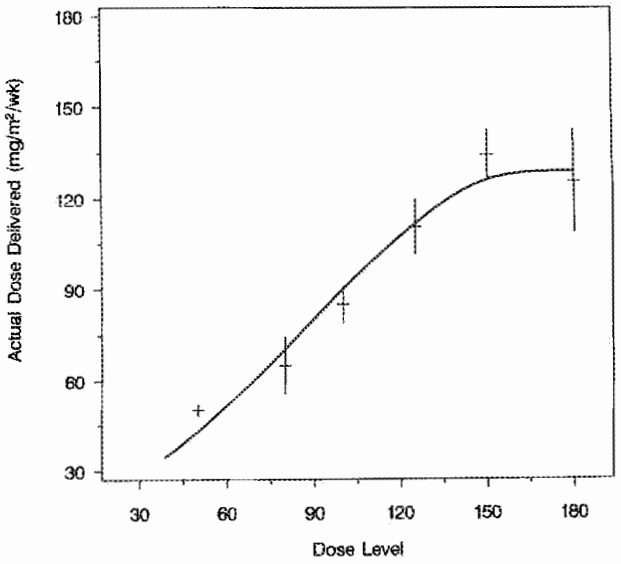

Fig 2. Dose-intensity relationship for patients during cycle no. 1 of treatment with CPT-11. The line was generated by the qualitative least-squares regression method. Maximal dose delivery was achieved at the 150-mg/m²/wk dose level.

section of her primary tumor. On tumor recurrence 8 months later, she underwent resection of a solitary hepatic metastasis followed by 1 year of adjuvant 5-FU and leucovorin. Eleven months after completion of adjuvant therapy, she developed abdominal carcinomatosis and multiple hepatic metastases. She had progressive disease following two cycles of another phase I agent and was enrolled on the CPT-11 phase I trial at the 125-mg/m² dose level. After two cycles of CPT-11, her measurable tumor had decreased from a total dimension of 51.0 cm² to 5.0 cm² (90.2% reduction) and her carcinoembryonic antigen level had normalized (60.0 ng/mL at baseline to 1.8 ng/mL) (Fig 3). This patient continued on treatment and maintained a partial response for 10 months.

Eleven patients had disease stabilization lasting from 5 to 12+ months. This included one patient with squamous cell carcinoma of the cervix, one patient with renal cell carcinoma, one patient with hepatoma, and eight patients with colorectal carcinoma. One patient with colon cancer remains on study with stable disease, 12+ months after initiation of CPT-11.

Pharmacokinetics

Plasma pharmacokinetic analysis was performed on samples obtained from 17 patients during and after administration of the first dose of CPT-11. This information is listed in Table 5. The mean terminal half-life of total CPT-11 was 7.9 ± 2.8 hours, while the lactone form of CPT-11 had a terminal half-life of 6.3 ± 2.2 hours. The mean terminal half-life of the active metabolite of CPT-11, SN-38 was longer: 13.0 ± 5.8 hours and 11.5 ± 3.8 hours for the total and lactone ring forms, respectively. A representative plasma elimination curve is shown in Fig 4. The lactone AUC to total AUC ratio remained relatively constant over the entire dosage range for both CPT-11 (mean, 33.9% ± 5.2%) and SN-38 (mean, 44.7% ± 10.2%). The peak plasma concentration ($C_p$max) for CPT-11 occurred at the end of the 90-minute infusion, while the $C_p$max for SN-38 occurred at more variable time points 30 to 90 minutes after the EOI. Plasma clearance of CPT-11 was unrelated to dose, with a mean clearance of 15.3 ± 3.5 L/h/m² for the total and 45.6 ± 10.8 L/h/m² for the lactone form. Over the dose range tested, linear relationships were identified between CPT-11 dose and both $C_p$max of CPT-11 and CPT-11 AUC (data not shown). No such relationship existed between CPT-11 dose and SN-38 $C_p$max or SN-38 AUC. Two patients had plasma pharmacokinetics repeated for the fourth (and final) week of treatment on cycle no. 1. No striking differences were detected between week 1 and week 4 pharmacokinetics for these patients (data not shown).

Patients undergoing plasma sampling for pharmacokinetics also had urine collected for 48 hours during and after CPT-11 administration. During this period, 13.9% ± 6.5% of CPT-11 and 0.26% ± 0.19% of SN-38 was recovered from the urine, suggesting that renal clearance is not a major route of elimination for these compounds (data not shown).

We had the unique opportunity to obtain simultaneous plasma and bile samples from a patient treated at the 100-mg/m² dose level. This patient developed extrahepatic biliary obstruction just before receiving his first dose of CPT-11. Attempted internal stent placement via endoscopic retrograde cholangiopancreatography (ERCP) was unsuccessful and a percutaneous biliary catheter was placed under ultrasound guidance. Simultaneous plasma and bile concentrations of total CPT-11 and total SN-38 are depicted in Fig 5A and B, respectively. Bile concentrations of CPT-11 were 10- to 60-fold higher than plasma concentrations during the first 6 hours following CPT-11 infusion, while bile concentrations of SN-38 were twofold to ninefold higher than plasma during this same time.

DISCUSSION

CPT-11 is a water-soluble, semisynthetic derivative of camptothecin developed in Japan in the mid-1980s. The

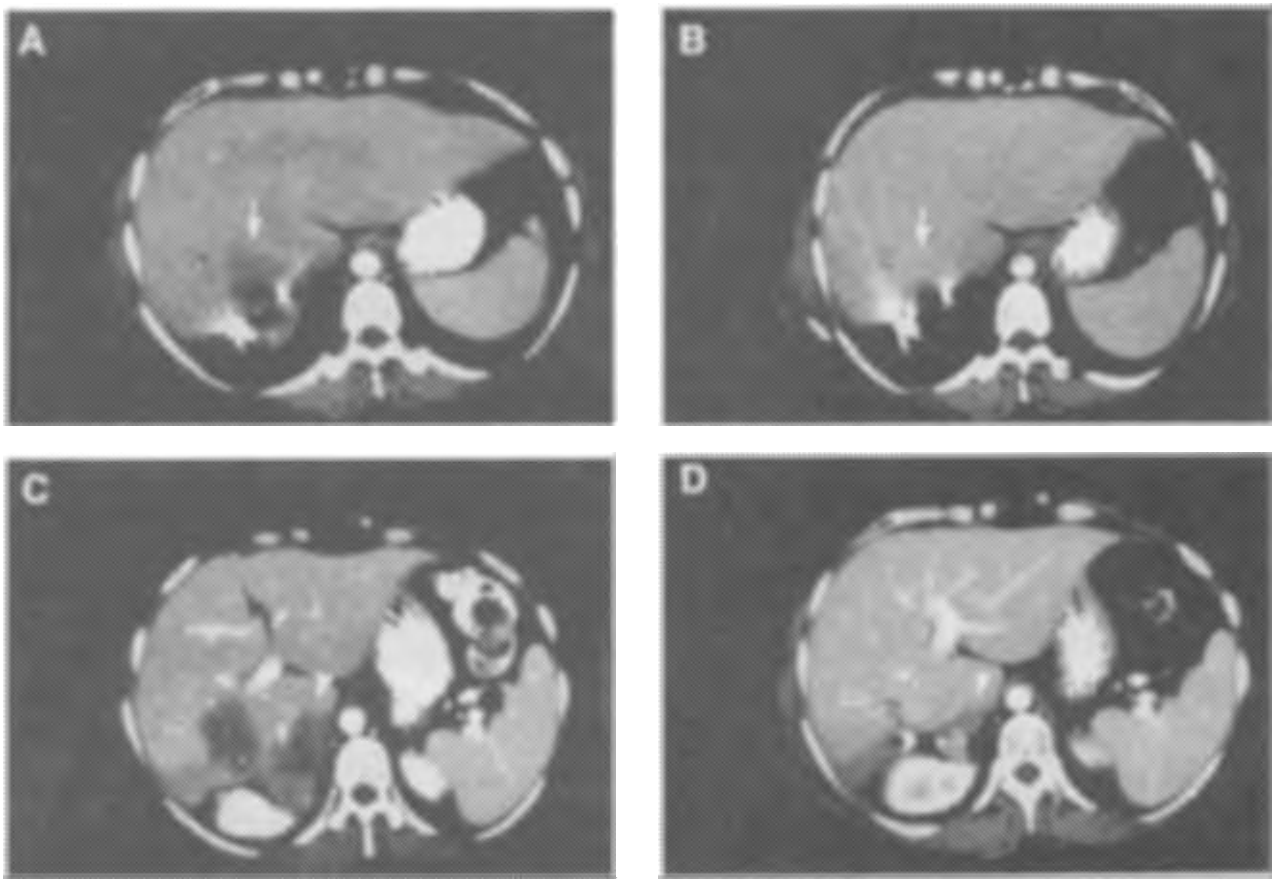

Fig 3. (A) Baseline computed tomographic (CT) scan shows 4.0 × 4.0-cm metastasis to the posterior right lobe of the liver (arrow). (B) Follow-up CT scan performed after 2 cycles of CPT-11 shows near total resolution of lesion (arrow). (C) Baseline CT scan demonstrates 5.0 × 3.0-cm lesion in lower right lobe of liver (outlined arrow) and 5.0 × 4.0-cm metastasis to the right adrenal gland (arrowhead). (D) Near-total resolution of both lesions following 2 cycles of treatment with CPT-11.

great interest surrounding CPT-11 and similar compounds stems from the fact that (1) camptothecin has significant antitumor activity in vitro and in vivo; (2) CPT-11 and the camptothecins exert their antitumor activity in a way that is distinct from all other anticancer compounds currently in use; (3) CPT-11 is more potent than camptothecin in virtually all model systems tested in vitro and in vivo; (4) CPT-11 has more predictable and manageable toxicity than camptothecin; and (5) higher levels of topoisomerase-I expression in tumor tissue compared with surrounding normal tissue may be exploited through treatment with this family of compounds.[16-18] Topoisomerase-I binds to DNA to form the cleavable complex that is responsible for release of the torsional strain imposed on the parent DNA strand by DNA replication and transcription. CPT-11 binds to and stabilizes this cleavable complex, preventing DNA reannealing after passage of the replication fork.

The DLT of CPT-11 in our trial was subacute diarrhea observed at the 180-mg/m^2 dose level. While the diarrhea experienced during CPT-11 infusion appeared to be due to increased cholinergic activity and was readily reversible with atropine, the diarrhea that occurred after the second or third week of treatment was relatively unresponsive to anticholinergic agents (ie, atropine or scopolamine) and appeared to be caused by a separate mechanism.[23] Diagnostic evaluation of this diarrhea in several of our patients failed to find specific pathogens. The most effective intervention against this subacute diarrhea was early recognition and intervention with antimotility agents (ie, loperamide [Imodium] or diphenoxylate hydrochloride with atropine sulfate [Lomotil]). However, once grade 4 diar-

Table 5. Pharmacokinetic Parameters

No. of Patients	Dose (mg/m²)	CPT-11 C_{max} (μg/mL)		CPT-11 $t_{1/2}$ (hours)		CPT-11 Cl (L/h/m²)		CPT-11 AUC (μg·h/mL)		SN-38 C_{max} (ng/mL)		SN-38 $t_{1/2}$ (hours)		SN-38 AUC (ng·h/mL)	
		Lactone	Total	Lactone	Total	Lactone	Total	Lactone	Total	Lactone	Total	Lactone	Total	Lactone	Total
2	50	0.45	0.89	5.4	5.7	44.3	18.0	1.13	2.79	13.3	26.4	9.0	8.6	62.2	215.2
2	80	0.49	1.12	6.6	8.3	39.3	11.2	2.12	7.18	12.4	31.6	11.6	16.1	162.1	321.5
4	100	0.69	1.29	7.3	11.5	45.5	14.7	2.23	6.83	15.3	34.3	13.4	17.0	214.8	369.5
2	125	0.94	1.70	9.0	9.3	31.2	12.0	2.97	7.67	16.1	39.3	14.4	12.6	195.5	449.8
4	150	0.66	1.56	6.2	9.0	54.5	18.4	2.81	8.44	13.1	36.7	12.2	15.4	123.2	409.8
3	180	0.83	1.97	8.3	9.1	47.8	15.4	3.83	11.75	11.8	26.2	21.0	19.7	232.8	367.6
Mean ± SD				6.3 ± 2.2	7.9 ± 2.8	45.6 ± 10.8	15.3 ± 3.5					11.5 ± 3.8	13.0 ± 5.8		

NOTE. A version of this table with SDs for all dose levels is available from the authors on request.

Abbreviations: $t_{1/2}$, half-life; CL, clearance.

rhea occurred, no measures were effective in reversing this process and care of these patients was mainly supportive in nature, consisting of intravenous fluids and electrolyte replacement, until the episode resolved in 5 to 7 days.

There were only three episodes of grade 4 neutropenia in 118 courses of treatment, all occurring in patients who had received three or more prior chemotherapy regimens and/or had received abdominal or pelvic irradiation. There were no episodes of neutropenic fever in our trial. Overall, the neutropenia was mild, short-lived, and not dose-limiting. CPT-11 did not appear to significantly suppress RBC or platelet counts.

One patient with colon carcinoma and interstitial pulmonary metastases died during the second cycle of therapy with bacteremia, persistent hypoxemia, and increased interstitial markings on chest x-ray. Since bronchoscopy and autopsy were not permitted by the family, the exact cause of this patient's death could not be determined. Our differential diagnosis for this patient's impaired pulmonary status included progressive spread of previously documented interstitial tumor, bacterial pneumonia, noncardiogenic pulmonary edema (ie, adult respiratory distress syndrome), or pulmonary toxicity from CPT-11. Of note is that there have been isolated reports of pneumonitis occurring in other patients receiving CPT-11.[10] Masuda et al[10] reported pulmonary toxicity in two of 16 patients with small-cell lung cancer treated with a weekly schedule of CPT-11. Both patients developed dyspnea, fever, and a diffuse reticulonodular pattern on chest x-ray. Transbronchial biopsy in one patient showed interstitial edema, fibroblastic proliferation, lymphoid cell infiltration, and fibrinous exudate. One patient responded to corticosteroids, but the other patient died of progressive respiratory insufficiency. Since we could not rule out an etiologic connection between drug administration and our patient's respiratory process, we considered the likelihood of a possible relationship between CPT-11 and the observed pulmonary impairment. Future clinical trials with CPT-11 should include close monitoring of pulmonary function with full work-up initiated at any sign of respiratory impairment.

In this study, we adopted a dose-modification schema so that we could continue to treat patients who experienced mild to moderate toxicity in the middle of a cycle by using a lower dose of CPT-11. For drugs delivered repetitively within a treatment cycle, determination of the dose level at which maximal dose-intensity is achieved may provide information complementary to determination of the MTD. We observed that a maximal dose-in-

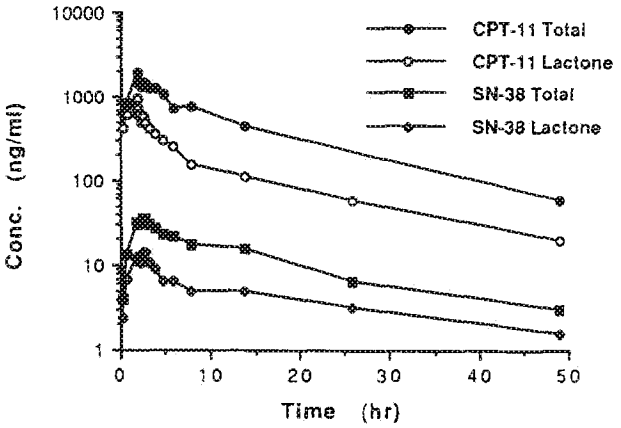

Fig 4. Plasma distribution curve for a patient treated with CPT-11 125 mg/m².

tensity of 134.4 mg/m²/wk was attained in patients treated at the 150-mg/m²/wk dose level, the same dose level at which MTD was achieved.

CPT-11 and SN-38 exist in equilibrium between lactone and carboxylate forms. By measuring lactone and total concentrations of each compound, we were able to gain insight into the nature of this equilibrium for patients treated with CPT-11. There is substantial data regarding the structure-activity relationship between camptothecin derivatives and antitumor activity in vitro and in vivo; only the closed lactone ring forms effectively inhibit topoisomerase-I function.[24,25] However, larger numbers of patients must be studied to determine whether separate measurement of both species is required to identify important pharmacodynamic relationships in patients.

We found that the mean terminal half-life of SN-38 in plasma was slightly longer than that for CPT-11: 13.0 ± 5.8 hours versus 7.9 ± 2.8 hours for the total forms, respectively, and 11.5 ± 3.8 hours versus 6.3 ± 2.2 hours for the lactone forms, respectively. Given the somewhat cell cycle–specific nature of topoisomerase-I function, the relatively long plasma half-lives are advantageous characteristics for antitumor activity. $C_p$maxs of CPT-11 occurred at the end of the 90-minute infusion, while peak concentrations of SN-38 occurred at variable time points 30 to 90 minutes after the EOI. This difference may be related to (1) the time it takes for endogenous carboxylesterases to convert CPT-11 to SN-38, and (2) interpatient variation in carboxylesterase level and/or activity.[26,27] Future efforts should include measurement of carboxylesterase levels to determine whether this may predict response or toxicity to CPT-11.

Murine studies conducted by Kawato et al[28] and Kaneda et al[29] suggest that the liver and gastrointestinal tract may concentrate and store CPT-11 and SN-38 and may also be important sites of conversion of CPT-11 to SN-38. Their observations suggest that this peripheral conversion may, in fact, be more important in determining the antitumor activity of CPT-11 than local conversion of CPT-11 to SN-38 that may occur within the tumor. Direct access to bile in one patient treated on our trial provided us with some limited insight into this issue. As shown in Fig 5A and B, simultaneous bile-to-plasma concentration ratios were as high as 60:1 for CPT-11 and 9:1 for SN-38. Within 6 hours of drug administration, 2.9% of the total dose of CPT-11 was detected in the bile. In rats, 55% of radioactively labeled CPT-11 was excreted unchanged in the bile within 24 hours, while 21.7% was

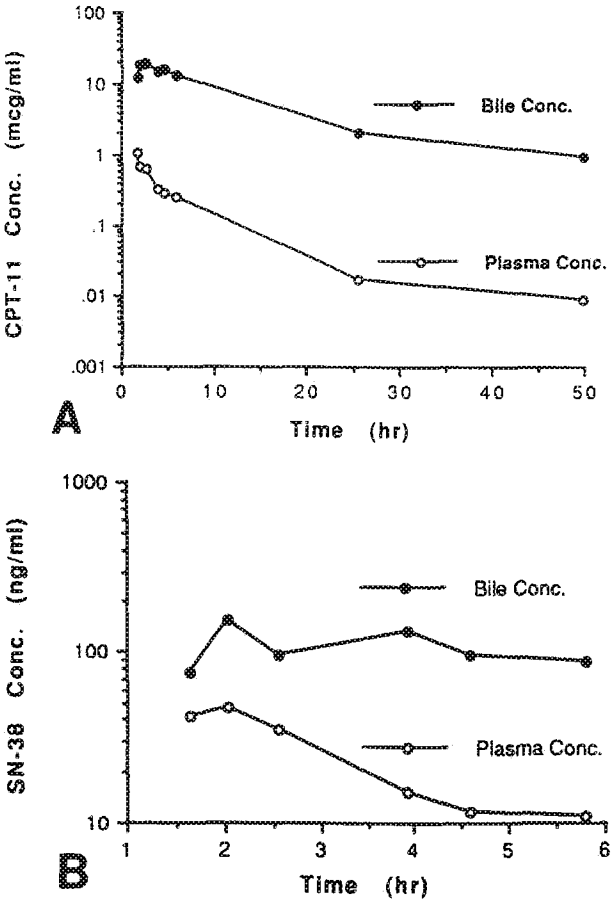

Fig 5. (A) Simultaneous plasma and bile concentrations of CPT-11 for a patient treated with CPT-11 100 mg/m². (B) Simultaneous plasma and bile concentrations of SN-38 for the same patient depicted in Fig 4A.

transformed to SN-38, and 9% appeared as SN-38 glucuronide. Overall, approximately 73% of the radioactivity could be recovered from the feces of rats and 25% from the urine within 72 hours following intravenous administration.[30]

CPT-11 is a complex drug. Future clinical trials should seek to correlate selected features of the tumor and host with response and normal tissue toxicity. In vitro, CPT-11 appears to be most effective against cells with high levels of topoisomerase-I expression. Conversely, cells with decreased levels or mutated forms of topoisomerase-I exhibit resistance to CPT-11 and the camptothecins.[31,32] It is likely that similar events occur in vivo, but data are lacking. In addition, pharmacokinetic parameters may correlate with CPT-11's DLT, diarrhea. Although there were relatively few episodes of grade 3 or 4 diarrhea in our study, there was a trend toward an association between SN-38 lactone AUC and diarrhea (data not shown). This association was by no means conclusive, and larger cohorts will need to be evaluated to confirm this trend.

In conclusion, the MTD of CPT-11 is 150 mg/m^2/wk when administered once a week for 4 weeks followed by a 2-week rest period in this patient population. The DLT is diarrhea at the 180-mg/m^2/wk dose level. Correlations were observed between drug dose, $C_p$max, and AUC for CPT-11, but not for SN-38. The clinical activity of CPT-11 using this schedule will be investigated through phase II trials now underway at multiple sites in the United States.

ACKNOWLEDGMENT

The authors express their appreciation to Judy Turner, RN, Kathleen Molpus, RN, and Judy Chandler, RN (phase I clinic), Suzanne Fields, PharmD and Julie Johnson, RPh (Investigational Drug Section), Jan Hyman (pharmacokinetic processing), Linda Higashi (data management), and all of the oncology fellows, nurses, and staff in the Drug Development Section for the excellent care provided to the patients on this study.

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Cancer Letters 127 (1998) 99–106

CANCER LETTERS

Effect of liposomalization on the antitumor activity, side-effects and tissue distribution of CPT-11

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Received 9 October 1997; received in revised form 5 January 1998; accepted 9 January 1998

Abstract

We have examined the efficacy of liposomalization and polyethyleneglycol (PEG) modification of liposomes on the antitumor activity, side-effects and tissue distribution of irinotecan hydrochloride (CPT-11). PEG-liposome was confirmed to elevate the plasma circulation of CPT-11 and SN-38 (active metabolite) concentrations. The tumor accumulation of CPT-11 and SN-38 was increased by the PEG-modified liposomes. The antitumor activity of CPT-11 increased due to the elevated tumor distribution of CPT-11 and SN-38 levels by the PEG-modified liposomes. In the tumor, CPT-11 was converted to SN-38. Thus, it is considered that passive targeting to the tumor by liposomalization elevated the SN-38 level in the tumor especially and increased the antitumor activity of CPT-11. Furthermore, intestinal disorder, a side toxicity of CPT-11, decreased dependent on the CPT-11 and SN-38 concentrations in the bile by liposomalization. Although the liposomes induce improved tissue distribution of the prodrug, the tissue distribution of active metabolites does not always improve. However, CPT-11-entrapped liposome was useful, as CPT-11 is converted to SN-38 in the tumor. These results suggested that the usefulness of CPT-11 could be extended. © 1998 Elsevier Science Ireland Ltd.

Keywords: Liposome; CPT-11; SN-38; Antitumor activity; Tissue distribution; Side toxicity

1. Introduction

Irinotecan hydrochloride (CPT-11), which possesses antitumor activity by the inhibition of topoisomerase I activity, has been shown to have superior efficacy on lung carcinoma [1–3]. However, CPT-11 has lethal side-effects such as myelosuppression and gastrointestinal disorders (mainly diarrhea) [4]. After it appeared on the market, there were reports of CPT-11-induced deaths and therefore its clinical usefulness has been severely restricted [5]. So although CPT-11 exhibits superior antitumor activity, it also exhibits severe side toxicity. One way to effectively utilize antitumor agents whose antitumor activity is prevented by its side-effects is to use a drug delivery system (DDS). Liposome is a typical DDS. We have reported on the efficacy of adriamycin-encapsulated liposomes [6–8].

The pharmacokinetic profiles of liposomes containing antitumor agents are influenced by the physical property of liposomes, the pharmacokinetic property of the antitumor agent and the factors of the body [9]. For effective usefulness, it is necessary to examine the best combination of these factors. Furthermore, CPT-11 is a prodrug which shows antitumor activity in the body after conversion to SN-38 from CPT-11

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0304-3835/98/$19.00
PII S0304-3835(98)00031-7

[10]. Liposomalization is not always useful on a pro-drug such as CPT-11. We have examined the effect of the composing liquid of liposomes and the efficacy of polyethyleneglycol (PEG) modification of liposomes on the antitumor activity and tissue distribution of CPT-11. To prepare liposomes, we have used dimyristoylphosphatidylcholine (DMPC), which has a phase transition temperature (T_c) lower than body temperature, and distearoylphosphatidylcholine (DSPC), which has a higher transition temperature than body temperature.

It has been reported that the liposomalization of an antitumor agent such as adriamycin not only increased antitumor activity but also decreased the side-effects [7,11]. Adding myelosuppression as a characteristic side-effect of the antitumor agent, CPT-11 creates severe gastrointestinal disorder, with dehydration and electrolyte disorder resulting in serious diarrhea [12,13]. It appears that this intestinal disorder is mainly caused by the excretion of SN-38 into the bile [14]. We have investigated the effect of liposomalization on the SN-38 level in the bile and the water content in the feces as indicators of CPT-11-induced delayed diarrheal symptoms.

2. Materials and methods

2.1. Drugs

Irinotecan hydrochloride (100 mg/5 ml vial), which was used as CPT-11 solution (Sol), was purchased from Daiichi Pharmaceutical (Tokyo, Japan). DMPC, DSPC and dimyristoylphosphatidylglycerol (DMPG), which were used to prepare liposomes, were purchased from Nichiyu Liposome (Tokyo, Japan). CPT-11, which was used to prepare liposomes, was a kind gift from Yakult Honsha (Tokyo, Japan). 1-Monomethoxypolyethyleneglycol-2,3-dimyristoylglycerol (PEG-DMG) with PEG of an average molecular weight of 2000 Da was a gift from Nippon Oil & Fats (Tokyo, Japan).

2.2. Preparation of liposomes

Liposome preparation was performed according to the method of Bangham et al. [15]. DMPC/CH/DMPG (100:100:60 μmol) and 10 mg CPT-11 (15 μmol) were dissolved in a chloroform/methanol mixture (2:1, v/v). The chloroform and methanol were evaporated to dryness under a stream of nitrogen gas. The thin lipid film was evacuated in a desiccator and then hydrated with 8.0 ml of 9.0% sucrose in 10 mM lactate buffer (pH 4.0) in a water bath at 50–60°C for 10 min. The suspension was sonicated for 20 min above the T_c after nitrogen gas bubbling. The liposome suspension was extruded through two stacked polycarbonate membrane filters with 0.2 μm pores, followed by five additional extrusions through filters with 0.1 μm pores above the T_c, in order to obtain homogeneously sized liposome suspension. M-Lip (liposomes composed of DMPC/cholesterol/DMPG/CPT-11) and M-PEG (PEG-coated M-Lip) were prepared by adding 2.0 ml of 9.0% sucrose in 10 mM lactate buffer (pH 4.0) with or without 5.0 mol% PEG-DMG to 8.0 ml of the liposome suspension and then sonicating the mixture. S-Lip (liposomes composed of DSPC/cholesterol/DMPG/CPT-11) and S-PEG (PEG-coated S-Lip) were prepared by the same method, except that DMPC was changed to DSPC as the composing lipid of this liposome. The liposome used to examine the side-effects was prepared by adding CPT-11 (45 μmol).

Liposome suspension was dialyzed in 9.0% sucrose in 10 mM lactate buffer (pH 4.0) for 16 h. The indicated trap ratio of CPT-11 in all liposomes was above 90%.

2.3. Animal experiments

Male CDF_1 mice (body weight 20–25 g, 5 weeks old) were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). Ehrlich ascites carcinoma (5×10^5 cells/animal) was transplanted onto the backs of the mice. On day 14 after transplantation, tumor-bearing mice were used in the experiments.

In the distribution study, tumor-bearing mice were injected via the tail vein with Sol, M-Lip, M-PEG, S-Lip and S-PEG at a dose of 10 mg/kg as CPT-11. At 1, 2 and 8 h after injection, the mice were sacrificed by cervical dislocation and the blood was collected from the heart. The liver, gall bladder and tumor were immediately removed and washed. CPT-11 and SN-38 concentrations in the plasma and each tissue were determined according to a previous paper [16].

In the antitumor activity study, Sol or liposome

(CPT-11, 10 mg/kg per day for 3 days) was administered intravenously 14, 17 and 20 days after tumor inoculation. On day 23 after inoculation, the animals were killed by cervical dislocation. Tumors were rapidly removed and weighed. The CPT-11 and SN-38 concentrations in the tumor were determined.

For the study of side-effects, Sol, S-Lip and S-PEG (CPT-11, 100 mg/kg per day for 4 days) were injected intraperitoneally into normal mice once a day for 4 days. The mice were killed by cervical dislocation on day 2 following the last injection. After observing the intestine with the naked eye, the wet and dry weights of the feces in the large intestine were determined. The water content in the feces was calculated from these weights.

2.4. SN-38 generation in tissue homogenate

The liver and tumor in the tumor-bearing mice were removed and a 5.0% homogenate was prepared in an isotonic saline. Sol (10 μg/ml CPT-11) was added to each homogenate and incubated at 37°C for 4 h. After incubation, the SN-38 concentration in this homogenate was determined.

2.5. Statistical analysis

Statistical analysis was carried out by ANOVA and Student's *t*-test.

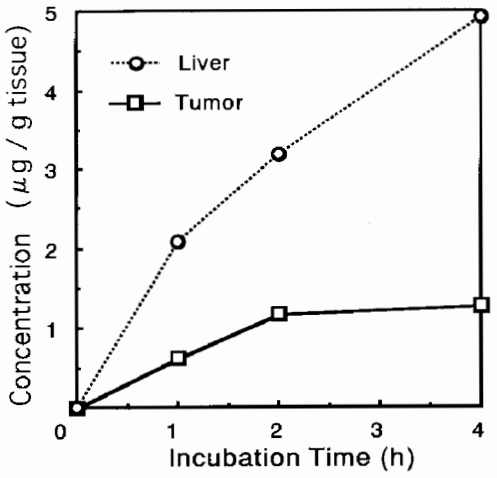

Fig. 1. Conversion to SN-38 from CPT-11 in the tumor and liver of mice (in vitro). Each point represents the mean of the duplicate of three samples.

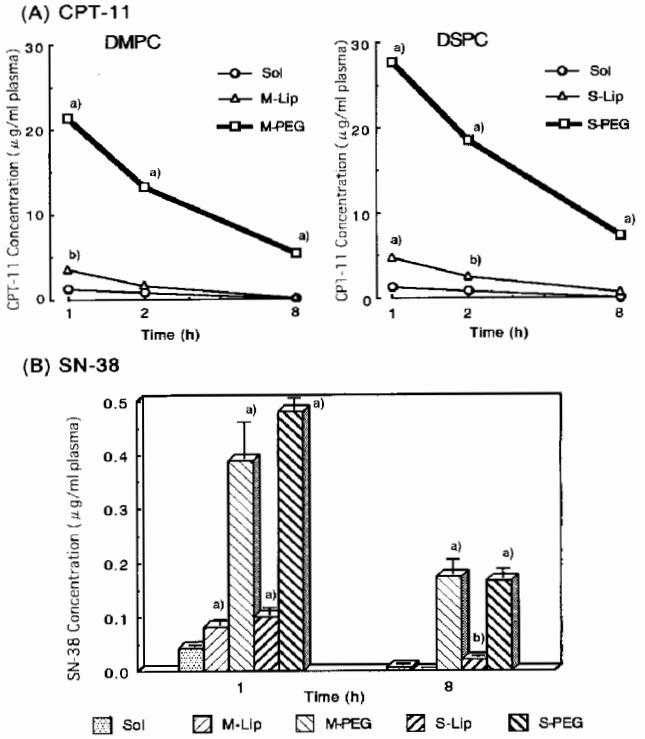

Fig. 2. CPT-11 and SN-38 concentrations in the plasma. Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. (A) Each point represents the mean of three mice, each with no more than 10% variation between them. (B) Each column represents the mean ± SD of three mice. Significant differences from the level of the Sol group are indicated by $^{a}P < 0.001$ and $^{b}P < 0.01$.

3. Results

3.1. Conversion to SN-38 from CPT-11 in vitro (Fig. 1)

CPT-11 was transiently converted to SN-38 by incubation in the liver homogenate. The SN-38 concentration was 3.19 μg/g liver and 4.92 μg/g liver 2 and 4 h after incubation, respectively. In the tumor homogenate, 37 and 26% SN-38 was generated at 2 and 4 h, respectively, compared to that in the liver.

3.2. Effect of liposomalization on tissue distribution of CPT-11

3.2.1. Plasma

In the plasma, the CPT-11 concentration 1 h after

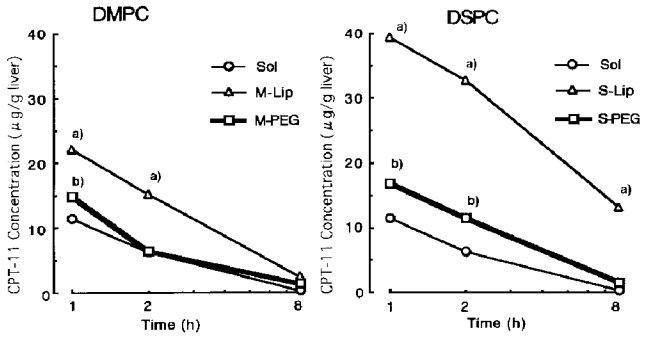

Fig. 3. CPT-11 concentration in the liver. Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. Each point represents the mean of three mice, each with no more than 10% variation between them. Significant differences from the level of the Sol group are indicated by $^{a}P < 0.001$ and $^{b}P < 0.01$.

injection in the M-Lip and S-Lip groups was increased 2.7-fold ($P < 0.01$) and 3.6-fold ($P < 0.001$), respectively, compared to that in the Sol group. Furthermore, each liposome prolonged the circulation in the plasma by PEG modification of liposomes and the CPT-11 concentration 8 h after injection in the M-PEG and S-PEG groups were shown to be increased 100-fold ($P < 0.001$) and 136-fold ($P < 0.001$), respectively, compared to that in the Sol group (Fig. 2A). The SN-38 concentration in the plasma was increased by liposomalization, suggesting the same tendency as in the CPT-11 concentration. In particular, the SN-38 concentrations in the M-PEG and S-PEG groups 8 h after injection were 11.4-fold ($P < 0.001$) and 9.2-fold ($P < 0.001$) that in the Sol group, respectively (Fig. 2B).

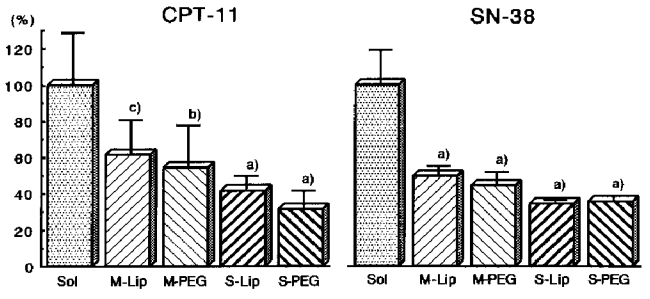

Fig. 4. CPT-11 and SN-38 concentrations in the bile. Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. Each column represents the mean ± SD of three mice. Significant differences from the level of the Sol group are indicated by $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$.

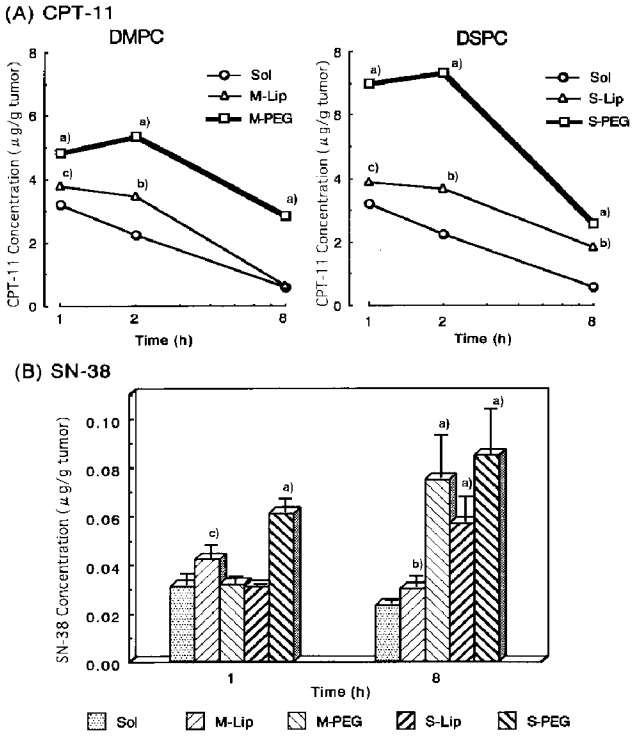

Fig. 5. CPT-11 and SN-38 concentrations in the tumor. Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. (A) Each point represents the mean of three mice, each with no more than 10% variation between them. (B) Each column represents the mean ± SD of three mice. Significant differences from the level of the Sol group are indicated by $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$.

3.2.2. Liver (Fig. 3)

The CPT-11 concentration in the liver of the M-Lip and S-Lip groups 2 h after injection was 1.9-fold ($P < 0.001$) and 3.4-fold ($P < 0.001$) that in the Sol group, respectively. The order of CPT-11 con-centration in the liver on the liposomes was M-Lip (S-Lip) > Sol = M-PEG (S-PEG). The change in SN-38 concentration showed the same tendency as that of the CPT-11 concentration. The SN-38 concentration in the M-PEG and S-PEG groups 8 h after injection was lower than that in the Sol group (data not shown).

3.2.3. Bile (Fig. 4)

The CPT-11 concentration in the bile was reduced by liposomalization. In particular, the concentration in the S-PEG group was only 30% ($P < 0.001$) of that in the Sol group. The change in the SN-38 concentra-

tion indicated the same tendency as the CPT-11 concentration in that the SN-38 concentration in the S-Lip and S-PEG groups was lower than that in the M-Lip and M-PEG groups, respectively.

3.2.4. Tumor (Fig. 5)

The CPT-11 concentration in the tumor was increased by liposomalization and PEG modification of the liposome. The order was S-PEG > M-PEG. The CPT-11 concentration 8 h after administration in the S-Lip and S-PEG groups increased by 3.2-fold ($P < 0.01$) and 4.5-fold ($P < 0.001$), respectively, compared to that in the Sol group (Fig. 5A). Furthermore, this time, the SN-38 concentration was increased 2.5-fold ($P < 0.001$) and 3.7-fold ($P < 0.001$) by S-Lip and S-PEG, respectively. The SN-38 concentrations in the M-PEG, S-Lip and S-PEG groups 8 h after injection were higher than those 1 h after injection (Fig. 5B).

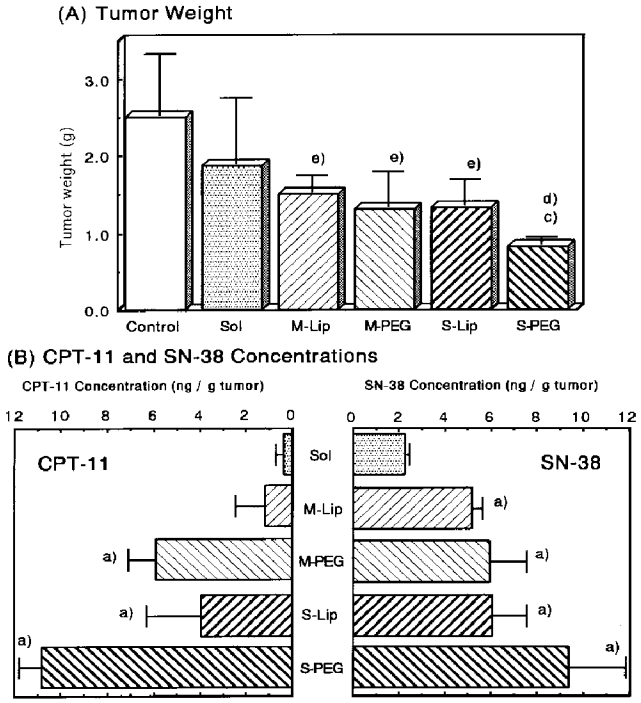

Fig. 6. Effects of liposomalization on (A) the changes in tumor weight induced by CPT-11 and (B) CPT-11 and SN-38 concentrations in the tumor. Mice were injected with CPT-11 (10 mg/kg per day (i.v.) for 3 days) in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. Each column represents the mean ± SD of eight mice. Significant differences from the level of the Sol group are indicated by $^{a}P < 0.001$ and $^{c}P < 0.05$. Significant differences from the level of the control group are indicated by $^{d}P < 0.01$ and $^{e}P < 0.05$.

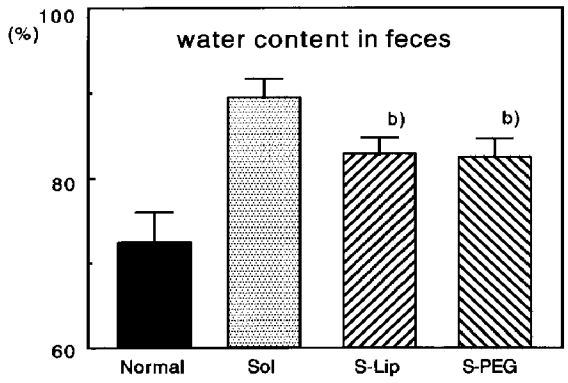

Fig. 7. Effect of liposomalization on CPT-11-induced delayed diarrheal symptoms in mice. Mice were injected with CPT-11 (100 mg/kg per day (i.p.) for 4 days) in the form of Sol, S-Lip and S-PEG. Each column represents the mean ± SD of six mice. A significant difference from the level of the Sol group is indicated by $^{b}P < 0.01$.

3.3. Antitumor activity of CPT-11-encapsulated liposomes

The tumor weight of the control level was 2.51 ± 0.79 g. The level in the Sol group was 1.87 ± 0.85 g, which decreased to 74.5% of the control level (Fig. 6A). There was an increased effect of liposomalization and PEG modification on the reduction of the tumor weight. The order of this effect was S-PEG > S-Lip = M-PEG > M-Lip > Sol. In particular, S-PEG enhanced by 2.6-fold (a significant difference from the level of the Sol group, $P < 0.05$) the CPT-11 inhibitory effect of tumor growth. Furthermore, the CPT-11 concentration in the tumor in the S-Lip and S-PEG groups was increased 10.6-fold ($P < 0.001$) and 29.0-fold ($P < 0.001$), respectively, compared to that in the Sol group. The SN-38 concentration in the tumor was elevated by liposomalization ($P < 0.001$), compared to the Sol level. The order of the SN-38 concentration was S-PEG > S-Lip = M-PEG > M-Lip > Sol (Fig. 6B).

3.4. Effect of liposomalization on CPT-11-induced intestinal toxicity

During the experimental period, the body weight of the mice in the Sol group decreased by 15%. However, the decreases in the S-Lip and S-PEG groups were smaller than that in the Sol group (data not shown). The water content in the feces on day 2

after the final administration in the Sol group was increased by 17% of the normal level. On the other hand, the increased ratio of this level in the S-Lip and S-PEG groups was inhibited to about 50% ($P < 0.01$) of that in the Sol group (Fig. 7).

4. Discussion

In these experiments, the interaction of CPT-11 in liposome was found to enhance the antitumor effect due to liver metabolism, but to decrease its effectiveness due to liposome uptake. CPT-11 was mainly converted to SN-38, an active metabolite, by the carboxylesterase in the liver. SN-38 has strong antitumor activity [16]. On the other hand, the liposome uptake by the reticuloendothelial system (RES) in the liver and spleen prevented the usefulness of liposome delivery. Therefore, PEG modification on the surface of the liposomes was attempted to avoid RES [6,17]. However, when a prodrug, such as CPT-11, is delivered in liposomes, PEG modification of the liposome is not always an improvement due to the elevated requirements involved in prodrug processing, especially in the case of processing of CPT-11 to SN-38 in these tumors. In this study, we confirmed the conversion of CPT-11 to SN-38 in the liver homogenate in vitro. Conversion to SN-38 in the tumor was also shown, with a conversion ratio that was a quarter of that in the liver. Thus, it is expected that the targeting of CPT-11 to the tumor by liposomalization may elevate the SN-38 level in the tumor and increase the antitumor activity of CPT-11. Furthermore, because SN-38 is not only associated with the antitumor activity of CPT-11 but also with the CPT-11-induced side toxicity, the SN-38 accumulation in the tumor may reduce its toxicity.

We have examined the effect of the composition of the phospholipid of the liposome containing CPT-11 on CPT-11 distribution in mice. In plasma, liposomalization and PEG modification of the liposomes increased the circulation of CPT-11. In particular, the CPT-11 concentration in the S-PEG group was markedly increased. Similarly, PEG modification of the liposome elevated the plasma circulation of SN-38. The order of liver accumulation by the liposomalization and the RES avoidance by the PEG modification was DSPC > DMPC. In the liver, the SN-38 concentration 8 h after the PEG-modified liposome injection decreased compared to that of the Sol group (data not shown). The decrease in the SN-38 concentration in the liver, where CPT-11 was mainly converted to SN-38, is expected to be connected with the reduction of side toxicity.

The order of the CPT-11 concentration in the tumor was M-PEG (S-PEG) > M-Lip (S-Lip) > Sol. This phenomenon is explained by the fact that PEG modification forms the fixed aqueous layer around the surface of the liposomes [18]. This fixed aqueous layer prevents an attack on liposomes by opsonins in the plasma and, therefore, avoids RES. The tumor accumulation of CPT-11 then increases by passive as opposed to active targeting. The change in the SN-38 concentration in the tumor was similar to that in the CPT-11 concentration and these levels 8 h after injection in the S-Lip and S-PEG groups increased to 2.5-fold ($P < 0.001$) and 3.7-fold ($P < 0.001$), respectively, of that in the Sol group. The increment of CPT-11 and SN-38 concentrations in the tumor by liposomalization and PEG modification suggests the possibility of an increase in CPT-11-induced antitumor activity. We examined the antitumor activity of these liposomes.

The weight of Ehrlich-solid tumor after S-Lip and S-PEG injection decreased 1.8- and 2.6-fold ($P < 0.05$), respectively, compared to the level in the Sol group. During this time, the CPT-11 and SN-38 concentrations in the tumor of the S-PEG group increased to 29-fold ($P < 0.001$) and 4.2-fold ($P < 0.001$), respectively, of the levels in the Sol group. Therefore, the increase in CPT-11-induced antitumor activity by liposomalization and PEG modification was corrected with the CPT-11 and SN-38 concentrations in the tumor. Because the therapeutic dose of CPT-11 in the mice was about 50 mg/kg (i.v.), the administered dose used in our experiment can be considered as a therapeutic dose. There was no CPT-11-induced death or decrease in body weight. Therefore, these results appear to show an increase in antitumor activity and no change in the side-effects.

The liposomalization of CPT-11 as the prodrug has been indicated to also increase antitumor activity. With regard to the liposomalization of the prodrugs, these results suggest that the targeting in the tumor is useful in the case of the generation of an active metabolite in the tumor.

CPT-11 has severe side toxicities such as myelosuppression and intestinal disorder in clinical use and there are reports of CPT-11-induced deaths [5]. The intestinal toxicity is connected with SN-38 [19], which is conjugated with grucronic acid in the liver and excreted in the bile. After deconjugation in the intestine, SN-38 was again regenerated. This SN-38 in the intestine is likely to cause intestinal disorder [14,20,21]. The CPT-11 and SN-38 concentrations in the bile were decreased by liposomalization (Fig. 4). Therefore, it may be possible to reduce the CPT-11-induced intestinal disorder by liposomalization of CPT-11.

During the experiments, the body weight of the mice in the Sol group was observed to decrease by 15%, whereas the decreases in the S-Lip and S-PEG groups were less than 15%. Swelled intestine and intestinal disorder were found on day 2 after the final administration in the Sol group. In contrast, these changes in the S-Lip and S-PEG groups were slight. The water content in the feces in the Sol group increased by 17% of the normal level, whereas the increase in the S-Lip and S-PEG groups was about 50%. Thus, liposomalization of CPT-11 appears to be able to suppress CPT-11-induced diarrhea as lethal toxicity.

The CPT-11 and SN-38 concentrations in the bone marrow after S-Lip treatment were 109 and 86%, respectively, compared to the level in the Sol group. Thus, there was no increase in the CPT-11 and SN-38 concentrations in the bone marrow by liposomalization. Furthermore, the CPT-11-induced reduction in the number of bone marrow cells was not enhanced by liposomalization (109% of that in the Sol group). Therefore, it is expected that CPT-11-induced myelosuppression was not amplified or improved by liposomalization.

This paper is the first to report on the usefulness of liposomalization of the prodrug. We are confident that this report will significantly contribute to liposomal study and will extend the efficacy of the clinical use of CPT-11.

Acknowledgements

We are grateful to Yakult Honsha Co. Ltd., Tokyo, Japan for supplying the irinotecan hydrochloride used in this study.

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IRINOTECAN PLUS FLUOROURACIL AND LEUCOVORIN FOR METASTATIC COLORECTAL CANCER

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ABSTRACT

Background The combination of fluorouracil and leucovorin has until recently been standard therapy for metastatic colorectal cancer. Irinotecan prolongs survival in patients with colorectal cancer that is refractory to treatment with fluorouracil and leucovorin. In a multicenter trial, we compared a combination of irinotecan, fluorouracil, and leucovorin with bolus doses of fluorouracil and leucovorin as first-line therapy for metastatic colorectal cancer. A third group of patients received irinotecan alone.

Methods Patients were randomly assigned to receive irinotecan (125 mg per square meter of body-surface area intravenously), fluorouracil (500 mg per square meter as an intravenous bolus), and leucovorin (20 mg per square meter as an intravenous bolus) weekly for four weeks every six weeks; fluorouracil (425 mg per square meter as an intravenous bolus) and leucovorin (20 mg per square meter as an intravenous bolus) daily for five consecutive days every four weeks; or irinotecan alone (125 mg per square meter intravenously) weekly for four weeks every six weeks. End points included progression-free survival and overall survival.

Results Of 683 patients, 231 were assigned to receive irinotecan, fluorouracil, and leucovorin; 226 to receive fluorouracil and leucovorin; and 226 to receive irinotecan alone. In an intention-to-treat analysis, as compared with treatment with fluorouracil and leucovorin, treatment with irinotecan, fluorouracil, and leucovorin resulted in significantly longer progression-free survival (median, 7.0 vs. 4.3 months; $P=0.004$), a higher rate of confirmed response (39 percent vs. 21 percent, $P<0.001$), and longer overall survival (median, 14.8 vs. 12.6 months; $P=0.04$). Results for irinotecan alone were similar to those for fluorouracil and leucovorin. Grade 3 (severe) diarrhea was more common during treatment with irinotecan, fluorouracil, and leucovorin than during treatment with fluorouracil and leucovorin, but the incidence of grade 4 (life-threatening) diarrhea was similar in the two groups (<8 percent). Grade 3 or 4 mucositis, grade 4 neutropenia, and neutropenic fever were less frequent during treatment with irinotecan, fluorouracil, and leucovorin. Adding irinotecan to the regimen of fluorouracil and leucovorin did not compromise the quality of life.

Conclusions Weekly treatment with irinotecan plus fluorouracil and leucovorin is superior to a widely used regimen of fluorouracil and leucovorin for metastatic colorectal cancer in terms of progression-free survival and overall survival. (N Engl J Med 2000;343:905-14.)

THE antimetabolite fluorouracil is widely used to treat metastatic colorectal cancer, the second-leading cause of death from cancer in North America.[1] The drug inhibits thymidylate synthase, an enzyme required for the synthesis of DNA.[2] It is commonly administered with leucovorin, a reduced folate (tetrahydrofolate) that increases the affinity of fluorouracil for thymidylate synthase. Among various schedules of administration, the efficacy of the Mayo Clinic bolus regimen, in which the two drugs are injected daily for five days every four weeks, has been validated in randomized trials[3-5] and is frequently used as first-line therapy for metastatic colorectal cancer.

Irinotecan (Camptosar, Pharmacia) is a potent inhibitor of topoisomerase I, a nuclear enzyme involved in the unwinding of DNA during replication.[6-8] Irinotecan has demonstrated antitumor activity against metastatic colorectal cancer when used alone as first-line treatment[9-12] or as second-line treatment after the failure of fluorouracil.[10-18] In randomized phase 3 trials, second-line irinotecan extended survival significantly as compared with supportive care[19] or infusions of fluorouracil and leucovorin as a second-line therapy.[20]

The mechanism of action of irinotecan and its activity against untreated and fluorouracil-resistant colorectal cancer were the rationale for combining irinotecan with fluorouracil and leucovorin as first-line therapy for this disease. A phase 1 study[21] developed a combination regimen based on the weekly irinotecan schedule that had been the most widely studied in the United States.[9,11,13,14,16,18,22] We conducted a phase 3 trial in which the combination of irinotecan, fluorouracil, and leucovorin was compared with the Mayo Clinic bolus regimen of fluorouracil and leucovorin as a first-line treatment for metastatic colorectal cancer. A third group of patients was treated with irinotecan alone to determine the activity of this drug as a single agent in a multicenter trial.

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*Additional principal investigators are listed in the Appendix.

METHODS

Study Design and Entry Criteria

We conducted a phase 3, randomized, open-label, multicenter trial. To be eligible, patients had to have histologically documented colorectal cancer and measurable metastatic disease; an Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2; and adequate organ function. Prior therapy for metastatic disease was not permitted; patients who had received adjuvant fluorouracil-based therapy were eligible if they had remained free of disease for at least one year after the completion of adjuvant therapy. Patients who had received pelvic irradiation were excluded. The protocol was approved by the institutional review boards of all participating institutions, and all patients gave written informed consent before enrollment.

Stratification, Randomization, and Therapy

Patients were stratified according to age (<65 years vs. ≥65 years), ECOG performance status (0 vs. 1 or 2), interval from diagnosis to enrollment (<6 months vs. ≥6 months), and history of adjuvant therapy with fluorouracil (yes vs. no) and were then randomly assigned to one of three regimens (Table 1). Treatment was continued until one of the following occurred: disease progression, unacceptable adverse effects, or the withdrawal of consent by the patient. After the first treatment, the doses were adjusted to accommodate individual levels of tolerance. The severity of adverse effects was evaluated with use of the National Cancer Institute Common Toxicity Criteria (version 1.0), in which a grade of 0 indicates no adverse effects, a grade of 1 minor effects, a grade of 2 moderate effects, a grade of 3 severe effects, and a grade of 4 life-threatening effects. The doses of irinotecan and fluorouracil (in the triple-drug group) were reduced by 20 percent during a cycle if a grade 2 adverse effect occurred and were omitted in the event of a grade 3 or 4 effect. Once the adverse effect resolved, treatment was resumed; the dose was reduced by 20 percent in the case of a grade 3 adverse effect and by 40 percent in the case of a grade 4 adverse effect or neutropenic fever. After grade 3 or 4 mucositis, only the doses of fluorouracil were reduced. For the Mayo Clinic bolus regimen, the doses of fluorouracil were reduced by 20 percent after a grade 3 adverse effect and by 40 percent after a grade 4 effect or neutropenic fever.

Supportive care included intensive treatment with loperamide[23] for late diarrhea. Atropine was given as needed for irinotecan-related cholinergic symptoms.[24,25] Antiemetic agents were provided at the discretion of the treating physician. The prophylactic use of colony-stimulating factors was not permitted.

Evaluation of Patients

Tumors were measured every 6 weeks through week 24 and then every 12 weeks until the tumor progressed. An objective response was defined as a reduction of at least 50 percent in the area of all measurable lesions on computed tomographic (CT) or other scanning. Confirmed objective responses were those for which a follow-up scan obtained at least four weeks later demonstrated the persistence of the response. Tumor progression was defined as an increase of at least 25 percent in the overall area of the tumor or the appearance of new lesions. The determination of responses and progression was based on investigator-reported measurements. Safety assessments and complete blood counts were performed weekly. Serum chemical values and the quality of life were assessed at the beginning of each treatment cycle. The Quality of Life Questionnaire of the European Organization for Research and Treatment of Cancer (version 2) was used to assess the quality of life. On this test, scores can range from 0 to 100, with higher scores on functional scales and lower scores on symptom scales indicating a better quality of life. Data on subsequent treatments for colorectal cancer and survival were collected approximately every three months after the end of the study treatment.

Statistical Analysis

The primary end point was progression-free survival. Progression-free survival was defined as the length of time from randomization to disease progression or to death from disease progression or unknown causes. For patients who were removed from the study or died of causes unrelated to colorectal cancer, progression-free survival was conservatively defined as the time from randomization to the last date on which the patient was known to be free of progressive disease.

Past experience suggested that the median progression-free survival with fluorouracil and leucovorin would be five months.[4,5] We estimated that 220 patients would be needed in each group in order to detect a 40 percent improvement in median progression-free survival, to seven months, with triple-drug therapy with a power of 0.85.

In the evaluation of efficacy end points, all patients enrolled in the study were included and analyzed according to the intention-to-treat principle. In the analysis of treatment administration and adverse effects, only the patients who actually received treatment were assessed. (Of the 683 patients who enrolled in the study, the 16 who were not treated were excluded from this part of the analysis and the adverse effects in the 4 patients who received the wrong treatment were attributed to the drugs they actually received.) We used two-tailed, unstratified, log-rank tests, with a P value of 0.05 or less considered to indicate statistical significance, in the analyses comparing time-to-event end points between the triple-drug group and the two-drug group, which were selected a priori as the only groups to be used in statistical hypothesis testing. For response rates, we used chi-square tests to compare these two groups. We assessed changes in subscale scores of the quality of life between groups using analysis of variance for repeated meas-

TABLE 1. TREATMENT REGIMENS.*

REGIMEN	STARTING DOSE	SCHEDULE
Irinotecan	125 mg/m² of body-surface area intravenously over a 90-minute period	Each one given weekly for 4 weeks every 6 weeks
Leucovorin	20 mg/m² as an IV bolus	
Fluorouracil	500 mg/m² as an IV bolus	
Leucovorin	20 mg/m² as an IV bolus	Each one given daily for 5 days (on days 1–5) every 4 weeks
Fluorouracil	425 mg/m² as an IV bolus	
Irinotecan alone	125 mg/m² intravenously over a 90-minute period	Given weekly for 4 weeks every 6 weeks

*For each regimen, the agents are listed in the order in which they were administered. IV denotes intravenous.

ures, whereas we used Student's t-tests to compare the greatest worsening in the quality of life from base line.

We used proportional-hazards modeling with forward selection to determine the influence of the patients' base-line characteristics on response, progression-free survival, and overall survival. A P value of less than 0.05 was considered to indicate statistical significance. Interactions between treatment and the various factors with a P value of less than 0.10 were assessed. Predefined base-line characteristics for this analysis included the four stratification factors and other potentially prognostic factors: sex, race or ethnic group, the site of the primary tumor, the time from diagnosis of disease to the occurrence of metastasis, the number of involved organs, the presence or absence of liver involvement, hemoglobin level, white-cell count, and serum levels of carcinoembryonic antigen, lactate dehydrogenase, and total bilirubin.

RESULTS

Characteristics of the Patients

A total of 683 patients were enrolled in the study and randomly assigned to one of the three treatments between May 1996 and May 1998 at 71 sites in the United States, Canada, Australia, and New Zealand. Data were collected for an additional 19 months after accrual ended, with data on survival collected through December 1999. The intention-to-treat population comprised 231 patients in the group assigned to receive irinotecan, fluorouracil, and leucovorin; 226 patients in the group assigned to receive fluorouracil and leucovorin; and 226 patients in the group assigned to receive irinotecan alone. After the exclusion of the 16 patients who never received therapy and the 4 who received the wrong treatment, the treated population included 225 patients who received irinotecan, fluorouracil, and leucovorin; 219 patients who received fluorouracil and leucovorin; and 223 patients who received irinotecan alone.

Table 2 shows the base-line characteristics of the patients, all of which were balanced among the treatment groups except for the proportion of men, which was greater in the triple-drug group than in the two-drug group (65 percent vs. 54 percent, P=0.02). The median age was just over 60 years. More than 50 percent of the patients had an ECOG performance status of 1 or 2 at base line. Approximately 35 percent of the patients in each group had at least two organs involved, with the liver being the most common site of metastatic disease. Because most patients had metastatic disease at diagnosis, only about 10 percent of them had received adjuvant therapy. In violation of the entry criteria, nine patients had received pelvic radiotherapy. There were no significant differences in base-line laboratory values among the groups.

Treatment

The median duration of treatment with irinotecan, fluorouracil, and leucovorin was 5.5 months. For patients who received fluorouracil and leucovorin and those who received irinotecan alone, the median durations of treatment were 4.1 months and 3.9 months, respectively. The median relative intensity of the dose of irinotecan (calculated as the actual dose delivered divided by the intended dose) was similar in the group given irinotecan alone and the group given irinotecan, fluorouracil, and leucovorin (75 percent vs. 72 percent). The median relative intensity of the dose of fluorouracil in the triple-drug group was lower than that in the two-drug group (71 percent vs. 86 percent). This lower dose intensity may have resulted, in part, from the weekly reductions in dose permitted with the triple-drug regimen.

Among patients with follow-up data, 52 percent of those who received irinotecan, fluorouracil, and leucovorin during the study, 70 percent of those who received fluorouracil and leucovorin, and 79 percent of those who were given irinotecan alone received additional chemotherapy after the study treatment ended. The majority of patients (56 percent) in the group given fluorouracil and leucovorin received an irinotecan-based regimen after the study. Oxaliplatin or other investigational agents were administered to fewer than 5 percent of patients in any group.

Efficacy

Progression-free survival, the primary end point of the study, was significantly longer among patients who were assigned to receive irinotecan, fluorouracil, and leucovorin than among those assigned to receive fluorouracil and leucovorin (median, 7.0 months vs. 4.3 months; P=0.004) (Table 3). Progression-free survival among patients who were assigned to receive irinotecan alone (median, 4.2 months) was similar to that among patients who were assigned to receive fluorouracil and leucovorin. The Kaplan–Meier estimates of progression-free survival in the three groups are shown in Figure 1.

The objective rate of response was 50 percent among patients who were assigned to receive irinotecan, fluorouracil, and leucovorin and 28 percent among those assigned to receive fluorouracil and leucovorin (P<0.001). The rates of objective responses that were confirmed by imaging tests four to six weeks later were also significantly higher among patients in the triple-drug group than among those in the two-drug group (39 percent vs. 21 percent, P<0.001). The rates of objective and confirmed responses with irinotecan alone (29 percent and 18 percent, respectively) were similar to those with fluorouracil and leucovorin (28 percent and 21 percent, respectively). A complete response was seen in six patients in the triple-drug group, two patients in the two-drug group, and four patients assigned to receive irinotecan alone. The median duration of confirmed response was approximately nine months in all groups.

The median survival of patients who were assigned to receive irinotecan, fluorouracil, and leucovorin was 14.8 months, as compared with 12.6 months among patients who were assigned to receive fluorouracil and leucovorin (P=0.04). The median survival of patients

TABLE 2. BASE-LINE CHARACTERISTICS OF THE PATIENTS.*

CHARACTERISTIC	IRINOTECAN, FLUOROURACIL, AND LEUCOVORIN (N=231)	FLUOROURACIL AND LEUCOVORIN (N=226)	IRINOTECAN ALONE (N=226)
Sex — no. (%)			
Male	151 (65)	123 (54)	145 (64)
Female	79 (34)	101 (45)	80 (35)
Not available†	1 (<1)	2 (1)	1 (<1)
Age‡			
Median — yr	62	61	61
Range — yr	25–85	19–85	30–87
<65 years — no. (%)	139 (60)	136 (60)	135 (60)
≥65 years — no. (%)	91 (39)	88 (39)	90 (40)
Not available — no. (%)†	1 (<1)	2 (1)	1 (<1)
ECOG performance status — no. (%)‡			
0	89 (39)	93 (41)	104 (46)
1	106 (46)	102 (45)	103 (46)
2	35 (15)	29 (13)	18 (8)
Not available†	1 (<1)	2 (1)	1 (<1)
Site of primary tumor — no. (%)			
Colon	188 (81)	192 (85)	189 (84)
Rectum	38 (16)	31 (14)	33 (15)
Not available†	5 (2)	3 (1)	4 (2)
No. of involved organs — no. (%)			
1	147 (64)	149 (66)	140 (62)
2	59 (26)	52 (23)	64 (28)
>2	24 (10)	23 (10)	21 (9)
Not available†	1 (<1)	2 (1)	1 (<1)
Liver involvement — no. (%)			
Yes	189 (82)	185 (82)	188 (83)
No	41 (18)	39 (17)	37 (16)
Not available†	1 (<1)	2 (1)	1 (<1)
Time from diagnosis to randomization — mo‡			
Median	1.9	1.7	1.8
Range	0.1–161	0.1–203	0.1–185
Prior adjuvant fluorouracil — no. (%)‡			
Yes	25 (11)	18 (8)	23 (10)
No	206 (89)	208 (92)	203 (90)
Prior radiotherapy — no. (%)			
Any	7 (3)	5 (2)	3 (1)
Pelvis or abdomen	4 (2)	2 (1)	3 (1)
Other sites	3 (1)	3 (1)	0
Base-line laboratory abnormalities — no./total no. (%)			
White-cell count ≥8×10^3/mm^3	119/227 (52)	115/217 (53)	113/221 (51)
Hemoglobin <11 g/dl	58/227 (26)	55/217 (25)	57/221 (26)
Lactate dehydrogenase > upper normal limit	126/210 (60)	112/201 (56)	104/195 (53)
Total bilirubin > upper normal limit	15/226 (7)	9/218 (4)	22/220 (10)
Carcinoembryonic antigen ≥100 ng/ml	89/224 (40)	82/213 (38)	81/219 (37)

*Because of rounding, percentages may not total 100. ECOG denotes Eastern Cooperative Oncology Group.

†Information was not available for some patients who underwent randomization but were not treated.

‡This was a stratification variable.

assigned to receive irinotecan alone was similar to that of patients assigned to receive fluorouracil and leucovorin (12.0 vs. 12.6 months). The Kaplan–Meier survival curves are shown in Figure 2.

Proportional-Hazards Modeling

Multiple regression modeling of the rates of objective response revealed no interactions between treatment and the stratification factors or other potentially prognostic factors. The addition of irinotecan to therapy with fluorouracil and leucovorin virtually doubled the response rates in all predefined subgroups of patients.

For progression-free and overall survival, we used Cox regression techniques to compare the effects of irinotecan, fluorouracil, and leucovorin with those of fluorouracil and leucovorin in the context of the stratification factors and other predefined base-line clinical characteristics (Table 4). Factors predictive of improved progression-free survival and overall survival were a normal lactate dehydrogenase level and an excellent performance status (a score of 0). A hemo-

TABLE 3. INTENTION-TO-TREAT ANALYSIS OF EFFICACY.

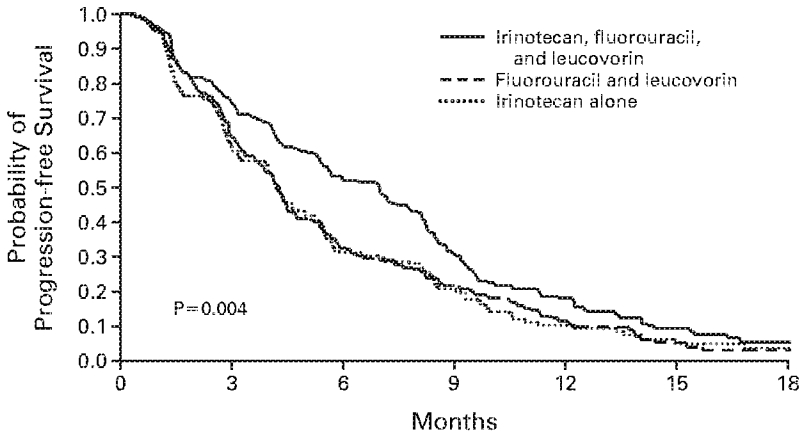
END POINT	IRINOTECAN, FLUOROURACIL, AND LEUCOVORIN (N=231)	FLUOROURACIL AND LEUCOVORIN (N=226)	P VALUE*	IRINOTECAN ALONE (N=226)
Median progression-free survival (mo)	7.0	4.3	0.004†	4.2
Objective response rate (%)	50	28	<0.001‡	29
Confirmed objective response rate (%)§	39	21	<0.001‡	18
Median duration of confirmed response (mo)	9.2	8.7	0.37†	9.0
Median overall survival (mo)	14.8	12.6	0.04†	12.0

*P values are for the comparison of the triple-drug group with the two-drug group.

†The log-rank test was used.

‡The chi-square test was used.

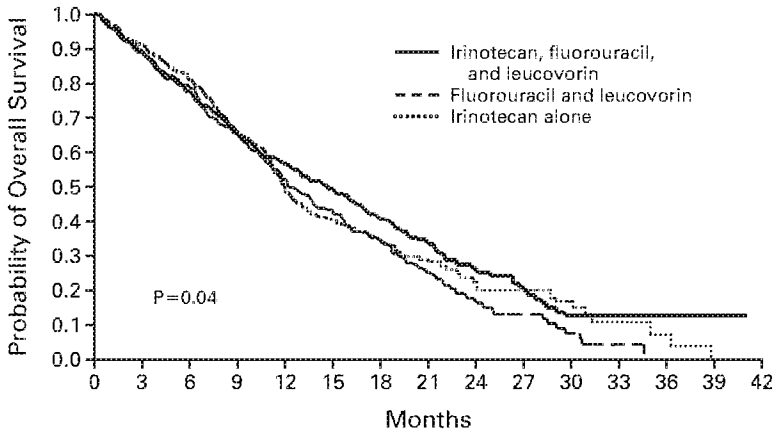
§Responses were confirmed by computed tomography or other scanning four to six weeks after the initial evidence of an objective response had been obtained.



NO. AT RISK							
Irinotecan, fluorouracil, and leucovorin	231	154	99	49	23	11	5
Fluorouracil and leucovorin	226	124	54	32	15	5	2
Irinotecan alone	226	112	51	29	12	4	1

Figure 1. Kaplan–Meier Estimates of Progression-free Survival.
The P value was derived from a log-rank test comparing the triple-drug group with the two-drug group.

globin level of at least 11 g per deciliter and a normal white-cell count were predictive of better progression-free survival and overall survival, respectively. Unexpectedly, an age of 65 years or older was also associated with a longer progression-free survival. Treatment with irinotecan, fluorouracil, and leucovorin remained a significant independent predictor of longer progression-free survival ($P<0.001$) and overall survival ($P=0.03$) when other significant base-line characteristics were taken into account. Treatment with irinotecan, fluorouracil, and leucovorin was associated with a 36 percent reduction in the risk of progression and a 22 percent reduction in the risk of death relative to treatment with fluorouracil and leucovorin alone (Table 4). No relevant interactions between treatment and other factors were identified for progression-free survival, indicating that progression-free survival was improved in all the predefined subgroups of patients. In the comparison of irinotecan, fluorouracil, and leucovorin with fluorouracil and leucovorin, the reduction in the risk of death among patients with a normal lactate dehydrogenase level was 43 percent, as compared with a reduction of 12 percent among those with an elevated lactate dehydrogenase level,



No. at Risk								
Irinotecan, fluorouracil, and leucovorin	231	178	129	84	33	10	3	1
Fluorouracil and leucovorin	226	175	116	72	23	5	0	0
Irinotecan alone	226	181	110	69	28	11	2	0

Figure 2. Kaplan–Meier Estimates of Overall Survival.
The P value was derived from a log-rank test comparing the triple-drug group with the two-drug group.

Table 4. Results of Cox Regression Analysis.*

Factor	Progression-free Survival		Overall Survival	
	Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)	P Value
Serum lactate dehydrogenase (≤UNL vs. >UNL)	0.60 (0.47–0.76)	<0.001	0.47 (0.36–0.60)	<0.001
No. of involved organs (1 vs. ≥2)	0.63 (0.50–0.80)	<0.001	0.67 (0.54–0.83)	<0.001
Performance status (0 vs. 1 or 2)	0.74 (0.59–0.93)	0.009	0.56 (0.44–0.70)	<0.001
Bilirubin level (≤UNL vs. >UNL)	0.56 (0.35–0.89)	0.01	0.53 (0.33–0.83)	0.005
White-cell count (<8×10^3/mm^3 vs. ≥8×10^3/mm^3)†	—	—	0.65 (0.52–0.82)	<0.001
Hemoglobin level (≥11 g/dl vs. <11 g/dl)‡	0.74 (0.58–0.95)	0.02	—	—
Age (≥65 yr vs. <65 yr)	0.78 (0.63–0.98)	0.03	0.82 (0.65–1.02)	0.08§
Treatment (irinotecan, fluorouracil, and leucovorin vs. fluorouracil and leucovorin)	0.64 (0.51–0.79)	<0.001	0.78 (0.63–0.97)	0.03

*CI denotes confidence interval, and UNL upper limit of normal.

†This variable was not included in the analysis of progression-free survival, because it was not significant.

‡This variable was not included in the analysis of overall survival, because it was not significant.

§Age was identified as a stratification factor in the study design and was therefore included in the model even though it was only marginally significant (P=0.08).

suggesting a possible interaction of the lactate dehydrogenase level with treatment with respect to survival (P=0.07).

Adverse Effects

As shown in Table 5, 22.7 percent of patients who were given irinotecan, fluorouracil, and leucovorin had diarrhea of grade 3 or 4, as compared with 13.2 percent of patients who were given fluorouracil and leucovorin and 31.0 percent of patients who were given irinotecan alone. The difference between the triple-drug group and the two-drug group was primarily in the incidence of grade 3 diarrhea; the incidence of grade 4 diarrhea was similar in the two groups (7.6

percent and 7.3 percent). The group given irinotecan alone had an incidence of grade 4 diarrhea of 12.6 percent. Vomiting of grade 3 or 4 was more common with combination regimens that included irinotecan. Mucositis of grade 3 or 4 occurred in only 2.2 percent of patients who received irinotecan alone or in combination. As expected, the Mayo Clinic bolus regimen of fluorouracil and leucovorin was associated with a much higher frequency of grade 3 or 4 mucositis (16.9 percent). Moreover, the frequency of grade 4 neutropenia during treatment with irinotecan, fluorouracil, and leucovorin was almost half that observed during treatment with fluorouracil and leucovorin (24.0 percent vs. 42.5 percent); neutropenic fever was also less common with irinotecan, fluorouracil, and leucovorin than with fluorouracil and leucovorin (7.1 percent vs. 14.6 percent). Irinotecan alone was associated with the lowest incidence of grade 3 or 4 neutropenia. The incidence of treatment-related death was approximately 1 percent in all three groups.

TABLE 5. ADVERSE EVENTS AMONG PATIENTS WHO RECEIVED THE ASSIGNED TREATMENT.*

ADVERSE EVENT	IRINOTECAN, FLUOROURACIL, AND LEUCOVORIN (N=225)	FLUOROURACIL AND LEUCOVORIN (N=219)	IRINOTECAN ALONE (N=223)
		percent	
Diarrhea			
Grade 3 or 4	22.7	13.2	31.0
Grade 3	15.1	5.9	18.4
Grade 4	7.6	7.3	12.6
Vomiting			
Grade 3 or 4	9.7	4.1	12.1
Grade 3	5.3	2.7	5.8
Grade 4	4.4	1.4	6.3
Mucositis			
Grade 3 or 4	2.2	16.9	2.2
Grade 3	2.2	14.6	1.8
Grade 4	0	2.3	0.4
Neutropenia			
Grade 3 or 4	53.8	66.2	31.4
Grade 3	29.8	23.7	19.3
Grade 4	24.0	42.5	12.1
Neutropenic complications			
Fever	7.1	14.6	5.8
Infection	1.8	0.0	2.2
Discontinuation related to adverse events	7.6	6.4	11.7
Drug-related deaths	0.9	1.4	0.9

*A grade of 3 indicated a severe event, and a grade of 4 a life-threatening event.

Quality of Life

Analyses of the quality of life showed that there were no significant differences between the group given irinotecan, fluorouracil, and leucovorin and the group given fluorouracil and leucovorin (Fig. 3). In univariate analyses in which we compared the greatest worsening in the quality of life from base line, the mean increases in the severity of symptoms were smaller in the triple-drug group than in the two-drug group with respect to fatigue (increase in severity, 8 percent vs. 20 percent), anorexia (1 percent decrease vs. 9 percent increase), and pain (increase, 1 percent vs. 8 percent) ($P<0.05$ for all comparisons, by Student's t-test). As indicated by the measurement of the greatest declines from base line in role functioning (the ability to perform the activities of daily living), the triple-drug group had a smaller decrease in function than the two-drug group (decrease, 6 percent vs. 13 percent; $P<0.05$ by Student's t-test). The extent of the changes in other subscales in this analysis was not significantly different between the groups.

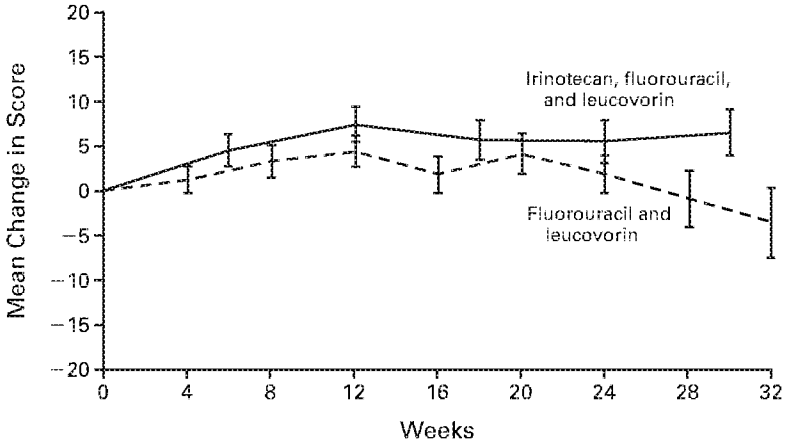
DISCUSSION

In this phase 3, randomized study, we compared the clinical benefits of a combination of irinotecan plus fluorouracil and leucovorin with those of fluorouracil and leucovorin alone as first-line therapy for metastatic colorectal cancer. The control regimen of fluorouracil and leucovorin that we used has been one of the most commonly used treatments for metastatic colorectal cancer in North America; thus, our findings can give practitioners insight into the relative efficacy and safety of the new regimen as contrasted with a familiar standard.

The base-line clinical characteristics of the treatment groups were similar except for a greater proportion of men in the group assigned to receive irinotecan, fluorouracil, and leucovorin than in the group assigned to receive fluorouracil and leucovorin. However, this difference did not appear to influence the results; when sex was tested in multivariate analyses, it was not significantly predictive of the outcome.

Our results show that, as compared with fluorouracil-based therapy, the combination of irinotecan, fluorouracil, and leucovorin significantly delays disease progression while reducing the size of tumors. Progression-free survival was significantly longer among patients who were assigned to receive irinotecan, fluorouracil, and leucovorin than among those assigned to receive fluorouracil and leucovorin (median progression-free survival, 7.0 and 4.3 months, respectively; $P=0.004$), with an average reduction of 36 percent in the risk of disease progression at any given time. The rates of response with irinotecan, fluorouracil, and leucovorin were close to double those with fluorouracil and leucovorin (50 percent vs. 28 percent, $P<0.001$). Improvements in response rates and progression-free survival were observed in every subgroup of patients in the triple-drug group, including those with a poor ECOG performance status, an age of 65 years or older, extensive disease, a history of adjuvant therapy, or abnormal laboratory values at base line.

Treatment with irinotecan, fluorouracil, and leucovorin was also associated with a statistically signif-



No. at Risk									
Irinotecan, fluorouracil, and leucovorin	222	166	145	125	107	87			
Fluorouracil and leucovorin	218	167	152	131	109	84	71	54	48

Figure 3. Mean (±SE) Changes from Base Line in Scores on the Global Health Status Subscale of the Quality of Life Questionnaire of the European Organization for Research and Treatment of Cancer.

On this scale, scores can range from 0 to 100, with higher scores indicating a better quality of life.

icant improvement in overall survival (median, 14.8 months, as compared with 12.6 months in the group assigned to fluorouracil and leucovorin; $P=0.04$). Evaluation of the hazard ratio for the three-drug group relative to the two-drug group indicated that, at any given time, the relative risk of death in the triple-drug group was an average of 22 percent lower. The improvement in survival in the patients who received the triple-drug combination is particularly notable because over half the patients in the control group eventually received irinotecan as second-line therapy. Thus, concurrent first-line administration of irinotecan and fluorouracil appears to be superior to sequential administration.

We performed a Cox regression analysis of prognostic factors identified in other trials[19,26,26-28] and confirmed that a good performance status, fewer metastatic sites, relatively normal laboratory results (normal lactate dehydrogenase and bilirubin levels, normal white-cell count, and a hemoglobin level of at least 11 g per deciliter) are associated with better outcomes. After adjustment for these factors, the difference between the group given irinotecan, fluorouracil, and leucovorin and the group given fluorouracil and leucovorin remained significant with respect to improvements in both progression-free survival ($P<0.001$) and overall survival ($P=0.03$).

As stipulated by the protocol, the irinotecan-alone group was not the focus of statistical testing. However, as expected, the efficacy results in this group were generally consistent with those observed with fluorouracil and leucovorin alone.

The results of our study complement the findings of another phase 3 trial comparing irinotecan, fluorouracil, and leucovorin with fluorouracil and leucovorin as first-line therapy for metastatic colorectal cancer.[29] That study, conducted primarily in Europe, randomly assigned 385 patients to receive either irinotecan plus infusions of fluorouracil and leucovorin or infusions of fluorouracil and leucovorin alone. The results of both studies are remarkably consistent; in the European trial, progression-free survival was significantly improved with the triple-drug therapy as compared with the two-drug therapy (median, 6.7 months vs. 4.4 months; $P<0.001$). Likewise, the confirmed objective response rate in the group given irinotecan, fluorouracil, and leucovorin was significantly higher than the rate in the group given fluorouracil and leucovorin (35 percent vs. 22 percent, $P<0.005$). The addition of irinotecan resulted in an improvement in survival of approximately 20 percent (median, 17.4 months vs. 14.1 months; $P=0.03$), results that were similar to those in our study.

In our study, the incidence of grade 3 diarrhea was greater with triple-drug therapy than with two-drug therapy. However, grade 4 diarrhea — largely defined by the need for hospitalization for supportive care — was infrequent (<8 percent) in both groups. The incidence of grade 4 diarrhea was lower with irinotecan, fluorouracil, and leucovorin than with irinotecan alone, perhaps because the neutropenia induced by fluorouracil and leucovorin in the three-drug group prompted early midcycle reductions in the dose of irinotecan and fluorouracil that lowered the risk of grade 4 diarrhea.

Vomiting of grade 3 or 4 was more common with

irinotecan, fluorouracil, and leucovorin than with fluorouracil and leucovorin, but it occurred in less than 10 percent of patients in the three-drug group, and its incidence might have been further reduced with the more frequent use of prophylactic serotonin-antagonist antiemetics. Mucositis of grade 3 or 4, grade 4 neutropenia, and neutropenic fever occurred less often with triple-drug therapy than with two-drug therapy. This finding most likely results from differences in the scheduling of fluorouracil and leucovorin treatments in the two regimens; the combination of fluorouracil and leucovorin is associated with lower rates of these adverse effects when it is given weekly.[4,5] Treatment-related death was rare (a rate of 1 percent in all groups). Furthermore, analysis of the quality of life indicated that the combination of irinotecan with fluorouracil and leucovorin did not worsen the quality of life as compared with that reported with fluorouracil and leucovorin.

In conclusion, we found that combining irinotecan with fluorouracil and leucovorin benefits patients with metastatic colorectal cancer. As compared with a widely used regimen of fluorouracil and leucovorin, the triple-drug therapy was associated with higher rates of tumor regression, progression-free survival, and overall survival without compromising the quality of life. The combination of irinotecan, fluorouracil, and leucovorin is now being compared with a weekly regimen of fluorouracil and leucovorin as adjuvant therapy for patients with stage III colon cancer to determine whether it will increase rates of cure in patients with an earlier stage of the disease.

Presented in preliminary form at the 19th Annual Meeting of the American Society of Clinical Oncology, Atlanta, May 15–18, 1999.

Supported by Pharmacia.

We are indebted to Bonnie Keller, Mary Anne Needham, Dawn Wikel, Jodi Tiffany, Margie Bruns, Dawn Price, and Sally Boos for meticulous data review; to Angelina Pastorelli and Luca Cuomo for conscientious data management; to Patricia Newman, Nicola Amos, Jo Gordon, and Monica McDonald for attentive site management; and to Bonnie Ayotte, Mary Fitzjohn, and Cheryl Krause for diligent administrative support.

APPENDIX

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Surg Today (2002) 32:1075–1080

SURGERY TODAY

Irinotecan Plus Low-Dose Cisplatin for α-Fetoprotein-Producing Gastric Carcinoma with Multiple Liver Metastases: Report of Two Cases

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Abstract
α-Fetoprotein (AFP)-producing gastric carcinoma generally causes multiple liver metastases and has an extremely poor prognosis. There is no standard chemotherapy for this disease. Two recent consecutive patients who had AFP-producing gastric carcinoma were treated with a novel chemotherapy regimen: irinotecan hydrochloride (100 mg/body over 90 min) plus low-dose cisplatin (10 mg/body) by intravenous infusion. Treatment was done weekly during admission and once every 2 weeks on an outpatient basis. Both patients had multiple liver metastases with high serum levels of AFP, and one demonstrated resistance to 5-fluorouracil. In both patients, liver metastases showed a dramatic complete response to chemotherapy, and the serum AFP levels returned to normal. No significant toxicities were observed. These preliminary results suggest that the present regimen may cause fewer side effects while retaining its synergistic antitumor activity. This regimen may therefore be worth trying as first-line chemotherapy for patients with metastatic AFP-producing gastric carcinoma.

Key words Gastric carcinoma · α-Fetoprotein production · Liver metastasis · Irinotecan · Cisplatin

Introduction

Since the first report of a patient with gastric cancer producing α-fetoprotein (AFP) by Bourreille et al. in 1970,[1] a considerable number of such patients has been found. AFP-producing gastric carcinoma is well known to generally cause multiple liver metastases and to also have an extremely poor prognosis.[2–4] There is no standard chemotherapy available for this disease, although there have been a few cases where different regimens were effective including EAP (etoposide, adriamycin, and cisplatin),[5] FEP (5-fluorouracil, epirubicin, and cisplatin),[6] and FAP (5-fluorouracil, adriamycin, and cisplatin).[7] It is therefore imperative to establish an effective first-line chemotherapy regimen for AFP-producing gastric cancer with unresectable metastases.

Irinotecan hydrochloride (CPT-11) is a water-soluble derivative of camptothecin, a plant alkaloid obtained from *Camptotheca acuminata*. It retains the original antitumor activity of the parent compound, but is less toxic.[8] The antitumor activity of CPT-11 is due to the inhibition of DNA topoisomerase I.[9–11] In patients with gastric cancer, the response rate for CPT-11 monotherapy was reported to be less than 20% in phase II studies.[12] However, CPT-11 shows a marked synergism with cisplatin, as well as a lack of cross-resistance due to different mechanisms of action, and a relatively different profile of adverse reactions.[13] A phase II study employing a combination of CPT-11 (70 mg/m^2) and cisplatin (80 mg/m^2) to treat metastatic gastric cancer achieved an overall response rate of 48%,[12] thus suggesting that a combination of CPT-11 and cisplatin might also be promising for patients with metastatic AFP-producing gastric cancer. However, since patients with progressive metastatic disease usually have a poor performance status, aggressive chemotherapy using the high doses mentioned above might not be appropriate. In addition, it is desirable to treat such patients on an outpatient basis, if possible. Recent in vitro data have suggested that the topoisomerase I inhibitory effect of CPT-11 against colorectal cancer[14] or a non-small cell lung cancer cell line[15] is enhanced by low-dose cisplatin.

Therefore, we attempted to perform a new regimen of chemotherapy with CPT-11 plus low-dose cisplatin for AFP-producing gastric cancer. To our knowledge, this is the first time that the present regimen (100 mg/body of CPT-11 plus 10 mg/body of cisplatin) achieved a

Reprint requests to: M. Ogawa
Received: October 10, 2001 / Accepted: May 7, 2002

complete remission of metastatic AFP-producing gastric cancer.

Case Reports

Patient 1

A 71-year-old Japanese man with a 1-month history of hepatic dysfunction and weight loss was referred to our department on June 14, 2000. Endoscopy revealed a type 2 lesion in the mid-portion of the stomach (Fig. 1A). AFP-positive cells were demonstrated by immunohistochemistry of biopsy specimens (Fig. 2), and the histologic diagnosis was hepatoid adenocarcinoma. Computed tomography of the whole body revealed multiple metastases in the liver (Fig. 3A). The serum AFP level was 1290 ng/ml prior to chemotherapy. His hepatic function was severely impaired (serum total bilirubin: 5.4 mg/dl; ICG R15: 36%). We initially tried chemotherapy using the oral administration of dihydropyrimidine dehydrogenase (DPD) inhibitory 5-fluorouracil (5-FU). However, no response was observed and the serum AFP level increased to 5190 ng/ml. We next tried a novel regimen of CPT-11 plus low-dose cisplatin at weekly intervals. After two courses of this treatment, the serum AFP level started to decrease and it declined rapidly with a half-time of

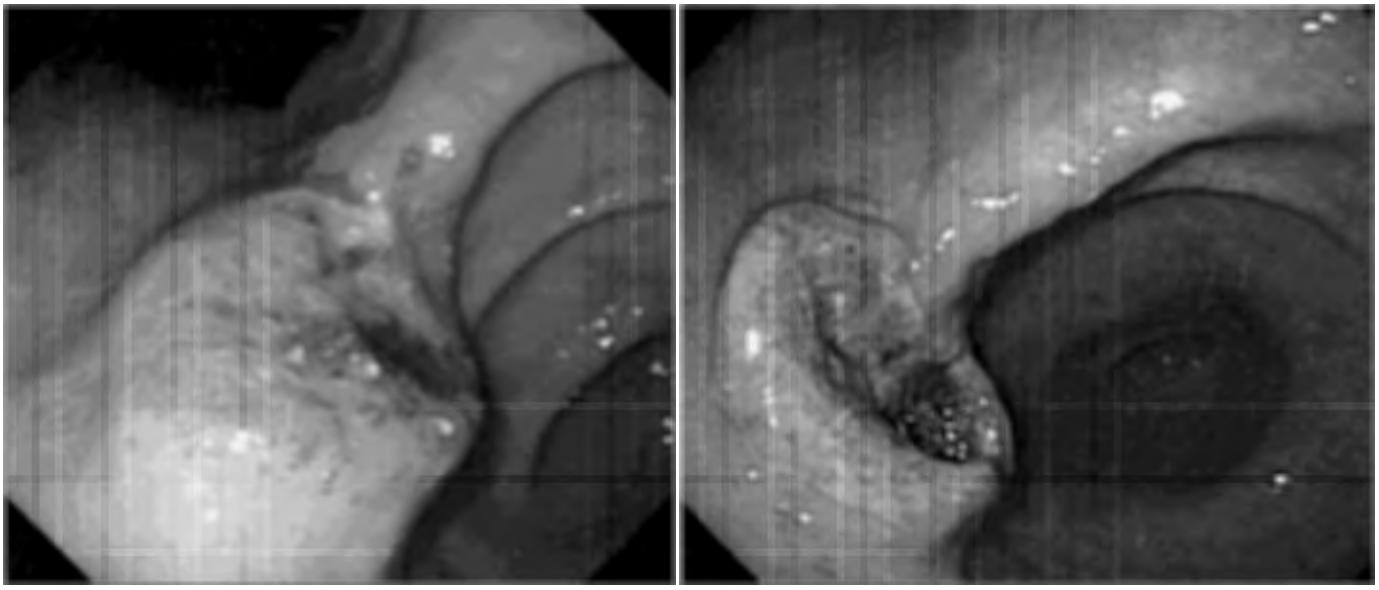

Fig. 1A,B. Case 1. **A** Endoscopic view of a type 2 tumor in the mid-portion of the stomach before chemotherapy. **B** A deep ulcer is observed after treatment

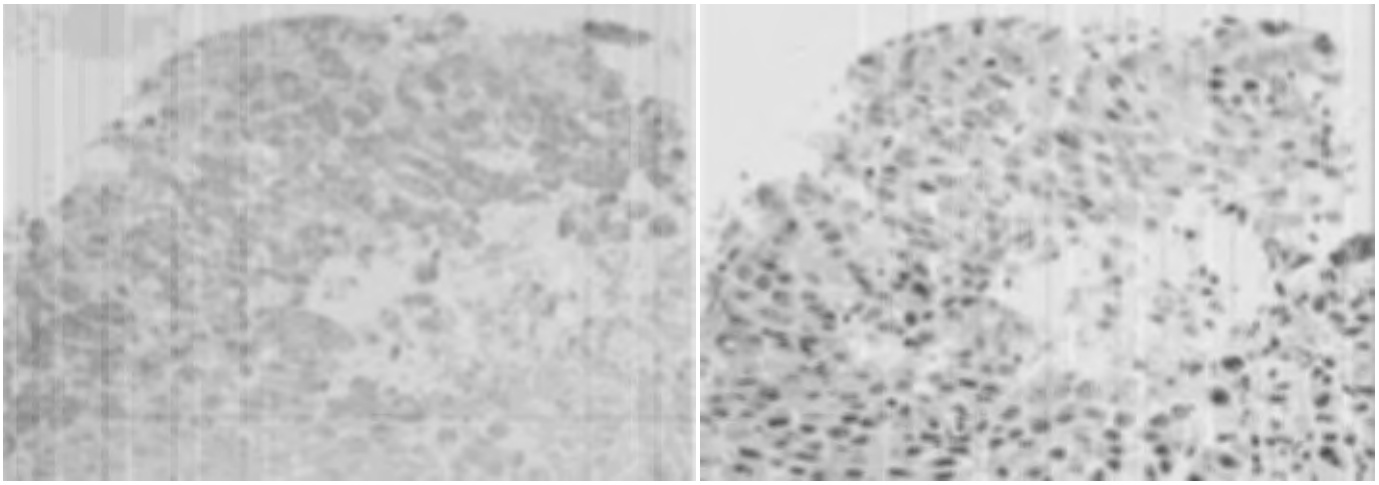

Fig. 2A,B. Case 1. **A** Immunohistochemical staining of a biopsy specimen with α-fetoprotein antibody. **B** hematoxylin–eosin staining

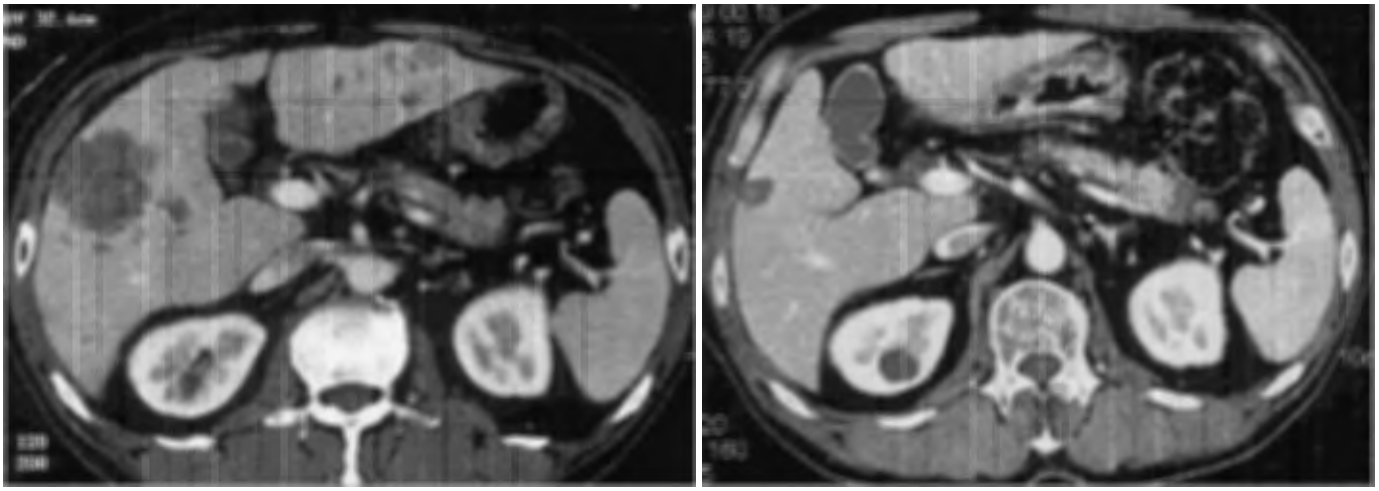

Fig. 3A,B. Computed tomography of multiple liver metastases in case 1, **A** before and **B** after combination chemotherapy

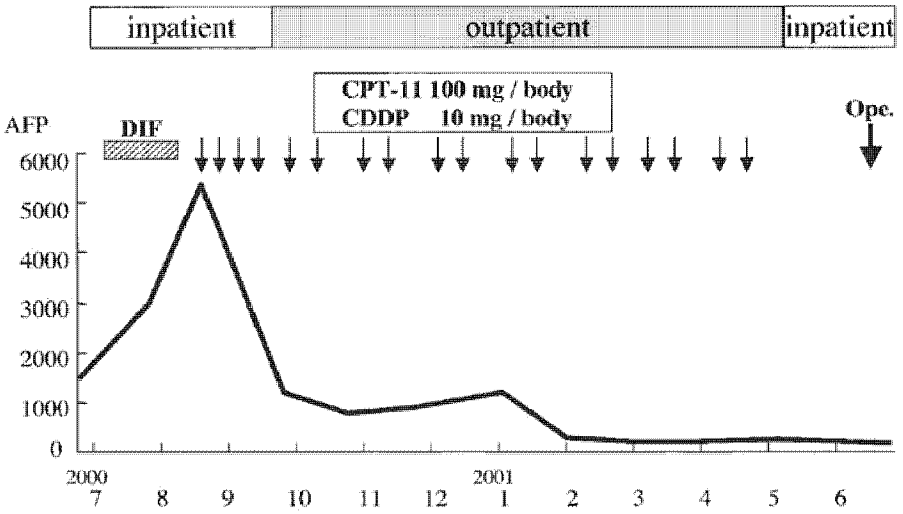

Fig. 4. Clinical course and changes in the serum level of α-fetoprotein (*AFP*) in case 1. *CPT*, Irinotecan hydrochloride; *CDDP*, cisplatin; *DIF*, dihydropyrimidine dehydrogenase inhibitory 5-fluorouracil

approximately 2 weeks. The patient was thereafter treated with chemotherapy every 2 weeks on an outpatient basis. After 18 courses of this regimen, the serum AFP level eventually decreased to 17 ng/ml (Fig. 4). During treatment, leukopenia was never severe enough for the administration of granulocyte colony-stimulating factor (G-CSF), and several episodes of leukopenia recovered after postponing the next treatment by 1 or 2 weeks, i.e., Grade 2 toxicity according to World Health Organization criteria.[16] No other side effects were observed, including diarrhea, anemia, or nausea/vomiting. The liver metastases almost completely disappeared (Fig. 3B) and the primary tumor in the stomach formed a deep ulcer (Fig. 1B). The gastric tumor was eventually removed by a distal gastrectomy with an extensive lymph node dissection, on June 5, 2001. Although biopsy specimens of the liver revealed no viable tumor cells in the metastases, microwave coagulation therapy was performed for the complete ablation of any potentially viable cancer cells. The postoperative course was uneventful. A histological examination confirmed metastasis in only three epigastric lymph nodes among a total of 48 dissected lymph nodes. The histological efficacy[16] was Grade 2 for both the primary tumor and the regional lymph nodes, while it was Grade 3 for liver metastasis.

Patient 2

A 63-year-old Japanese man who had a giant tumor in the gastric cardia (Fig. 5A) with numerous large liver metastases (Fig. 5C) was admitted to our institution on December 19, 2000. Since AFP-positive cells were demonstrated immunohistochemically in biopsy specimens and the serum AFP level was 156 ng/ml, AFP-producing gastric carcinoma was diagnosed. The tumor histology was hepatoid adenocarcinoma. CPT-11 and low-dose cisplatin were administered by weekly intravenous infu-

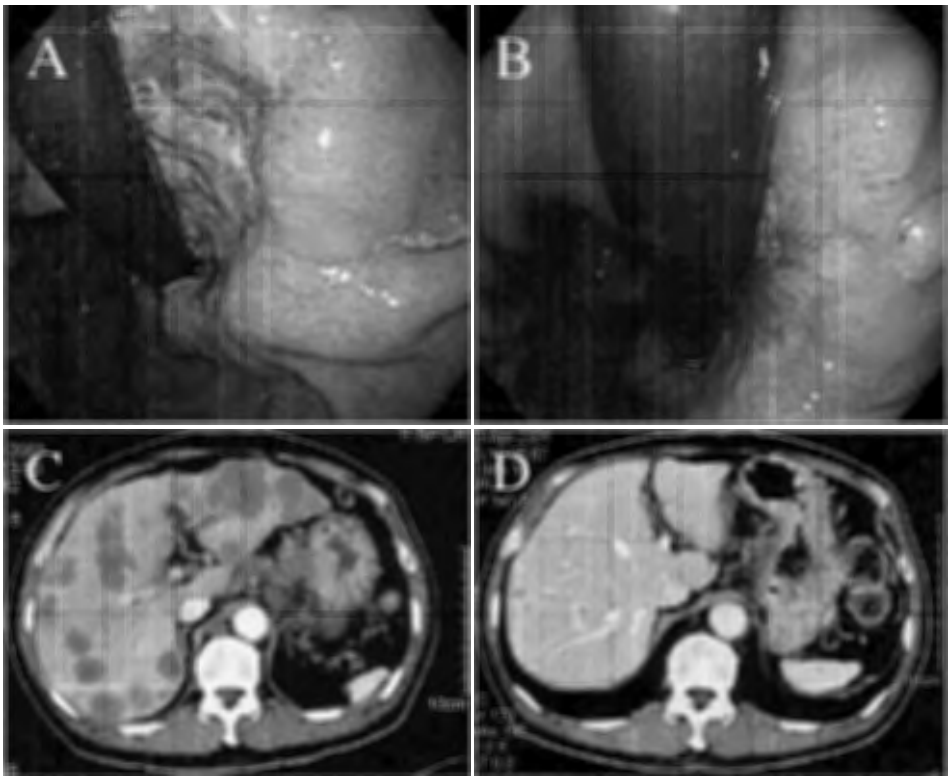

Fig. 5A–D. Case 2. **A** Endoscopic view of a Borrmann type 2 tumor in the upper portion of the stomach before chemotherapy. **B** The tumor decreased in size and formed an ulcer scar after the treatment. Computed tomography of multiple liver metastases in case 2, **C** before and **D** after undergoing the combination chemotherapy

sion. After the third course of this therapy, the tumor volume and the serum AFP level started to decrease dramatically. His hepatic metastases almost completely disappeared (Fig. 5D) and the primary gastric cancer also decreased in size by more than 90% and thereafter formed an ulcer *scar* (Fig. 5B) on May 15, 2001. Since no side effects have so far been observed and the patient maintains a very good quality of life, this treatment is being continued every 2 weeks on an outpatient basis. No progression of the liver metastases or the primary tumor has been observed.

Chemotherapy Regimen and Evaluation of Response

These two patients with AFP-producing gastric carcinoma were treated by a novel chemotherapy regimen comprising CPT-11 combined with low-dose cisplatin. This regimen was given as a second-line therapy for case 1 and as first-line therapy for case 2. CPT-11 was given at a dose of 100 mg per body over 90 min combined with 10 mg per body of cisplatin by intravenous infusion. Treatment was done on a weekly basis during admission and every 2 weeks on an outpatient basis. If any side effects were observed, the chemotherapy was postponed until such toxicity subsided. Tumor response and toxicities were classified in accordance with the World Health Organization criteria.[16]

Histological and Immunohistochemical Studies

All specimens were fixed in 10% buffered neutral formalin and embedded in paraffin. The sections were then cut (3 μm thick), stained with hematoxylin–eosin, and examined under a light microscope. The histological diagnosis of gastric carcinoma was done according to the classification of Motoyama et al.[2] Namely, the tumors were classified into three subtypes: (i) hepatoid type, (ii) yolk sac tumor-like type, and (iii) fetal gastrointestinal type. Immunohistochemical studies were conducted on 3-μm sections using the avidin-biotin-peroxidase complex method. ABC Elite kits (Vector Laboratories, Burlingame, CA, USA) for rabbit IgG and anti-AFP antibody (DAKO, Carpinteria, CA, USA) were used.

Discussion

Motoyama et al.[2] proposed that AFP-producing gastric carcinomas should be divided into three subtypes, namely, hepatoid type, yolk sac tumor-like type, and fetal gastrointestinal type. It has been suggested that hepatoid type and yolk sac tumor-like type are respectively derived from hepatocellular metaplasia and yolk sac cell metaplasia of the common poorly differentiated medullary adenocarcinoma. In contrast, fetal gastrointestinal type seems to arise from the imitation of fetal

gastrointestinal epithelium by common tubular adenocarcinoma. The hepatoid type is the most common type of AFP-producing gastric carcinoma. Unfortunately, most hepatoid tumors tend to be highly malignant.[2,17] Both of our patients had tumors that were classified as having a hepatoid subtype. AFP-producing gastric cancer has a high malignant potential (high proliferative activity, little apoptosis, and rich neovascularization) when compared with AFP-negative gastric cancer. These biological characteristics of AFP-producing gastric cancer reflect its aggressive behavior and the poor prognosis of patients with such tumors.[3] Increased expression of c-Met might be another possible explanation for the poor prognosis of AFP-producing gastric cancer.[4]

AFP-producing gastric cancer is associated with a high incidence of multiple liver metastases and the presence of such metastases makes a resection impossible. Furthermore, AFP-producing gastric cancer is reported to respond poorly to various chemotherapy regimens. There has only been one report of a successful surgical resection of liver metastases as well as the AFP-producing primary gastric cancer with a good outcome.[18] Therefore, more effective chemotherapy is needed to improve the prognosis of patients with AFP-producing gastric cancer. 5-Fluorouracil is still the mainstay of systemic treatment for patients with metastatic gastric cancer, but one of our patients showed no response to this drug. A phase II study of combination chemotherapy with cisplatin and CPT-11 was previously conducted to assess its efficacy and feasibility in patients with metastatic gastric cancer.[12] This regimen was found to be active and well tolerated. Accordingly, CPT-11 plus cisplatin was used as a second-line therapy in case 1 after the failure of 5-fluorouracil. Our regimen achieved a complete remission of metastatic liver tumors even though the patient had AFP-producing gastric cancer. It is assumed that low-dose cisplatin enhanced the inhibition of topoisomerase I by CPT-11, thus resulting in the synergistic effect of this combination.

Diarrhea and leukopenia are serious dose-limiting toxicities of CPT-11.[19–21] The incidence of grade 3 or 4 diarrhea is 20% and that of grade 4 neutropenia is 57% when CPT-11 is given with high-dose cisplatin. The main toxic effects of cisplatin include myelosuppression, renal impairment, and nausea and vomiting.[22,23] When cisplatin is administered at high doses, forced diuresis is necessary, which is both inconvenient and also impairs the patient's quality of life. We therefore used cisplatin at a low dose for outpatient therapy. In the present two cases, no diarrhea, severe leukopenia, renal dysfunction, or nausea and vomiting were observed. The principles of chemotherapy are considered to be (1) a high tumor effectiveness, (2) a good performance status during chemotherapy, (3) the ability to continue the oral intake of food, and (4) the ability to be administered on an outpatient basis. Our two cases both met these four principles. Our results suggest that the dose and administration schedule of this regimen may reduce the number of side effects without any loss in the synergistic antitumor activity, and these findings are very encouraging for patients with metastatic AFP-producing gastric cancer.

In conclusion, this is the first report of successful treatment with CPT-11 and low-dose cisplatin combination chemotherapy for AFP-producing gastric cancer patients with multiple liver metastases. Using the present cases as a trigger, it is hoped that this novel regimen will prove to be an important advance in the treatment of gastric cancer, and that the mechanisms underlying the above-described combination chemotherapy can be elucidated in the near future.

Acknowledgment. This work was supported in part by Grants-in-Aid (Nos. 11671254 and 12877194) for Scientific Research from the Japanese Ministry of Education.

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0090-9556/00/2804-0423--433$02.00/0
DRUG METABOLISM AND DISPOSITION
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Vol. 28, No. 4
Printed in U.S.A.

PHARMACOKINETICS, METABOLISM, AND EXCRETION OF IRINOTECAN (CPT-11) FOLLOWING I.V. INFUSION OF [^{14}C]CPT-11 IN CANCER PATIENTS

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(Received July 23, 1999; accepted December 1, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

This study determined the disposition of irinotecan hydrochloride trihydrate (CPT-11) after i.v. infusion of 125 mg/m^2 (100 μCi) [^{14}C]CPT-11 in eight patients with solid tumors. Mean ± S.D. recovery of radioactivity in urine and feces was 95.8 ± 2.7% (range 92.2–100.3%, *n* = 7) of dose. Radioactivity in blood, plasma, urine, and feces was determined for at least 168 h after dosing. Fecal excretion accounted for 63.7 ± 6.8 (range 54.2–74.9%, *n* = 7) of dose, whereas urinary excretion accounted for 32.1 ± 6.9% (range 21.7–43.8%; *n* = 7) of dose. One patient with a biliary T-tube excreted 30.1% of dose in bile, 14.2% in feces, and 48.2% in urine. Quantitative radiometric HPLC revealed that CPT-11 was the major excretion product in urine, bile, and feces. Aminopentane carboxylic acid (APC) and SN-38 glucuronide (SN-38G) were the most significant metabolites in urine and bile, whereas SN-38 and NPC, a primary amine metabolite, were relatively minor excretion products. SN-38 and APC were the most significant metabolites in feces. The relatively higher amount of SN-38 in feces compared with bile is presumably due to hydrolysis of SN-38G to SN-38 by enteric bacterial β-glucuronidases. There was close correspondence between quantitative fluorescence HPLC and mass balance findings. CPT-11 was the major circulating component in plasma (55% of the mean radiochemical area under the curve), and CPT-11, SN-38, SN-38G, and APC accounted for 93% of the mean radiochemical AUC. These results show that the parent drug and its three major metabolites account for virtually all CPT-11 disposition, with fecal excretion representing the major elimination pathway.

The antineoplastic agent irinotecan hydrochloride trihydrate (CPT-11, Camptosar, PNU-101440E; (*S*)-[1,4′-bipiperidine]-1′-carboxylic acid, 4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinolin-9-yl ester, monohydrochloride, trihydrate; $C_{33}H_{38}N_4O_6{\cdot}HCl{\cdot}3H_2O$) is a semisynthetic derivative of the natural product camptothecin (Kunimoto et al., 1987; Sawada et al., 1991). CPT-11 was recently approved by the U.S. Food and Drug Administration for the treatment of patients with metastatic carcinoma of the colon or rectum whose disease has recurred or progressed following 5-fluorouracil-based therapy. The recommended starting dosage of CPT-11 is either 125 mg/m^2 i.v. over 90 min once a week for 4 weeks, followed by a 2-week rest, or 350 mg/m^2 given once every 3 weeks. Dosage modifications after the initial dose are based on individual patient tolerance.

The metabolism of CPT-11 in humans has been studied extensively using nonradiometric methodology (Kono and Hara, 1991; Gupta et al., 1994; Lokiec et al., 1995, 1996; Rivory and Robert, 1995; Rivory et al., 1996, 1997; Slatter et al., 1997; Dodds et al., 1998; Haaz et al., 1998a,b; Sparreboom et al., 1998). The structures of CPT-11 and its known metabolites are shown in Fig. 1. CPT-11 is converted in vivo by carboxylesterase enzymes (Haaz et al., 1997; Slatter et al., 1997) into SN-38, a potent inhibitor of topoisomerase I, which is a nuclear enzyme that plays a critical role in DNA replication and transcription (Hsiang et al., 1985). Differences in the in vitro cytotoxicity of SN-38 and CPT-11 can be as low as 130- to 570-fold (human colon cancer cell lines; Jansen, 1997) or as high as 2973-fold (human KB cell line; Kaneda et al., 1990; Lavelle et al., 1996). However, relative in vitro potency data are of limited usefulness due to the in situ formation of SN-38 from CPT-11 and a variety of other experimental factors. Accordingly, CPT-11 is generally considered to be effectively inactive and serves only as a soluble prodrug of SN-38 (Kawato et al., 1991; Yoshida et al., 1993; Lavelle et al., 1996). The active metabolite SN-38 is in turn excreted intact, or as a glucuronide metabolite (SN-38G) (Gupta et al., 1994; Rivory and Robert, 1995). Other known human metabolites of CPT-11 include an aminopentane carboxylic

Presented in part at the 35th Annual Meeting of the American Society of Clinical Oncology (ASCO Proceedings), Volume 18, Abstract 633, Page 164a, Atlanta, May 15–18, 1999. Methodological aspects were presented at the 12th Central US Meeting of the International Isotope Society (IIS), Kalamazoo MI, May 20–21, 1999 (*J Labelled Compd Radiopharm* 1999; **42**: 915–916.)

[1] Abbreviations used are: CPT-11, irinotecan hydrochloride trihydrate; APC, aminopentane carboxylic acid metabolite of CPT-11; ARE, amount remaining to be excreted; cMOAT, canalicular multiple organic anion transporter; MS, mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; LSC, liquid scintillation counting; NPC, primary amine metabolite of CPT-11; SN-38, active metabolite of CPT-11; SN-38G, SN-38 glucuronide; SPE, solid-phase extraction; AUC, area under the curve; Rt, retention time; QC, quality control; CL, total systemic clearance; V_z, terminal phase volume of distribution, LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation.

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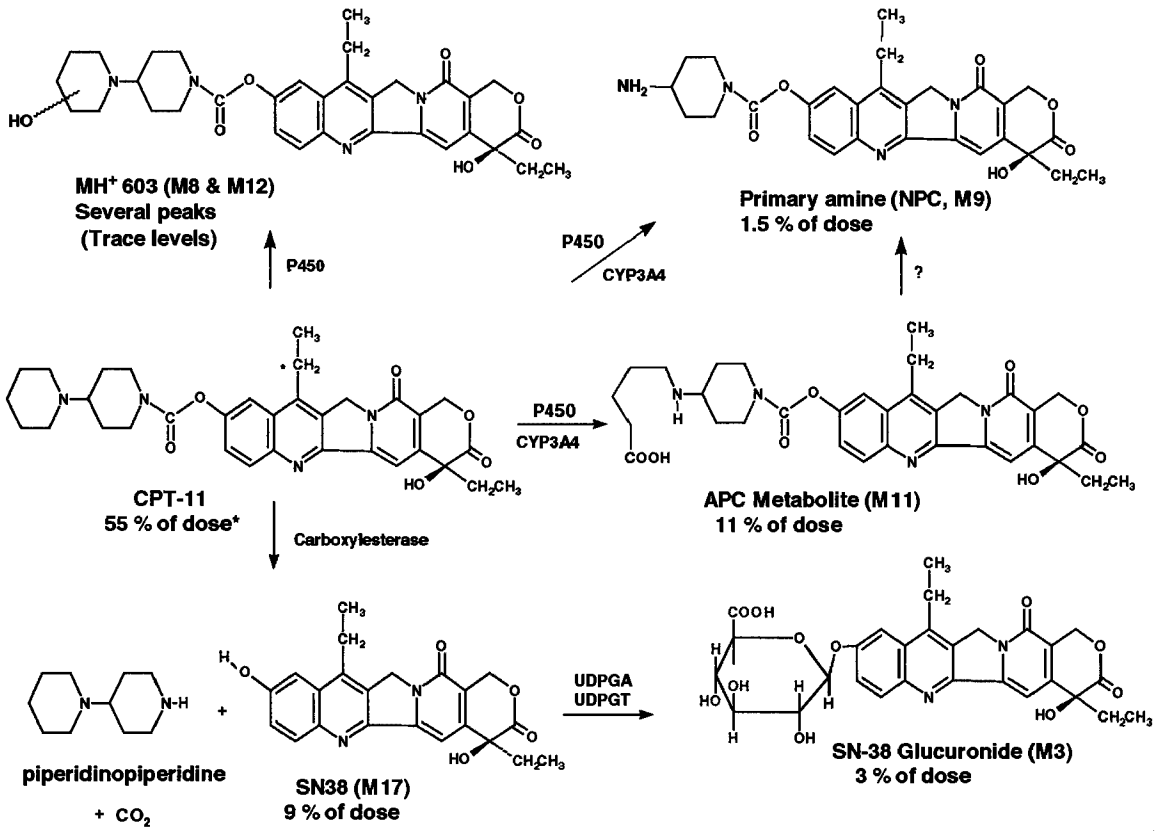

FIG. 1. *Metabolism scheme for [^{14}C]CPT-11.*

Nonextractable (mostly fecal) radioactivity = 11% of dose; late excretion products not profiled = 4% of dose; unidentified peak M7 = 1% of dose; M5 (CPT-11 hydroxy acid-artifact) = 0.2% of dose; sum of all other 10 peaks = 1.2% of dose. * denotes radiolabel location.

acid (APC) arising from CYP 3A-mediated α-carbon oxidation of the outer piperidine ring of CPT-11 (Lokiec et al., 1996; Rivory et al., 1996) and a primary amine metabolite (NPC; RPR-132595A; Dodds et al., 1998; Haaz et al., 1998a) derived from the CYP 3A-mediated oxidative cleavage of both α-carbons of the outer piperidine ring. A variety of other minor metabolites and decomposition products have been identified by mass spectrometry (MS) and chemical synthesis (Lokiec et al., 1996; Dodds et al., 1997).

Biliary excretion represents the major elimination pathway for CPT-11 and its metabolites in preclinical species (Kaneda and Yokokura, 1990; Kaneda et al., 1990). CPT-11 and its metabolites exist in a pH dependent equilibrium between active lactone and inactive hydroxy acid anion (carboxylate) forms at physiological pH. The carboxylate forms of CPT-11 and SN-38 and both the carboxylate and lactone forms of SN-38G (Chu et al., 1997a) are all anions at physiological pH. In vitro and in vivo studies in rats have demonstrated that the biliary excretion of the carboxylate forms of CPT-11 and SN-38 and the carboxylate and lactone forms of SN-38G are mediated by the active transporter cMOAT, which is located on the bile canalicular membrane (Chu et al., 1997a,b, 1998). The carboxylate forms were also shown to have a higher biliary excretion clearance than the lactone forms (Chu et al., 1998). These results are consistent with other studies which have demonstrated that the clearance of the lactone forms of other camptothecin analogs is generally lower than that of the hydroxy acid anion forms (Scott et al., 1993a,b).

The objective of this study was to quantitatively determine the pharmacokinetics, metabolism, and excretion of [^{14}C]CPT-11 and its metabolites in male and female human cancer patients.

Materials and Methods

Study Design. The study enrolled eight Caucasian patients, (four male/four female, 51–74 years of age) with histologically confirmed diagnoses of advanced solid tumor malignancy for which there were no clearly established standard treatment options. Patients had an Eastern Cooperative Oncology Group performance status of 0 to 2 and adequate organ function. Prior chemotherapy or radiation therapy was allowed. All subjects provided written informed consent before enrollment. Radiation exposure estimates for human tissues were predicted from rat [^{14}C]CPT-11 distribution and excretion data with the use of maximum internal radiation dose software (Loevinger et al., 1991). The 100-μCi dose chosen for this study is similar to that given in most other human ^{14}C trials conducted in the United States (Dain et al., 1994).

Each patient received a single 90-min i.v. infusion (500 ml total volume) containing a target dose of 125 mg/m^2 of CPT-11 and 100 μCi of [^{14}C]CPT-11 on day 1. Urine, feces, blood, plasma, and, in one patient, bile were collected over the next 7 to 9 days for radioanalysis, pharmacokinetic analysis, and metabolite profiling. Patient demographics and dosimetry are summarized in Table 1. Actual radiation exposure, calculated for each patient with the maximum internal radiation dose method (Loevinger et al., 1991), was well below the limits permitted by the U.S. Food and Drug Administration (Code of Federal Regulations 21, Part 361.1, 1998). CPT-11 administration at this dose level was well tolerated, with no instances of vomiting or immediate-onset diarrhea.

Formulation and Dose Administration. CPT-11 was labeled with ^{14}C on the proximal carbon of the 11-ethyl substituent of the camptothecin ring. The radiolabel was formulated as a sterile solution concentrate and packaged in 5-ml amber ampules containing [^{14}C]CPT-11 (0.235 mg/ml, 20 μCi/ml), ethanol to dissolve (~0.06 ml/ml), and 5% dextrose (q.s. ad 1 ml). At this low concentration, the sterile solution could be stored frozen without risk of self-association or precipitation (Aiyama et al., 1992). The radiopurity was 97% over the duration of the study. Nonradioactive CPT-11 was obtained from

TABLE 1

Summary of patient demographics and dosimetry

	Gender/Age	Tumor Type	Baseline Bilirubin	Weight	Surface Area	Dose		
	yr		*mg/dl*	*kg*	m^2	*μCi*	*mg/m²*	*mg/kg*
Patient								
1	Male/73	Lung	0.3	109.1	2.2	93.0	113.0	1.0
2	Male/67	Colon	0.9	89.1	2.0	91.1	129.4	1.5
3	Male/51	Colon	0.7	78.6	2.0	93.6	130.3	1.7
4	Male/70	Esophageal	0.4	98.2	2.2	94.1	112.9	1.1
5[a]	Female/64	Bile duct	0.7	84.4	1.9	93.3	118.7	1.4
6	Female/74	Endometrial	0.4	88.0	2.0	93.2	121.6	1.4
7[b]	Female/52	Lung	0.5	52.7	1.6	93.6	117.0	2.2
8	Female/71	Ovarian	0.6	83.2	2.1	92.0	116.0	1.4
Males (n = 4)								
Mean ± S.D.			0.6 ± 0.3	93.8 ± 13.0	2.1 ± 0.1	93.0 ± 1.3	121.4 ± 9.8	1.3 ± 0.3
% CV			50	13.9	4.9	1.4	8.0	21.4
Females (n = 4)								
Mean ± S.D.			0.6 ± 0.1	77.1 ± 16.4	1.9 ± 0.2	93.0 ± 0.7	118.3 ± 2.4	1.6 ± 0.4
% CV			17	21.2	11.2	0.8	2.1	25.8
All Patients (n = 8)								
Mean ± S.D.			0.6 ± 0.2	85.4 ± 16.3	2.0 ± 0.2	93.0 ± 1.0	119.9 ± 6.8	1.5 ± 0.4
% CV			33	19.1	9.5	1.0	5.7	24.6

% CV, percent coefficient of variation.
[a] Patient had bile duct *t*-tube.
[b] Patient was a smoker.

commercial Camptosar. For each patient, radioactive and nonradioactive concentrates were weighed and aseptically transferred into a 500-ml bag of sterile dextrose 5% in water just before administration. The administered dose was determined gravimetrically. The mean concentration of CPT-11 in the formulation (n = 8) was 0.46 ± 0.05 mg/g, and the specific activity was 0.40 ± 0.05 μCi/mg.

Biological Specimen Collection. Blood samples were collected at −90 min (predose); 0 min (end of infusion); and 5, 15, and 30 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 24, 36, 48, 72, 96, 120, 144, and 168 h after the end of the infusion. Urine was collected and pooled over −24 h to −90 min (preinfusion); −90 min to 0 h (during the infusion); 0 to 2, 2 to 4, 4 to 8, 8 to 12, 12 to 24, 24 to 36, 36 to 48, 48 to 60, 60 to 72, 72 to 84, 84 to 96, 96 to 120, 120 to 144, and 144 to 168 h (postinfusion); and in 24-h increments thereafter as needed. Feces were collected as voided and pooled in 0- to 12-, 12- to 24-, and 24-h increments thereafter. In one patient (5F) with a biliary T-tube, bile was collected quantitatively over the first 72 h postdose and selectively thereafter as determined by cholestasis and the clinical status of the patient. Excretion data for this patient were omitted from the calculation of means for all excretion-based data.

Radioactivity Analysis. All assays were done gravimetrically by direct capture of sample weights by Debra version 4.1c (LabLogic Systems Ltd., Sheffield, UK). Radioactivity analysis was performed using Packard Tri-Carb liquid scintillation spectrometers (Packard Instrument Co., Meriden, CT). Fecal homogenates and blood were combusted with a Packard Tri-Carb sample oxidizer (model 387) and analyzed by liquid scintillation counting (LSC) in Carbosorb E/Permafluor E+ (Packard Instrument Co.). Radioactivity in plasma, bile, and urine was determined by LSC in Ultima Gold.

Radioactivity Data Analysis. Dose weights recorded in mg of CPT-11 hydrochloride trihydrate, specific activity in μCi/mg, body weight in kg, sample weights in g, aliquot weights in g, uncorrected LSC results in dpm, LSC background in predose matrix, and patient numbers were processed by Debra version 4.1c. Excretion data for each matrix (urine, feces, and bile) and for the sum of all matrices were expressed as recovered percentage of administered radioactive dose per collection. The percentage of dose excreted during each collection interval calculated by Debra was transformed to cumulative percentage of dose excreted and percentage of dose remaining to be excreted [amount remaining to be excreted (ARE)] with Excel version 5.0c (Microsoft Corp., Redmond WA). ARE half-lives were calculated for urine data by linear regression of the log-linear terminal phase. Blood and plasma radioanalysis data were expressed as μg-eq of CPT-11 hydrochloride trihydrate/g of sample matrix. For comparison to plasma levels of CPT-11 and metabolites, μg-eq/g were converted to μM-eq. Hematocrit data from each patient were used to calculate the blood/plasma partition coefficient K_p (Sun et al.,1987) written in Microsoft Excel as: K_p = {Cb − {Cp·(1 − HCT)}}/HCT/Cp, where HCT is the hematocrit expressed as a fraction, Cb is concentration in blood, and Cp is the concentration in plasma.

Sample Extraction and Concentration for Metabolite Profiling. Methods were developed to maximize extraction efficiency and concentrate radioactivity from excreta sufficient to allow flow HPLC radiometric quantitation of minor radioactive metabolites. Excreta collections were pooled in proportion to total sample weight. Recoveries were monitored by LSC throughout sample preparation and during HPLC analysis.

Urine. Thawed urine was pooled, diluted 4- to 5-fold with water, stirred for 30 min to dissolve precipitated radioactivity, and concentrated on C18 solid-phase extraction (SPE) cartridges (Varian Inc., Harbor City, CA). Cartridges were washed sequentially with water and 5% acetonitrile/water, and radioactivity was eluted from the SPE columns with methanol/ammonia (pH 9.0). The eluate was adjusted to pH ~4 to 5 with acetic acid and concentrated to near-dryness under nitrogen. The concentrate was dissolved in mobile phase (see below) for HPLC analysis.

Feces. The procedures necessary to obtain good recoveries of radioactivity from feces were tedious. Initial solubilization of radioactivity was difficult, requiring large volumes of diluent. Numerous extraction and concentration strategies using liquid/liquid extraction and/or SPE and/or dialysis failed due to low extraction efficiency or coprecipitation and binding of solubilized radioactivity to solids during the concentration steps. The extraction procedure required careful optimization, with recovery determined at each step.

Fecal homogenates were diluted at least 10-fold with 50 mM acetic acid (pH 3.0) and mixed in a Vortex mixer. To remove fats, each sample was extracted twice with 10 ml of hexane and centrifuged. The hexane extract was verified to be free of radioactivity and discarded. The aqueous phase was diluted with acetonitrile/methanol (3:1 v/v) and centrifuged; these steps were repeated three times. Supernatants were combined and concentrated under vacuum, washed into a vial, and evaporated to near-dryness under nitrogen. The concentrate was redissolved in 1 ml of acetonitrile/methanol (1:1 v/v) with sonication and then diluted with 50 mM ammonium acetate (pH 4.5) with sonication. The extract was washed into a microcentrifuge tube and centrifuged, and the supernatant was analyzed by HPLC.

Bile. Thawed bile was diluted with 4 volumes of 0.05 M ammonium acetate (pH 4.5) containing D-saccharic 1,4-lactone monohydrate (Aldrich Co., Milwaukee, WI) to prevent hydrolysis of the β-glucuronide conjugate (SN-38G). The solution was shaken for 30 min to dissolve precipitated radioactivity and extracted with an equal volume of hexane to remove precipitated cholesterol. The emulsions were centrifuged, and the hexane layer was removed, found free of radioactivity, and discarded. Samples were diluted with ammonium acetate and extracted again. The aqueous phase was processed by SPE as described for

urine, and radioactivity was eluted with acidic methanol (0.1% HCl). The extract was adjusted to pH 4 to 5 with dilute ammonium hydroxide. Samples were evaporated to near-dryness and dissolved in mobile phase (see below) for HPLC analysis.

Plasma. Plasma samples (0.5 ml, collected 0–4 h) from individual patients were pooled, diluted with an equal amount of water, centrifuged, and applied to Varian SPE columns. Radioactivity was eluted with 4 × 10 ml of pH 4 methanol/acetic acid. The eluent was concentrated to near-dryness and redissolved in the initial mobile phase. Radioactivity was measured before HPLC analysis. Mean extraction recoveries of radioactivity from urine, feces, bile, and plasma were 96 ± 5, 82.6 ± 5.9, 92.0 ± 7.1, and 72 ± 11%, respectively.

Radiometric Methodology. Radiochromatographic analyses were done with a Perkin Elmer HPLC system, a Waters 474 fluorescence detector, and a Radiomatic Flow-One beta radio-chromatography Series A-500 detector. Fluorescence was monitored at an excitation wavelength of 368 nm and an emission wavelength of 500 nm. Separations were done on a Zorbax SB-CN, 4.6- × 150-mm (5 μ) column (first column), serially connected to a Waters Symmetry C8, 4.6- × 250-mm (5 μ) column, plus guard columns. The mobile phase was a gradient of ammonium acetate buffer (0.05 M, pH 4.50; B) and acetonitrile/methanol (3:1 v/v; A) at a flow rate of 1.0 ml/min. Initial conditions of 25% A and 75% B were held over 0 to 2 min, followed by a linear gradient to 33% A:67% B over 1 min, then held isocratic at 33% A:67% B over 3 to 22 min, followed by a linear gradient to 98% A:2% B over 22 to 23 min, held isocratic at 98% A:2% B over 23 to 30 min, and then re-equilibrated under isocratic starting conditions from 31 to 40 min. Radioactivity recoveries from the HPLC column were quantitative, and quenching effects due to the mobile phase gradient were negligible. The HPLC method described by Lokiec et al. (1996) was compared with this method to screen for any coeluting peaks; none were observed.

Metabolite abundance was expressed as percentage of recovered dose. The percentage of dose lost during each sample extraction was quantified. Thereafter, radiochromatograms of each extract were integrated, and each measurable radioactive peak was expressed as the percentage of total radioactive peaks. Peak percentage was converted to percentage of dose based on the amount of radioactivity in the extracted sample and the total sample weight. Data were analyzed with Excel version 5.0c.

HPLC Analysis of Plasma Concentrations of Intact CPT-11 and Its Metabolites. CPT-11, SN-38, and APC plasma concentrations were determined by validated HPLC methods. Concentrations measured were the sum of lactone and hydroxy acid forms. Aliquots (100-μl) of plasma were mixed with 50 μl of freshly prepared β-glucuronidase (50,000 U/ml), and the mixture was heated at 37°C for 30 min. Proteins were precipitated with 300 μl of acetonitrile, followed by mixing and centrifugation. Supernatant (300 μl) was mixed with 300 μl of 10% glacial acetic acid and incubated at 40°C for 30 min to convert analytes to the lactone form. Aliquots (100-μl) were then chromatographed on an HPLC system consisting of a Brownlee C8 Newguard precolumn (3.2- × 10-mm, 7 μm) and a Zorbax CN (4.6- × 150-mm, 5 μm) analytical column with a mobile phase of acetonitrile/methanol/0.05 M ammonium acetate buffer (pH 4.5, 1.5/1.5/7, v/v/v) at a flow rate of 1.5 ml/min. Two fluorescence detectors (372 nm λ_{ex}, 535 nm λ_{em} to monitor SN-38, and 368 nm λ_{ex}, 432 nm λ_{em} to monitor CPT-11 and APC) were used. Quantitation of concentrations was achieved by inverse prediction from the slope of a best fit with an intercept linear curve with concentration^{-2} weighting determined from fortified plasma calibration standards.

Determination of nonconjugated CPT-11, SN-38, and APC concentrations was done as above, except that 50 μl of water was added to plasma in place of freshly prepared β-glucuronidase enzyme, and the mixture was not heated. All analyte concentrations in plasma were reported as free base or free acid equivalents. The plasma concentration of SN-38G was calculated by subtracting the concentration of nonconjugated SN-38 (denoted as SN-38) from the concentration of total SN-38 (nonconjugated + conjugated SN-38).

For CPT-11, the mean interassay precision of the method between the lower limit of quantitation (LLOQ) (1.4 ng/ml) and the upper limit of quantitation (ULOQ) (1370 ng/ml) was ±4.4%. Mean interassay recovery of quality control (QC) samples was 92 to 109%. For assays of nonconjugated SN-38, the mean interassay precision of the method between the LLOQ (0.464 ng/ml) and the ULOQ (460 ng/ml) was ±5.1%. The mean interassay precision of the system was ±6%. Mean interassay recovery of QC samples was 101 to 111%. In assays for total SN-38, the mean interassay precision of the method between the LLOQ (0.464 ng/ml) and the ULOQ (460 ng/ml) was ±4.9%. Interassay recovery of QC samples was 104 to 112%. For APC, the mean interassay precision of the method between the LLOQ (0.398 ng/ml) and the ULOQ (410 ng/ml) was ±7.5%. Mean interassay recovery of QC samples was 103 to 118%.

Pharmacokinetic Analysis. Pharmacokinetic parameters for intact CPT-11, SN-38, SN-38G, and APC in plasma and drug-related material in plasma and whole blood were calculated with noncompartmental methods by the Clinical Pharmacokinetics Analysis Package version 1.0 (Pharmacia & Upjohn, Inc., Kalamazoo, MI). Peak concentrations (C_{max}) and the corresponding T_{max} were determined from individual subject concentration-time curves. Area under the curve (AUC_{0-T}) was determined by trapezoidal approximation. Terminal elimination rate constants (λz) were estimated with least-squares regression of values in the terminal log-linear region of plasma concentration-time curves. Area under the curve from time zero to infinity ($AUC_{0-\infty}$) was estimated by adding AUC_{0-T} and $C_t/\lambda z$, where C_t is the last detectable plasma concentration and T is the time at which this concentration occurred. Total systemic clearance (CL) of CPT-11 was calculated as dose/$AUC_{0-\infty}$ where the dose of CPT-11 was expressed in free base equivalents (i.e., dose of CPT-11 hydrochloride trihydrate was multiplied by 0.8664, mol. wt. of the anhydrous free base = 586.69, and mol. wt. of the hydrochloride trihydrate = 677.19). The apparent volume of distribution (Vz/F) was calculated as CL/λz. Pharmacokinetic parameters determined for each gender were compared by *t* test analyses. All statistical tests were performed with the SAS System version 6 (SAS Institute Inc., Cary, NC). A *P* value of ≤.05 was considered to be statistically significant.

Liquid Chromatography–Mass Spectrometry (LC-MS). HPLC analyses were performed on a Perkin Elmer Series 200 HPLC system (pump and autoinjector). Mass spectrometry was performed with a tandem quadrupole mass spectrometer (TSQ 7000, Finnigan-MAT, San Jose, CA). The LC-MS interface was an atmospheric pressure ionization source operated in the positive ion electrospray ionization mode. The capillary temperature was 235°C, and the spray voltage was 3.0 kV. The sheath and auxiliary nitrogen gas flows were set to 80 psi and 60 ml/min, respectively. For acquisition of repetitive scanning MS data, the resolution was set to unit (10% valley), and the instrument was scanned from *m/z* 100 to 1000 every 2 s (MS1, R_f-only; MS2, mass selection). Collision-induced dissociation of MH^+ in the source region was accomplished by a 15-V offset. For product ion spectra, Q1 was operated as a high pass filter (*m/z* 500), and Q3 was scanned from 10 to 1000 every 2 s (argon, 1 mTorr, −30 V E_{lab}). Mass spectra of major and minor metabolites and degradation products of CPT-11 are adequately described elsewhere (Rivory and Robert, 1995; Lokiec et al., 1996; Rivory et al., 1996; Dodds et al., 1997, 1998). Ion-monitoring experiments to identify major and minor radioactive metabolites relied on the appearance of the correct protonated molecular ion at an appropriate relative retention time, on the absence of the same ion in identically treated blank (predose) sample matrix, and, when sensitivity permitted, on an appropriate Q2 product ion spectrum (data not shown).

Results

Excretion and Recovery of Total Radioactivity. The cumulative excretion of radioactivity is summarized in Table 2 and illustrated in Figs. 2 and 3. The cumulative recovery of radioactivity for 168 to 192 h after dosing was 95.8 ± 2.7% of dose. Fecal excretion was the dominant route of elimination, accounting for 63.7 ± 6.8% of the dose. Urinary excretion accounted for 32.2 ± 6.9% of the dose, with a harmonic mean terminal ARE half-life of 25.4 ± 2.5 h. Figures 2 and 3 show that urinary excretion was almost complete by 48 h. Delayed and variable fecal recoveries were a consequence of sporadic bowel habits in some patients. One female patient with a biliary T-tube excreted 30.1% of dose in bile. The 0- to 8-h postdose ARE half-life of radioactivity in bile was 3.7 h. Higher radioactivity recovery in urine and a shorter urine ARE half-life in this patient (18.9 h) suggest a shift away from biliary elimination.

Quantitative Metabolite Profiles in Urine, Feces, and Bile. Radiometric HPLC profiles of urine and feces from a representative

TABLE 2

Summary of cumulative recovery of radioactivity from cancer patients after i.v. administration of [^{14}C]CPT-11

Differences across gender were not significant. Bile-exteriorized Patient 5 was omitted from calculation of means.

	Percent of Radioactive Dose Excreted			
	Urine	Feces	Bile	Total
Patient				
1	27.7	67.0	N.D.	94.7
2	32.2	68.1	N.D.	100.3
3	43.8	54.2	N.D.	98.0
4	30.6	61.5	N.D.	92.2
5	48.2	14.2	30.1	92.5
6	21.7	74.9	N.D.	96.6
7	33.3	60.8	N.D.	94.1
8	35.8	59.1	N.D.	94.8
Mean (±) S.D. (n = 7)	32.2 ± 6.9	63.7 ± 6.8	N.D.	95.8 ± 2.7
% CV	21.4	10.8	N.D.	2.8

N.D., not done, only patient 5 was bile-exteriorized.

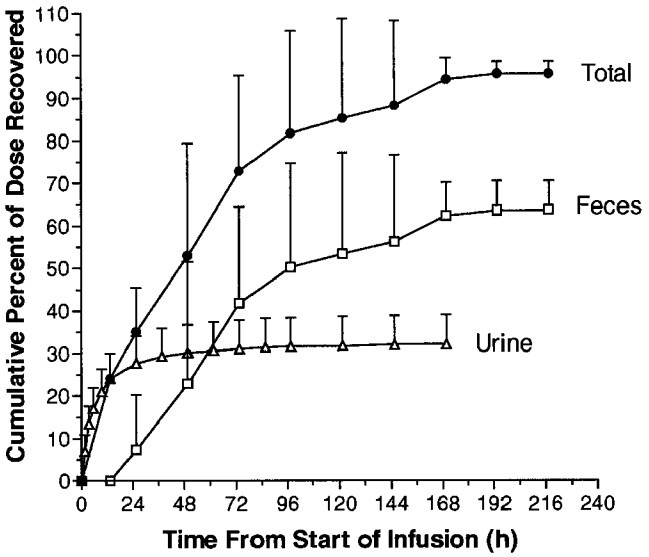

FIG. 2. *Mean (± S.D.) plot of cumulative excretion of [^{14}C]CPT-11-related radioactivity by matrix for male (patients 1–4) and female (patients 6–8) cancer patients.*

Patient 5F was bile-exteriorized and is shown separately in Fig. 3.

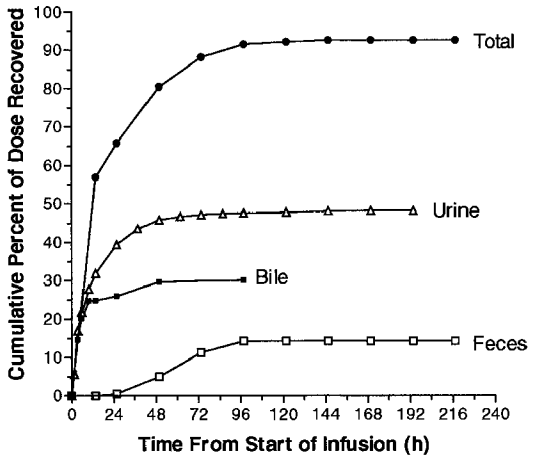

FIG. 3. *Plot of cumulative excretion of [^{14}C]CPT-11-related radioactivity by matrix for bile-exteriorized cancer patient 5F.*

patient (1M) are shown in Fig. 4. Comparable radiochromatograms for urine, feces, and bile from the patient with the biliary T-tube (5F) are shown in Fig. 5. Overall, in four male and three female patients, 92.2% of dose was profiled, with 3.7% of dose not chosen for profiling due to low relative concentration of radioactivity in excreta collected at later time points. Losses of radioactivity during the extraction and concentration procedures were 11.1% of dose and occurred predominantly from feces (9.86% of dose) as a result of difficult sample preparation. Four major and up to 14 minor radioactive peaks, as well as the parent drug, were quantified.

The overall abundance of quantitatively significant metabolites is summarized in Table 3. Major peaks were identified by retention time similarity to synthetic standards of CPT-11, SN-38, SN-38G, APC, and NPC and confirmed by mass spectrometry. CPT-11 was the major excretion product in urine, bile, and feces. APC and SN-38G were the most significant metabolites in urine and bile, but were much less abundant than CPT-11. SN-38 and the primary amine NPC were relatively minor excretion products. The ratio of SN-38 to SN-38G in feces was much higher than that observed in urine and bile, presumably due to in vivo or ex vivo hydrolysis by bacterial β-glucuronidases.

Characterization of Minor Drug-Related Peaks. Partial characterization of detectable minor metabolites, impurities, and some analytical artifacts is summarized in Table 4. Polar peak, M1, was tentatively identified as a quinoline *N*-oxide of SN-38, based on a strong 393 ion corresponding to SN-38 and an apparent MH^+ at *m/z* 409. SN-38 *N*-oxide is an intermediate used to introduce the phenolic 10-hydroxy group into the camptothecin nucleus during radiosynthesis (Sawada et al., 1991), although metabolic generation of this minor peak cannot be ruled out.

Extracts were screened for the MH^+ 491-, 559-, 561-, 575-, and 603-Da pseudomolecular ions of biotransformation products described by Lokiec et al. (1996). The MH^+ 561- (Lokiec metabolite 6) and 559-Da (Lokiec metabolite 5) compounds have also been described as photodegradation products PDP-1 and PDP-2 by Dodds et al. (1997). PDP-2 is a known degradation product in the CPT-11 bulk drug. HPLC-MS of urine revealed ions at *m/z* 491 [Retention time (Rt) 15.5 min, M13], 559 (Rt 17.8 min, M14) and 561 (Rt 13.5 min, observed by MS only). It is reasonable to speculate that M13 (MH^+ 491) may have been formed by metabolism of the PDP-2 impurity (M14) or may have arisen from degradation ex vivo of NPC (M9). Traces of ions corresponding to photodegradation products PDP-3, PDP-4, and PDP-5 (Dodds et al., 1997) were observed in urine extracts at 27.45 min. These ions correlated with trace-level radioactive peak M19.

Four MH^+ 603 peaks corresponding to monohydroxylated CPT-11 were observed in bile. The first peak was not detectable in urine and

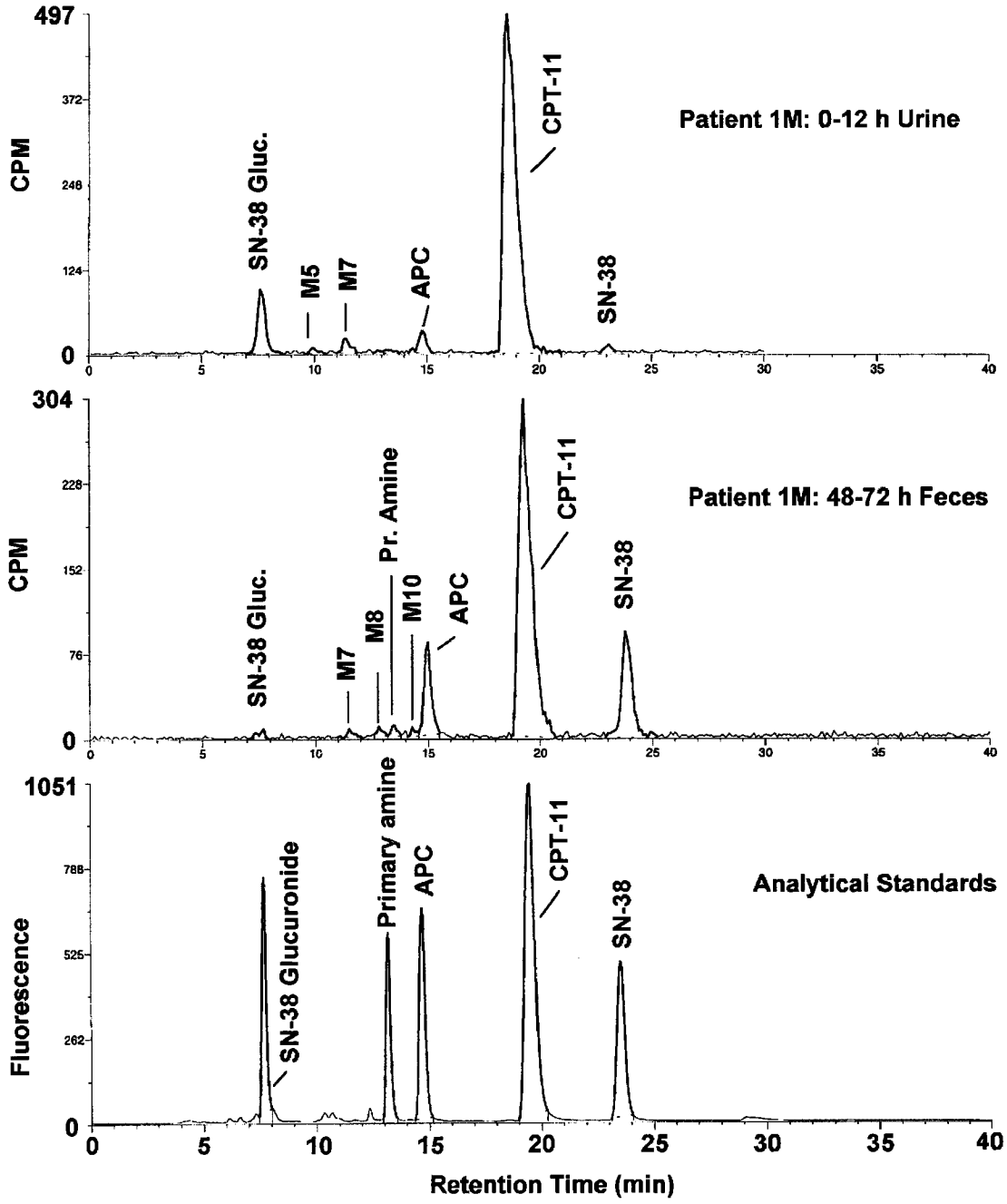

FIG. 4. *HPLC-radiochromatograms of excreta extracts from patient 1M.*

Top: 0- to 12-h urine concentrate 20.4% of dose, 100% extraction efficiency. Middle: 48- to 72-h feces extract (37.7% of dose, 85.4% extraction efficiency). Bottom: fluorescence HPLC chromatogram showing a mixed analytical standard containing SN-38 glucuronide (~7.6 min), primary amine metabolite NPC (~13.2 min), metabolite APC (~14.7 min), CPT-11 (~19.5 min), and SN-38 (~23.5 min). Trace-level radioactive peaks were observed at ~11.5 min (M7), ~12.8 min (M8), and ~14.3 min (M10). Fractions containing the highest possible percentage of dose were chosen to illustrate the overall metabolic disposition of CPT-11.

correlated with radioactive peak M8. The other three peaks matched the retention times of MH^+ 603 ions in urine. Radioactive urine metabolites M10 and M12 corresponded in retention time to the first and last of these *m/z* 603 peaks; the middle peak eluted under the APC peak.

These peaks are probably related to the MH^+ 603 peaks described by Lokiec et al. (1996) as metabolites 3, 7, 8, 9, and 13. Lokiec metabolite 7 is a degradation product present in CPT-11 bulk drug. Similarly, a degradation product, hydroxylated α to the nonbasic piperidine carbamoyl nitrogen, is a trace impurity in CPT-11 bulk drug and may account for either Lokiec metabolite 3 or 9. Nonetheless, we speculate that two of the MH^+ 603 metabolites described by Lokiec (8 and 9, based on similar retention time) represent trace-level metabolites arising from hydroxylation of positions 3 or 4 of the outer piperidine ring.

An *m/z* 605 ion corresponding to M5 represents the hydroxy acid anion of CPT-11 that is formed by lactone hydrolysis. Higher mass aggregate/multiply charged ions such as *m/z* 908 (3 MH^+/2) were formed in the MS source. It is now well established that CPT-11 exists in a pH dependent equilibrium between lactone and hydroxy acid anion forms, with acidic pH favoring the lactone form (Burke and Mi, 1994). Samples were acidified during analysis to convert drug-related

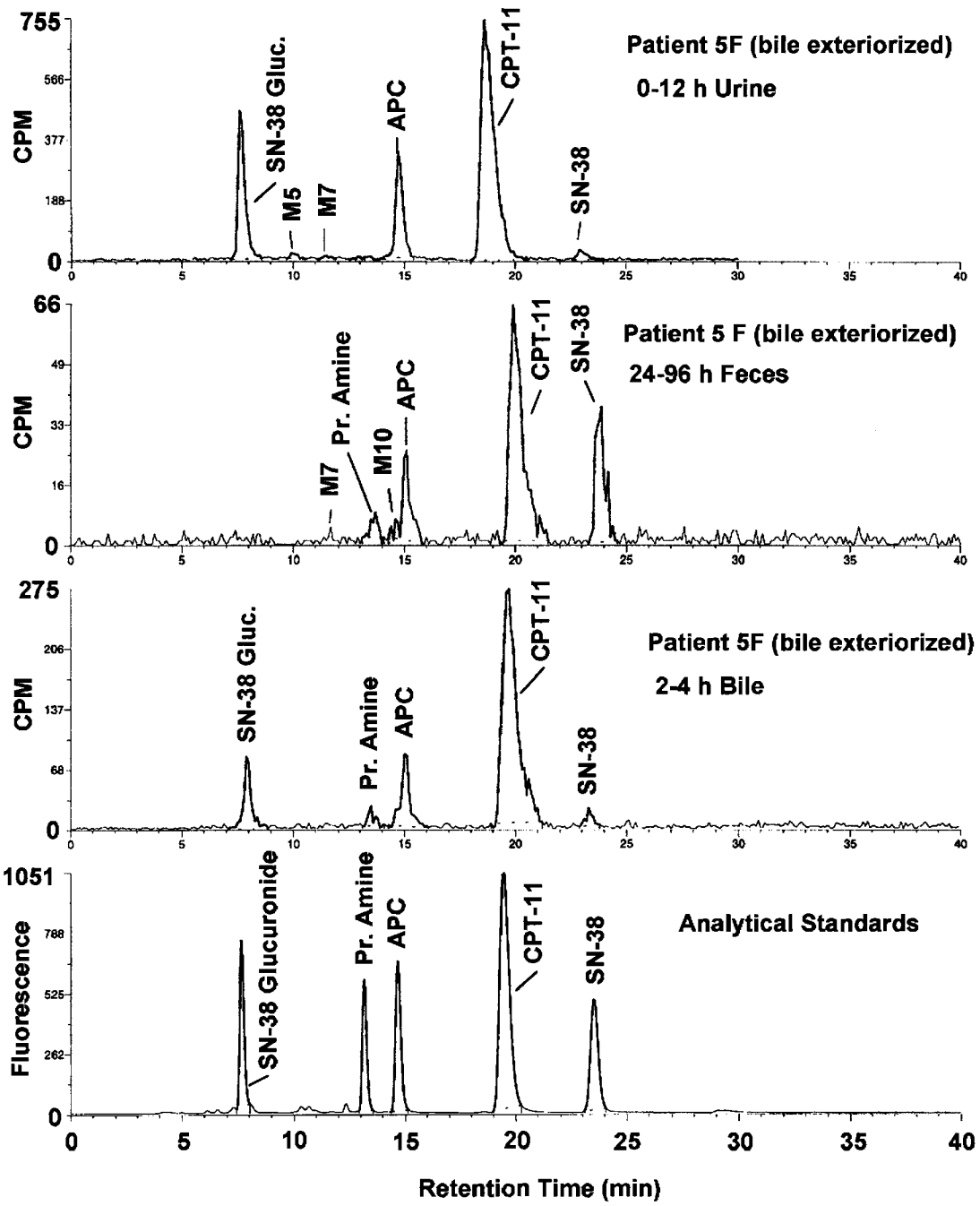

FIG. 5. *HPLC radiochromatograms of excreta extracts from bile-exteriorized patient 5F.*

Upper trace: 0- to 12-h urine concentrate (31.9% of dose, 93.9% extraction efficiency). Second trace: 24- to 96-h feces extract (13.7% of dose, 80.3% extraction efficiency). Third trace: 2- to 4-h bile concentrate (5.6% of dose, 90.6% extraction efficiency). Lower trace: fluorescence HPLC chromatogram showing a mixed analytical standard containing SN-38 glucuronide (~7.7 min), primary amine metabolite NPC (~13.2 min), metabolite APC (~14.7 min), CPT-11 (~19.5 min), and SN-38 (~23.5 min). Trace-level radioactive peaks were observed at 10.0 min (M5), ~11.5 min (M7), and ~14.3 min (M10). Fractions giving the highest recovery (urine and feces) or best signal-to-noise ratio (bile) were chosen to illustrate the overall metabolic disposition of CPT-11.

materials to the lactone form. Accordingly, M5 was only observed in highly concentrated extracts of excreta.

LC-MS of metabolite M7 revealed no interpretable ions, presumably due to coelution with a band of interferences. Masses were calculated for an array of known piperidine biotransformations (Gole et al., 1987; Masumoto et al., 1990); however, ions at *m/z* 577, 563, 599, 591, and 601 were not observed.

Radioactivity Profiles in Plasma. A comparison of radiometric and fluorometric HPLC profiles of pooled plasma (0–4 h) in Fig. 6 demonstrates that CPT-11 was the major circulating radioactive compound in plasma. APC and, to a much lesser extent, SN-38G and SN-38 were the only detectable radioactive metabolites in pooled plasma. NPC and a variety of other minor peaks were only detectable by fluorescence.

Pharmacokinetics in Whole Blood and Plasma. Concentrations of drug-related radioactivity in plasma were similar to those in whole blood. In vivo hematocrit-adjusted blood cell-to-plasma partition coefficient (K_p) values were ~1, indicating equal partitioning of radio-

TABLE 3

Summary of relative abundance of radioactive metabolites accounting for >1% of dose in urine, feces, and bile

Parent drug and metabolite abundance are expressed as percentage of administered dose and were determined by quantitative radiometric HPLC. Two minor radioactive peaks denoted M5 and M7 accounted for 0.23 and 0.98% of dose, respectively. M5 was the hydroxy acid form of CPT-11 in equilibrium with the lactone form and was observed as a consequence of sample concentration. All other minor radioactive peaks (12 of 19 total peaks) collectively accounted for a mean of less than 1.18% of dose. Differences across gender were not significant. Bile exteriorized patient 5 was excluded from calculation of means.

Excretion Matrix	Recovery Data		Drug or Metabolite Name				
	Percent of dose profiled	Percent of dose not extracted[a]	SN-38G (M3)	NPC (M9)	APC (M11)	CPT-11	SN-38 (M17)
All patients (n = 7)							
Urine	30.2 ± 6.6	1.25 ± 1.55	3.02 ± 0.77	0.14 ± 0.08	2.23 ± 1.53	22.40 ± 5.50	0.43 ± 0.12
Feces	62.0 ± 7.6	9.86 ± 3.77	0.27 ± 0.17	1.36 ± 0.94	8.29 ± 2.95	32.31 ± 4.47	8.24 ± 2.51
Total	92.2	11.1	3.29	1.50	10.5	54.7	8.67
Patient No. 5---Bile Duct Exteriorized							
Urine	45.8	2.67	12.01	0.09	7.73	21.77	0.90
Feces	13.7	2.71	0.00	0.32	1.66	6.26	2.78
Bile	29.7	3.18	2.67	0.30	3.90	18.46	0.44
Total	89.3	8.56	14.7	0.71	13.3	46.5	4.12

[a] Based on extraction efficiency and recovery after SPE and concentration steps.

TABLE 4

Correlation summary of radiometric HPLC and HPLC MS peaks characterized in human excreta

Radiomatic peak integrations of peaks representing <2.5% of peaks in a chromatogram were variable; therefore, minimum and maximum range data are valid as estimates only and represent the lowest and highest observed percentage of dose in individual patients. Ranges are more reliable for metabolites representing >2.5% of dose (CPT-11, SN-38, APC and SN-38 glucuronide).

Metabolite or Peak	Name	^{14}C Retention Time	MH$^+$	MS Retention Time	Lowest/Highest Individual % of Dose*	No. patients with ^{14}C peak[a]	Metabolite or Artifact
		min		*min*			
M1	SN-38 *N*-oxide	5.1–5.3	409 with 393 fragment	6.05	N.D./0.43	4/8	Radioimpurity or metabolite
M2	Unknown	6.0–6.5			N.D./0.12	4/8	Unknown
M3	SN-38 glucuronide	7.4–7.8	569	8.11	2.54/4.68	8/8	Major Metabolite
M4	Unknown	8.6			N.D./0.57	4/8	Unknown
M5	CPT-11 hydroxy acid	9.9–10.3	605	9.12	0.13/0.38	8/8	Parent-equilibrium artifact
M6	Unknown	10.9			N.D./0.02	1/8	Unknown
M7	Unknown	11.3–11.6			0.34/2.0	8/8	Unknown
M8	Hydroxy CPT-11	11.9–12.9	603	11.01	0.02/0.53	8/8	Possible metabolite in bile
M9	Primary amine (NPC)	12.9–13.6	519	11.24	0.22/2.66	8/8	Metabolite
M10	Hydroxy CPT-11	13.6–14.2	603	12.27	0.04/0.97	8/8	Possible metabolite
M11	APC	14.3–15.1	619	12.53	6.19/14.01	8/8	Major Metabolite
M12	Hydroxy CPT-11/Lokiec 7[b]	16.6	603	14.39	N.D./0.11	2/8	Impurity
	PDP-1[c]/Lokiec 6		561	13.45	N.D./N.D.	0/8	Impurity-MS only
M13	Lokiec 15[b]	17.1–17.4	491	15.45	N.D./0.33	2/8	Degradation artifact of NPC or metabolite of impurity M14
M14	PDP-2/Lokiec 5[b]	17.8	559	16.38	N.D./0.37	4/8	Impurity/degradation artifact
	CPT-11	18.4–19.7	587	17.10	45.39/63.06	8/8	Parent Drug
M15	Unknown	20.1–21.0			N.D./0.18	2/8	Unknown
M16	Unknown	21.0–22.0			N.D./0.14	1/8	Unknown
M17	SN-38	22.0–23.5	393	21.41	6.19/12.7	8/8	Active metabolite
M18	Unknown	24.9			N.D./0.12	1/8	Unknown
M19	PDP-3, -4, -5[c]	25.9–26.0	559/529/543	27.45	N.D./0.46	3/8	Impurities-coelute

[a] The number of patients that had a measurable radioactive peak for this metabolite or impurity, compared with total number of patients.
[b] Peak designation in Lokiec (1996).
[c] Peak designation in Dodds (1997).
N.D., not done, only patient 5 was bile-exteriorized.

activity between blood cells and plasma. Mean pharmacokinetic parameters derived for radioactivity in whole blood and plasma and for CPT-11, SN-38, SN-38G, and APC in plasma are summarized in Table 5. Plasma concentration versus time plots for drug-related radioactivity, CPT-11, and major circulating metabolites are compared in Fig. 7.

Whole blood and plasma concentrations of radioactivity peaked at the end of the infusion and were near the limit of quantitation by 24 to 36 h. Whole blood concentrations were generally lower in males, and plasma concentrations were higher in females. Because of intersubject variability, particularly in females, and the small sample size, differences in radioactivity pharmacokinetic parameters were not statistically significant.

Quantitative fluorescence HPLC determination of plasma concentrations of CPT-11, SN-38, SN-38G, and APC showed that CPT-11 was the major circulating component in plasma [55% of the mean radiochemical area under the curve (AUC)]. CPT-11 and the three major metabolites (SN-38, SN-38G, and APC) accounted for 93% of the mean radiochemical AUC.

Plasma concentrations of CPT-11 and the three CPT-11 metabolites, SN-38, SN-38G, and APC, generally appeared to be lower in male subjects compared with females. Because differences in pharmacokinetic parameters between males and females were not statistically significant, mean pharmacokinetic data for all patients are reported in Table 5. Gupta et al. (1997) has previously demonstrated no significant gender-based differences in CPT-11 pharmacokinetics and pharmacodynamics. Mean CPT-11 CL for all patients was 12.4 ± 3.02 l/h/m^2. Mean SN-38 $AUC_{0-\infty}$ values represented 4.3 ± 1.8% of

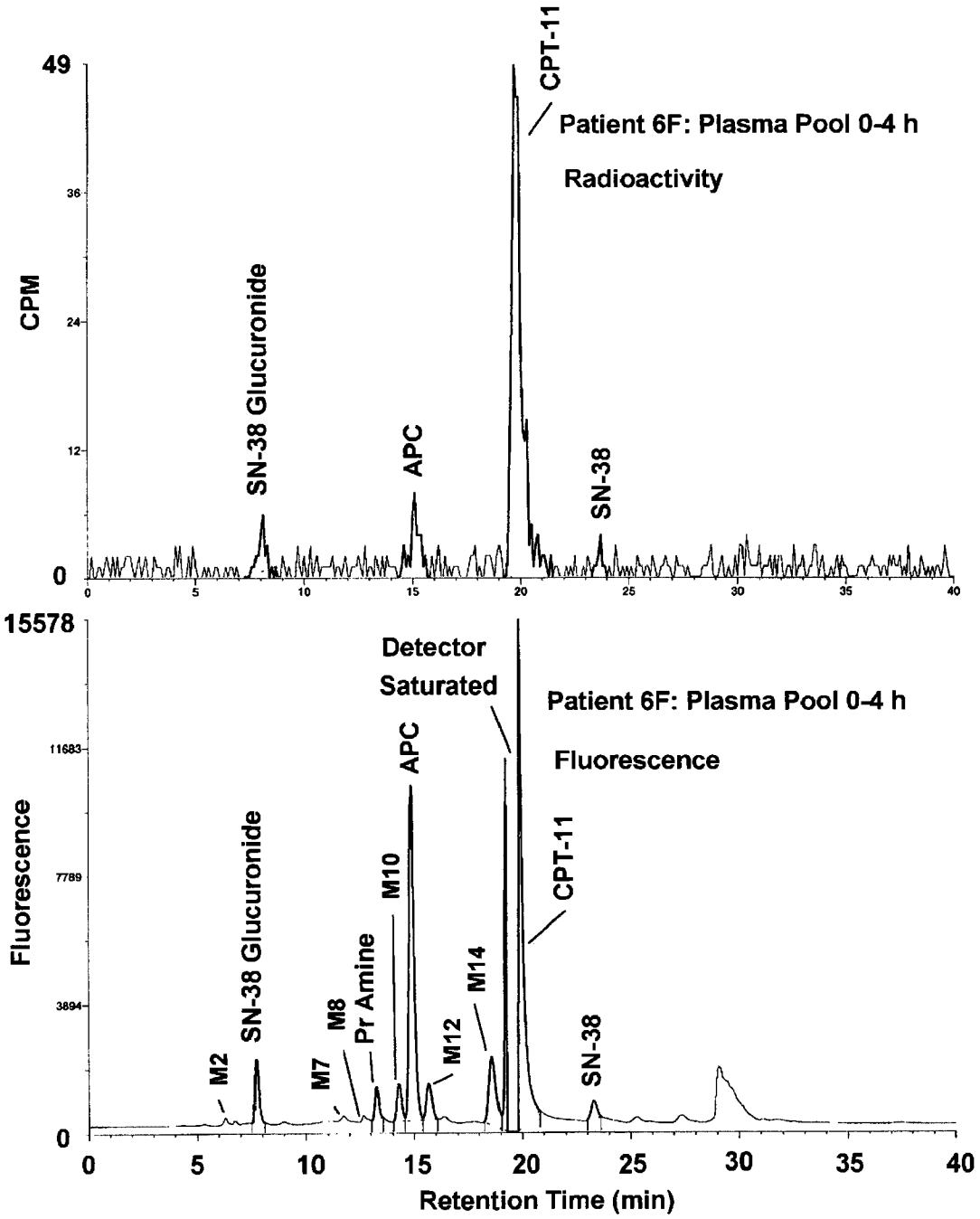

FIG. 6. *Representative HPLC chromatograms of 0- to 4-h pooled plasma extracts from patient 6F.*

Top: radiochromatogram showing CPT-11 as the major circulating component. Only traces of metabolite APC and SN-38 glucuronide were detectable radiometrically. Bottom: the corresponding HPLC-fluorescence chromatogram showing trace-level peaks of SN-38 glucuronide (~7.8 min), primary amine metabolite NPC (~13.2 min), metabolite APC (~14.9 min), CPT-11 (~19.5 min, detector saturated), and SN-38 (~23.3 min). Trace-level fluorescent peaks were observed at ~6.2 min (M2), ~11.8 min (M7), ~12.7 min (M8), ~14.3 min (M10), ~15.6 min (M12), and ~18.6 min (M14) but were not detectable radiometrically and, therefore, cannot be confirmed to be derived from CPT-11. Fractions containing the highest possible concentrations of radioactivity were chosen for profiling to attain an adequate signal-to-noise ratio for low abundance radioactive peaks.

the corresponding mean CPT-11 $AUC_{0-\infty}$ values. The plasma concentrations of SN-38G and APC exceeded those of SN-38. When expressed as ng·h/ml, the $AUC_{0-\infty}$ for SN-38G was ~4-fold higher than SN-38, whereas the $AUC_{0-\infty}$ for APC was ~7-fold greater. Although plasma concentrations of NPC were not determined, chromatograms demonstrated that the peak corresponding to this metabolite was resolved from APC under the HPLC conditions used for quantitation, and the NPC peak was small relative to that of circulating APC.

Discussion

Near-complete recovery of the radioactive dose was obtained in this study (95.8% of dose). Methods were developed to maximize extraction efficiency before quantitative metabolite profiling. In seven patients, a mean total of 92.2% of dose was quantitatively profiled. This included a loss during extraction of 11.1% of dose, primarily from feces. Our experience during the development of these extraction

TABLE 5

Mean ± S.D. (n = 8) pharmacokinetic parameters determined using [^{14}C]CPT-11-related radioactivity in whole blood and plasma and plasma concentrations of CPT-11, SN-38, SN-38G, and APC determined using fluorescence HPLC

Parameter	Total Radioactivity	
	Whole blood	Plasma
T_{max} (h)	1.5 ± 0	1.50 ± 0
C_{max} (μg-eq/g)	2.34 ± 0.314	2.14 ± 0.359
$AUC_{0-25.5}$(μg-eq · h/g)	13.7 ± 3.88	14.2 ± 6.66
$AUC_{0-49.5}$ (μg-eq · h/g)	15.7 ± 5.80	16.1 ± 9.20
$t^1/_2$ (h)‡	13.6	8.7

Parameter	CPT-11—Plasma	SN-38—Plasma	SN-38G—Plasma	APC—Plasma
T_{max} (h)	1.50 ± 0.00	2.32 ± 1.02	2.81 ± 0.458	3.44 ± 1.78
C_{max} (ng/ml)	1534 ± 143	27.1 ± 11.6	87.9 ± 42.5	203 ± 184
$AUC_{0-25.5}$ (ng · h/ml)	7765 ± 1876	228 ± 149	1047 ± 847	2400 ± 2460
$AUC_{0-\infty}$ (ng · h/ml)	8808 ± 2215	400 ± 242	1745 ± 1417	3271 ± 3481
CL (l/h/m^2)	12.4 ± 3.02	N.D.	N.D.	N.D.
Vz (l/m^2)	297 ± 119	N.D.	N.D.	N.D.
$t^1/_2$ (h)‡	14.6	28.5	35.5	17.8

N.D., not done, only patient 5 was bile-exteriorized.
‡ harmonic mean.

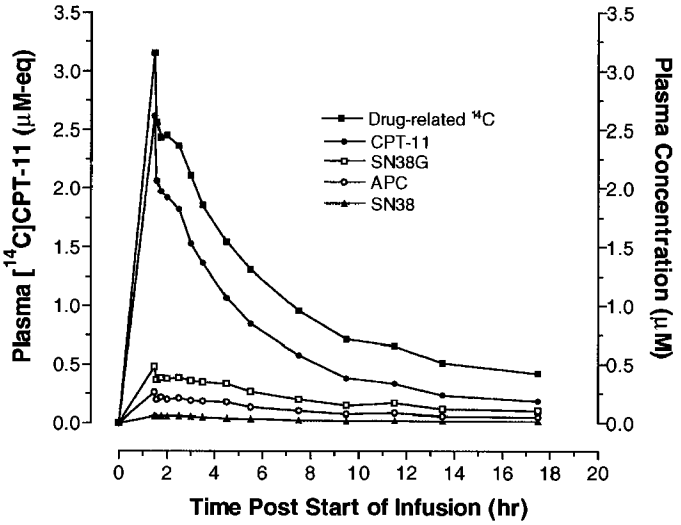

FIG. 7. *Plot of mean plasma concentrations of [^{14}C]CPT-11-related radioactivity and intact CPT-11, SN-38, SN-38G, and APC after single dose i.v. infusion of CPT-11 to male and female cancer patients.*

methods indicates that this loss was nonspecific, and, in our opinion, does not represent an unextracted metabolite.

Other investigators have attempted to quantify CPT-11 and metabolites in urine, bile, and feces with nonradiometric methods (Lokiec et al., 1995; Sparreboom et al., 1998). These studies generally obtained low total recoveries of drug-related materials. During this study, we discovered that there was a significant risk of precipitation of drug-related material in feces, bile, and urine during freezing. This made necessary the appropriate dilution of the specimen to assure dissolution of all drug-related material before SPE. Methods developed to prevent radioactivity losses during extraction and concentration steps were tedious. Optimization of these methods was facilitated by the radiolabel.

Based on parent drug and metabolite mass balance, and considering the well documented effect of lactone hydrolysis on both antineoplastic activity and systemic clearance of camptothecin drugs, we propose that exposure to the active antineoplastic metabolite SN-38 lactone is primarily dependent on the rates of lactone hydrolysis and excretion of CPT-11 (55% of dose). CPT-11 lactone AUC is therefore the primary driver of SN-38 lactone AUC. SN-38 is in turn excreted intact, lactone-hydrolyzed and excreted, or glucuronidated.

Studies in rodents have demonstrated that the activity of canalicular multiple organic anion transporter (cMOAT) is markedly inhibited by the presence of biliary tract obstruction and recovers slowly after restoration of bile flow (Kothe et al., 1993). This may explain the decreased biliary excretion (i.e., sum of recovery in bile and feces) and increased urinary excretion of radioactivity observed in the female patient with the biliary T-tube.

The mean CPT-11 systemic clearance and volume of distribution values determined in these patients (12.4 ± 3.02 l/h/m^2 and 297 ± 119 l/m^2) are comparable with those reported previously (Rothenberg et al., 1993; Chabot et al., 1995; Chabot, 1997).

The harmonic mean half-lives for CPT-11, SN-38, SN-38G, and APC determined from intact drug and metabolite plasma concentrations were 14.6 h (range 8.6–21.8 h), 28.5 h (range 16.7–54.5 h), 35.5 h (range 30.8–49.9 h), and 17.8 h (range 8.8–34.0 h), respectively. These values are longer than values reported previously (Chabot et al., 1995; Chabot, 1997; Gupta et al., 1997; Rivory et al., 1997). These literature studies used less sensitive methods of detection that were unable to quantify concentrations well into the elimination phase. Thus, some earlier literature analyses may have underestimated the half-lives of CPT-11 and its metabolites. In this study, the half-lives estimated from plasma concentrations determined by fluorescence detection were also longer than those based on radiometric detection. The longer half-lives reflect the higher sensitivity of fluorescence detection relative to radiometric detection. This resulted in a duration of analytical detectability that spanned several half-lives for both the parent drug and the metabolites.

The kinetics of conjugation of the active metabolite SN-38 to afford the inactive metabolite SN-38G has been proposed to be a significant factor in the etiology of CPT-11-induced diarrhea (Gupta et al., 1994). This glucuronide conjugate is a major metabolite of SN-38. Based on the presence of SN-38G in the bile of patient 5F, the low percentage of SN-38 in feces compared with urine can be explained by the action of enteric bacterial β-glucuronidases, although the relative amount of ex vivo versus enteric hydrolysis is not known. The latter process may increase concentrations of SN-38 in the gut lumen.

Several peaks described as metabolites by Lokiec et al. (1996) were observed at trace levels in this study. Many of these were actually impurities present in the bulk drug. Instability of CPT-11 during

sample preparation in both this study and Lokiec's study is also possible (Dodds et al., 1997). The observation of these trace-level products serves as a reminder that artifacts may be observed when significant amounts of drug-related material in excreta are concentrated for analysis by sensitive analytical techniques.

Whereas many fluorescent peaks were observed by HPLC, only CPT-11 and three metabolites (SN-38, SN-38G, and APC) individually accounted for greater than 1 to 2% of the dose recovered in excreta. Therefore, in our opinion, NPC, MH$^+$ 603 peaks, and other trace-level metabolites have no clinical or toxicological significance.

In conclusion, near-complete recovery of radioactivity was obtained after i.v. administration of [^{14}C]CPT-11. CPT-11 was the major excretion product, followed by much lower percentages of SN-38G, APC, SN-38, NPC, and an unidentified metabolite M7. All other transiently observed, trace-level radioactive peaks collectively accounted for ~ 1% of dose. These results show that the parent drug and its three major metabolites (SN-38, SN-38G, and APC) account for virtually all of CPT-11 disposition, with fecal excretion representing the major elimination pathway.

Acknowledgments. We are grateful to study contributors John Easter, Dorothy Wenzel, Dr. Dennis Avery (deceased), Dave Seybert, Barbara Gulotti, Karle Tackwell, and the staff at Pharmacia & Upjohn Clinical Research Unit and West Michigan Cancer Center. We also thank the cancer patients who gave of their valuable time to participate in this study.

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Annals of Oncology 18: 498–503, 2007
doi:10.1093/annonc/mdl427
Published online 8 December 2006

FOLFIRI.3, a new regimen combining 5-fluorouracil, folinic acid and irinotecan, for advanced pancreatic cancer: results of an Association des Gastro-Entérologues Oncologues (Gastroenterologist Oncologist Association) multicenter phase II study

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Received 21 September 2006; revised 17 October 2006; accepted 17 October 2006

Background: The purpose of the study was to prospectively evaluate the efficacy and tolerability of the FOLFIRI.3 regimen in patients with unresectable pancreatic adenocarcinoma.

Patients and methods: Chemotherapy-naive patients with histologically proven advanced pancreatic adenocarcinoma were treated with the FOLFIRI.3 regimen, consisting of irinotecan 90 mg/m^2 as a 60-min infusion on day 1, leucovorin 400 mg/m^2 as a 2-h infusion on day 1, followed by 5-fluorouracil (5-FU) 2000 mg/m^2 as a 46-h infusion and irinotecan 90 mg/m^2, repeated on day 3, at the end of the 5-FU infusion, every 2 weeks.

Results: Forty patients were enrolled, of whom 29 (73%) had metastatic disease. A total of 441 cycles were delivered (1–53). Grade 3–4 neutropenia occurred in 35% of the patients, accompanied by fever in two cases. Other relevant grade 3–4 toxic effects were nausea-vomiting (27%) and diarrhea (25%). Grade 2 alopecia occurred in 48% of the patients. There were no treatment-related deaths. The confirmed response rate was 37.5%. Stable disease was observed in 27.5% of the patients. The median progression-free and overall survivals were 5.6 months and 12.1 months, respectively. The 1-year survival rate was 51%.

Conclusion: The FOLFIRI.3 regimen seems to be active on advanced pancreatic cancer and to have a manageable toxicity profile. The lack of cross-resistance between FOLFIRI.3 and gemcitabine-based regimens allows efficient second-line therapies.

Key words: irinotecan, pancreatic cancer, systemic chemotherapy

introduction

Pancreatic cancer causes about 50 000 deaths annually in Europe and is the fourth leading cause of death by cancer in the Western countries; the mortality and incidence rates are similar [1–3]. The overall 6-month and 1-year survival rates among patients with advanced disease are, respectively, 35% and <10% in most studies [1–3]. About 80% of patients have unresectable or metastatic forms at diagnosis [4]. Systemic chemotherapy protocols for unresectable pancreatic cancer have given disappointing results during the last 20 years. Several drugs, given alone or in combination, have been tested in phase II and III trials, with objective response rates ranging from 0% to 20% and median survival times not exceeding 6 months [5]. One randomized trial showed the superiority of single-agent gemcitabine over single-agent 5-fluorouracil (5-FU) therapy and established gemcitabine as the reference for advanced pancreatic cancer [6]. The objective response rates, however, in large randomized trials of gemcitabine ranged from 4% to 16%, and the median survival time was only 4.6–6 months [7–10].

Irinotecan (Aventis, France), a camptothecin analogue, has a stronger growth-inhibiting effect than cisplatin, mitomycin C and fluorouracil on cultured pancreatic adenocarcinoma cells [11]. Irinotecan is also highly active on pancreatic tumor cells in culture and in xenograft models. [12, 13] Irinotecan monotherapy has been tested in patients with previously untreated pancreatic cancer, yielding response rates of 9%–27% [14, 15]. Second-line irinotecan monotherapy has also shown a degree of activity [16, 17]. In most trials, however, the response rates were low (<10%) and survival was poor.

In vitro studies indicate that synergism between irinotecan and 5-FU is sequence dependent, cytotoxicity being stronger when irinotecan is administered before 5-FU [18–20]. *In vivo*,

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however, a phase II randomized study of colorectal cancer patients indicated that cytotoxicity was stronger when irinotecan was administered after 5-FU [21]. These studies gave rise to the FOLFIRI.2 regimen, consisting of a simplified LV5FU2 administration, followed by irinotecan 180 mg/m^2 at the end of 5-FU infusion [22]. The latter phase II study, involving heavily pretreated colorectal cancer patients, showed encouraging efficacy but major toxicity. The same team subsequently designed a regimen (FOLFIRI.3) in which the irinotecan dose is administered in two halves, one before 5-FU and the other at the end of the 5-FU infusion. This regimen was then tested in a multicenter phase II study involving patients with metastatic colorectal cancer who had previously received FOLFOX. The response rate was 26% and the median progression-free and overall survival times were 5.1 and 10 months, respectively [23].

Here, in a multicenter phase II study, we evaluated the FOLFIRI.3 regimen in previously untreated patients with advanced pancreatic cancer.

patients and methods

patients

The following criteria were used for patient selection: histologically or cytologically proven pancreatic ductal adenocarcinoma; unresectable locally advanced or metastatic disease; at least one measurable lesion (response evaluation in solid tumors (RECIST) criteria); no previous chemotherapy or radiotherapy; age between 18 and 75 years; World Health Organization (WHO) performance status (PS) of less than three; initial morphologic assessment at least 3 weeks before treatment; adequate bone marrow status (polymorphonuclear neutrophils >1.5 g/l, platelets >100 g/l and hemoglobin >10 g/dl), renal function (serum creatinine level <125 μmol/l) and liver function [serum bilirubin level <1.5 × the upper limit of normal (ULN), alkaline phosphatase (ALP) and transaminase levels <3 × ULN] and estimated life expectancy >2 months. Surgical unresectablility was observed during laparotomy or decided by a multidisciplinary staff meeting in each participating center. The study was approved by the Pitié Salpêtrière Hospital ethics committee, and written informed consent was obtained from each patient. Patients were fully informed of the type and modalities of the treatment, as well as possible adverse effects and expected benefits. The pretherapeutic work-up included a complete physical examination, WHO PS, body weight, symptoms, abdominal computed tomography (CT) scan, CA 19-9 assay, standard chest X-ray examination and, if required, thoracic CT scan.

the FOLFIRI.3 regimen

FOLFIRI.3 consists of irinotecan 90 mg/m^2 administered as a 60-min infusion on day 1, together with leucovorin 400 mg/m^2 over 2 h, 5-FU 2000 mg/m^2 administered as a 46-h infusion and irinotecan 90 mg/m^2 repeated on day 3, at the end of the 5-FU infusion (Figure 1). The chemotherapy cycles were repeated every 2 weeks if the polymorphonuclear neutrophil count was >1500/mm^3, the platelet count >100 000/mm^3 and the serum bilirubin level <1.5 × ULN.

The use of antiemetic prophylaxis was decided locally. Patients who developed a severe cholinergic syndrome received preventive treatment with atropine (0.25 mg subcutaneously) during all subsequent cycles. Patients who developed late-onset diarrhea received high-dose loperamide following specific guidelines. If severe neutropenia occurred and/or if neutropenia did not recover to grade 1 or 0 on day 14, a granulocyte colony-stimulating factor (G-CSF) could be used during subsequent cycles.

Figure 1. The FOLFIRI.3 regimen.

The irinotecan dosage was reduced to 80 mg/m^2 and the 5-FU dosage was reduced by 20% if grade 3–4 toxicity occurred; other dose adjustments were decided on an individual basis. Dose reescalation was not permitted.

Treatment was interrupted if the tumor progressed or severe toxicity occurred, and at the patient's request. Second-line chemotherapy with gemcitabine, oxaliplatin, 5-FU and cisplatin was offered if the chemotherapist considered it appropriate.

assessment of therapeutic efficacy and symptom relief

The primary end point for efficacy was the tumor response rate, defined as the sum of complete and partial responses based on the RECIST criteria [24]. Tumor responses were assessed by means of helicoidal CT every 2 months (four cycles) or earlier in patients with suspected progression. Complete responses were defined as complete disappearance of all assessable disease. Partial responses were defined as a decrease of >30% in the sum of the largest diameters of target lesions. Stable disease was defined as a decrease of <30% or an increase of <20% in measurable lesions. Progressive disease was defined as an increase of at least 20% in measurable lesions or the appearance of new malignant lesions.

A second CT scan was carried out 4 and 8 weeks after the first scan to confirm complete and partial responses. All CT scans for responder patients were reviewed by an external response review committee (ERRC), composed of two independent radiologists who were not otherwise involved in the study. Secondary end points for efficacy included the time to progression and the progression-free and overall survival times. Body weight, WHO PS and symptoms were recorded at the beginning of each chemotherapy session.

toxicity

Toxicity was assessed with the National Cancer Institute Common Toxicity Criteria (version 3.0). A full blood count was carried out each week to assess hematological toxicity, and the patients had a complete physical examination and serum bilirubin, transaminase, ALP and creatinine assays before each treatment cycle. The patients were interviewed before each session, focusing on pain, nausea, vomiting, mucositis, diarrhea, asthenia, weight loss and neurological disorders. All patients who received at least one treatment session were considered assessable for toxicity.

statistical analysis

The main purpose of this study being to assess the response rate to the FOLFIRI.3 regimen, Simon's two-stage method was used for statistical analysis [25]. The population size was calculated to demonstrate treatment efficacy for an objective response rate ≥30% and treatment inefficacy for an objective response rate ≤10%, with a 5% alpha risk and 90% power. At the end of the first phase (18 patients included), the trial was to be stopped for treatment inefficacy if the number of objective responses was zero or one. If more than one objective response was observed, the trial was to be continued until a total of 35 patients had been enrolled. Assuming that 15% of the patients would be inassessable, 40 patients needed to be

enrolled. All analyses have been carried out on intention-to-treat. The results are expressed as means ± standard deviation or as ranges, as appropriate. Follow-up started at the outset of treatment. The censoring event for responses was the start of disease progression. The censoring event for survival was the date of death. Overall and progression-free survivals were determined using the Kaplan–Meier method.

results

From June 2003 to June 2005, 40 patients with advanced pancreatic adenocarcinoma were enrolled by seven French centers participating in this prospective study. The patients' clinical features and laboratory findings are shown in Table 1. Median age was 58 years (range 42–74) and the male–female sex ratio was 1.67 (25 men and 15 women). Twenty-nine patients (73%) had metastatic disease. Twenty patients had undergone surgery before inclusion, seven for curative treatment and 13 for palliative treatment or exploration. Concerning the seven patients who underwent a previous curative surgery, they all relapsed within 3–12 months after surgery and five of them had more than one metastatic site at relapse. One patient with metastatic relapse had received external irradiation (45 Gy) >6 months before the study treatment was initiated. Thirty-nine patients were assessable for toxicity and 34 for the tumor response.

Table 1. Characteristics of the patients before treatment

	No. of patients (%)
General	
Enrolled	40
Measurable lesions	40
Assessable for response	34
Assessable for toxicity	39
Age, median (minimum–maximum), years	58 (42–74)
Male/female	25/15
WHO PS	
0	9 (26)
1	19 (40)
2	12 (34)
Pancreas tumor sites	
Head	18 (45)
Body	12 (30)
Tail	10 (25)
Disease stage	
Stage III/IVa	11 (27)
Stage IVb	29 (73)
Disease sites	
Pancreas	33 (82.5)
Liver	25 (63)
Lymph nodes	6 (15)
Peritoneum	4 (10)
Lung	4 (10)
Others	2 (5)
Prior treatment	
None	20 (47.5)
Surgery	20 (47.5)
Palliative radiotherapy	1 (2.5)
Adjuvant chemotherapy	1 (2.5)
Palliative chemotherapy	0 (0)
Initially symptomatic	27 (67)

WHO, World Health Organization; PS, performance status.

tumor responses and survival

Six objective responses were observed in the first 18 assessable patients, authorizing further recruitment. The overall results are shown in Table 2. Objective tumor responses were observed in 37.5% of the 40 patients [95% confidence interval (CI) 24% to 53%]. There was one complete response and 14 partial responses. Eleven patients (27.5%) had stable disease. Tumor progression occurred in eight patients (20%) and six patients (15%) were not assessable, mainly because death occurred before the first planned evaluation. Three patients were classified as responders by the investigators but not by the ERRC, who considered that the sum of the largest diameters of the target lesions had fallen by <30% (24%–28%). Finally,

Table 2. Efficacy results (n = 40)

	Assessed by the ERRC	Assessed by the investigators
Objective response rate		
No.	15	18
%	37.5	45
95% CI	24–53	30–60
Metastatic		
No.	12	12
%	41	41
95% CI	25–59	25–59
Locally advanced		
No.	4	7
%	36	63
95% CI	15–65	35–85
Duration of response (months)		
Median	9.1	10.2
95% CI	5.5–11.9	6.8–13.9
Progression-free survival (months)		
All		
Median	5.6	
95% CI	3.7–8.7	
Metastatic		
Median	4.8	
95% CI	3.7–9.4	
Locally advanced		
Median	6.4	
95% CI	5–9.1	
Overall survival (months)		
All		
Median	12.1	
95% CI	5.8–16.4	
Metastatic		
Median	12.1	
95% CI	5.2–17.9	
Locally advanced		
Median	10.3	
95% CI	5.2–13.8	

ERRC, external response review committee; CI, confidence interval.

the overall response rate was 41% (95% CI 25% to 59%) in metastatic patients and 36% (95% CI 15% to 65%) in patients with locally advanced disease.

With a median follow-up of 21.5 months, the mean progression-free and overall survival times were 5.6 and 12.1 months, respectively. The 1-year survival rate was 51% (Figure 2). As usually observed in this setting, median overall survivals were 12.1 (95% CI 5–12.1), 15.6 (95% CI 8–17.9) and 5.8 months (95% CI 4–10.3) in patients with WHO PS of zero, one and two, respectively.

Two patients underwent surgical resection of their tumor remnants. The first patient was treated for metachronous liver metastases ($n = 5$) and had a durable (2 year) major response (>90%) to the FOLFIRI.3 regimen. He underwent right hepatectomy followed by a further 6 months of FOLFIRI.3 and is still alive with no detectable disease 8 months after surgery. The second patient was treated for a single pathologically proven metachronous lung metastasis. She had a partial response lasting for 6 months, then underwent lobectomy of the right lung and received a further 3 months of adjuvant FOLFIRI.3. A metastasis appeared in the left lung 6 months later and the pulmonary resection was again carried out. She refused adjuvant chemotherapy, and is alive and free of detectable disease 6 months after the last surgical procedure.

PS improved in 16 (51%, 95% CI 35–68) of the 31 patients whose initial WHO PS was more than zero. Weight gain was observed in 50% of the patients with initial weight loss and initial signs such as pain, asthenia or anorexia declined in 14 (52%) of the 27 initially symptomatic patients. Median delay to symptom relief was 4 weeks.

Six patients were still being treated with the FOLFIRI.3 regimen at the time of the final analysis. Three patients with locally advanced disease were given concomitant radiochemotherapy after 8, 11 and 12 FOLFIRI.3 cycles. Another 22 patients were given second-line chemotherapy consisting of gemcitabine + oxaliplatin ($n = 13$), gemcitabine alone ($n = 6$) or 5-FU + cisplatin C ($n = 3$). Six patients received a third line of chemotherapy with 5-FU or gemcitabine.

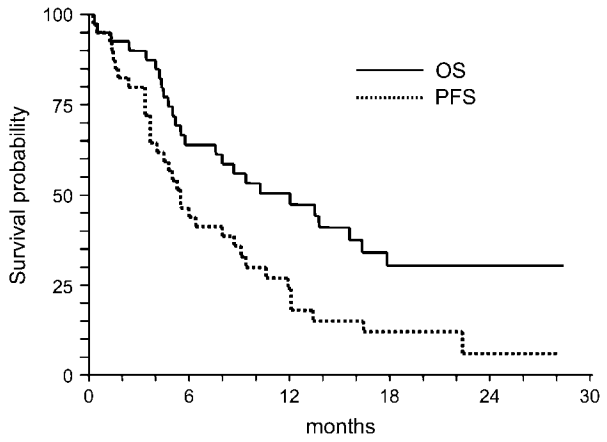

Figure 2. Overall survival (OS) and progression-free survival (PFS).

toxicity

Thirty-nine patients were included in the toxicity assessment (Table 3). A total of 441 chemotherapy sessions were administered, with a median of eight per patient (range 1–53). There were no treatment-related deaths. Fourteen patients (35%) developed grade 3–4 neutropenia. Grade 4 febrile neutropenia occurred in two patients who were not receiving growth factors. Five patients received G-CSF, for a total of 13 cycles. No new grade 3–4 toxic events were observed after cytotoxic dose reduction and/or G-CSF initiation.

Nonhematologic grade 3–4 toxic effects mainly consisted of gastrointestinal (GI) disorders. Despite routine prophylaxis with corticosteroids and setrons, grade 3 nausea-vomiting (considered to be one event) was the most frequent adverse effect, being observed in 11 patients (27%). Nausea-vomiting generally began 3 h after starting the infusion and lasted 1–3 days. Ten patients (25%) experienced grade 3 diarrhea, leading to hospitalization in two cases. All but one of the patients were able to continue treatment after a cytotoxic dose reduction and/or symptomatic treatment intensification. Aprepitant was necessary to control nausea and vomiting in three patients.

discussion

Until recently, pancreatic cancer was considered to be chemoresistant. This apparent chemoresistance was partly attributed to overexpression of the multidrug resistance and glutathione *S*-transferase genes in the normal and tumor-bearing pancreas [26, 27]. Despite disappointing results overall, chemotherapy has, over the last 10 years, improved the survival and quality of life of some patients with advanced pancreatic cancer [28]. 5-FU was widely used before 1997 to treat locally advanced and metastatic cancer of the pancreas [29]. In 1997, gemcitabine, which is easy to administer and well tolerated, was shown to be superior to 5-FU and became the new reference standard for this disease, although combinations based on platinum analogues and 5-FU are still widely used in France. Randomized trials of gemcitabine in combination with a second cytotoxic agent have failed to demonstrate any superiority over gemcitabine monotherapy, except for the gemcitabine plus capecitabine combination, but the final results of the two promising phase III studies are still awaited [7, 8, 10, 30–35].

Table 3. Toxicity

Toxicity (NCI-CTC)	Grade 1, N	Grade 2, N	Grade 3, N	Grade 4, N
Neutropenia	2	10	7	7
Thrombocytopenia	3	0	1	–
Anemia	10	19	3	–
Alopecia	12	19	–	–
Diarrhea	10	13	10	–
Nausea-vomiting	10	12	11	–
Mucositis	12	5	1	–
Neurotoxicity	5	1	–	–
Hand-foot syndrome	3	–	–	–
Maximum/patient (%)	5 (12.5)	10 (25)	18 (45)	7 (17.5)

NCI-CTC, National Cancer Institute Common Toxicity Criteria.
–, not observed.

Recently, the addition of anti-EGFR (epidermal growth factor) and anti-vascular endothelial growth factor to gemcitabine therapy was reported to yield response rates of 12%–21% and overall survival times of 7.1–8.8 months [36, 37]. Therefore, better systemic treatments using more efficient therapeutic regimens are still needed to treat advanced pancreatic cancer patients.

Irinotecan-based chemotherapies have previously been used for palliative treatment of pancreatic cancer [17, 31, 38–40]. The gemcitabine–irinotecan combination (IRINOGEM) gave promising results in phase II trials [40, 41], with objective response rates of 20%–25% and survival times of 5.7–7 months, but a subsequent phase III trial versus gemcitabine monotherapy gave negative results, with a response rate of only 16% and a median overall survival time of 6 months [31]. More recently, Conroy et al. [38] reported the results of a multicenter phase II trial testing 5-FU, oxaliplatin and CPT-11 combination therapy (FOLFIRINOX) in patients with locally advanced and metastatic pancreatic cancer. The objective response rate was 26%, as confirmed by an ERRC. The overall survival time was 10.2 months, the time to progression was 8.2 months and the median progression-free survival time is not given in the final publication.

We observed a 37.5% objective response rate in our trial. Furthermore, two of our patients with metachronous metastatic relapses were able to undergo secondary surgical R0 resection after long-lasting objective responses to the FOLFIRI.3 regimen. The tumor response is often difficult to assess in patients with locally advanced disease because of a frequent desmoplastic reaction around the organ. Major differences between the assessments of the investigators and the ERRC were observed in this subgroup, with three patients out of 11 classified as responders by the investigators and as stable by the ERRC. Moreover, the overall response rate was a little bit better in metastatic patients (41%) than in patients with locally advanced disease (36%). Concerning survival, it is noteworthy that the progression-free survival time was about half the overall survival time. Although second-line chemotherapy is classically considered ineffective on advanced pancreatic cancer, more than two-thirds of our patients received gemcitabine- or platinum-based second-line chemotherapy, and 45% of them had an objective response or disease stabilization. Thus, second-line chemotherapy with drugs showing no cross-resistance with the FOLFIRI.3 regimen might have improved the overall survival rate in this study. These results are in keeping with the trend in routine practice to offer further chemotherapy to patients with unresectable pancreatic cancer whose tumor progresses after first-line chemotherapy, as reported in other phase II and phase III trials [17, 39, 42–44]. Finally, although quality of life was not specifically assessed in this trial, about 50% of the patients gained weight, experienced symptom relief and had an improvement in their PS. These good results are not due to a patient selection bias, as about one-third of our patients had PS of two (WHO), one-quarter had more than two metastatic sites and five patients died before the first assessment of treatment efficacy. Thus, the objective response rate (37.5%, as confirmed by an ERRC) and the median overall survival time (12.1 months) observed in this study compare very favorably with the results of the latter two trials [31, 38] of irinotecan-based chemotherapies in pancreatic cancer.

The FOLFIRI.3 regimen has acceptable tolerability despite hematological and GI toxicity. These toxic effects were manageable in all the patients, and only 12.5% of patients had to stop the treatment because of severe adverse effects. No toxic deaths occurred. In future, however, patients with poor PS and other factors of poor prognosis such as a low albumin level, loss of appetite and high ALP or lactate dehydrogenase levels [7, 32, 45] may not be eligible for this regimen. Indeed, 50% of our patients with an initial PS of two experienced grade 3–4 neutropenia, 30% died before the first efficacy assessment and no tumor responses were observed in this subgroup of patients (only three had stable disease). Overall, 35% of the patients had grade 3 nausea-vomiting (taken as one event) and/or diarrhea. Only four of these patients had to be hospitalized for a few days and only one had to stop treatment of a GI adverse event. Concerning hematotoxicity, with grade 3–4 neutropenia in 35% of patients and only one case of grade 3 thrombocytopenia, the FOLFIRI.3 regimen seems to be more toxic than gemcitabine monotherapy but to be better tolerated than the FOLFIRINOX [38] and IRINOGEM regimens [31]. These results may be further improved by more frequent use of G-CSF prophylaxis.

In conclusion, with an objective response rate of 37.5%, a median overall survival time of 12 months and acceptable tolerability, the FOLFIRI.3 regimen seems to be active in patients with previously untreated advanced pancreatic cancer. The lack of cross-resistance between FOLFIRI.3 and gemcitabine-based regimens allowed efficient second-line therapy at treatment failure in this work. The FOLFIRI.3 regimen should now be tested in a randomized phase III trial versus gemcitabine.

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Electronic Acknowledgement Receipt

EFS ID:	38227949
Application Number:	15809815
International Application Number:	
Confirmation Number:	5137
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Customer Number:	153749
Filer:	Mary Rucker Henninger/Richard King
Filer Authorized By:	Mary Rucker Henninger
Attorney Docket Number:	263266-421428
Receipt Date:	07-JAN-2020
Filing Date:	10-NOV-2017
Time Stamp:	18:51:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Other Reference-Patent/App/Search documents	EP2861210_Opp_D11_Hoskins_2007.pdf	913759 94752cf19c698de34058354786478ba98b0ba1f0	no	6

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Eliel BAYEVER et al.	Group Art Unit: 1612
Application No.: 15/809,815	Examiner: Celeste A. Roney
Filed: November 10, 2017	Confirmation No.: 5137
For: Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin	

AMENDMENT AND RESPONSE TO NON-FINAL OFFICE ACTION

Via EFS-WEB
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Examiner Roney:

In reply to the Office Action mailed July 8, 2019, the period for response having been extended to January 8, 2020, by a request for extension of three months and fee payment filed concurrently herewith, please amend the above-identified application as follows:

Amendments to the Claims begin at page 2.

Remarks begin at page 6.

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): 01208-0007-01US

Application Number: 15/809,815

Filed: November 10, 2017

For: Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin

Art Unit: 1612

Examiner: Celeste A. Roney

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☑ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 1400
☐ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☐ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number _____.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 56,992.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Mary R. Henninger/ — Signature

January 7, 2020 — Date

Mary R. Henninger — Typed or printed name

404-891-1400 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Inventors:	Group Art Unit: 1612
Eliel BAYEVER et al.	Examiner: Celeste A. Roney
Application No.: 15/809,815	Confirmation No.: 5137
Filed: November 10, 2017.	
Title: Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin	

VIA EFS WEB
Commissioner of Patents
Mail Stop - Amendment
P.O. Box 1450
Arlington, VA 22313-1450

Commissioner:

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. § 1.97(c)

Pursuant to 37 C.F.R. §§ 1.56 and 1.97(c), Applicant brings to the attention of the Examiner the documents listed on the enclosed IDS Form PTO/SB/08. This Information Disclosure Statement is being filed after the mailing of an Office Action on the merits, but to Applicant's knowledge, prior to the mailing of a Final Office Action, *ex parte Quayle* Action, or Notice of Allowance. This Information Disclosure Statement is accompanied by $240, as required by 37 C.F.R. §1.97(c).

Copies of the listed foreign patent documents and non-patent literature documents are enclosed.

Applicant respectfully requests that the Examiner consider the listed documents and indicate that they were considered by making appropriate notations on the attached form.

This submission does not represent that a search has been made or that no better art exists and does not constitute an admission that each or all of the listed documents are material or

constitute "prior art." If the Examiner applies any of the documents as prior art against any claim in the application and Applicant determines that the cited documents do not constitute "prior art" under United States law, Applicant reserves the right to present to the U.S. Patent and Trademark Office the relevant facts and law regarding the appropriate status of such documents.

Applicant further reserves the right to take appropriate action to establish the patentability of the claimed invention over the listed documents, should one or more of the documents be applied against the claims of the present application.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account 506488.

Respectfully submitted,

MCNEILL BAUR PLLC.

Dated: January 7, 2020

By: /Mary R. Henninger/
Mary R. Henninger, PhD
Reg. No. 56,992
404-891-1400

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	15809815
Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	10350201	B2	2019-07-16	Hong et al.	
	2	10413510	B2	2019-09-17	Hong et al.	
	3	4604463	A	1986-08-05	Miyasaka et al.	
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	9	5593622	A	1997-01-14	Yoshioka et al.	
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	31	9364473	B2	2016-06-14	Bayever et al.	
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20020102298	A1	2002-08-01	Needham	
	2	20020146450	A1	2002-10-10	Slater et al.	
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	11	20160030342	A1	2016-02-04	Hong et al.	
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	1	1997028156	WO	A1	1997-08-07	Schering Aktiengelsellschaft Patente		
	2	2003030864	WO	A1	2003-04-17	Neopharm, Inc.		
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	6	2012146610	WO	A1	2012-11-01	Sanofi		

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	1	ABRAXANE package insert, revision December 23, 2011, retrieved from https://www.accessdata.fda.gov/drugsatfda_docs/label/2011/021660s025s026s029lbl.pdf, 13 pages.	
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	Attorney Docket Number	01208-0007-01US

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Mary R. Henninger/	Date (YYYY-MM-DD)	2020-01-07
Name/Print	Mary R. Henninger	Registration Number	56992

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(Not for submission under 37 CFR 1.99)

Application Number	15809815
Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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	1	AMODEO S, et al., "Can we downstage locally advanced pancreatic cancer to resectable? A phase I/II study of induction oxaliplatin and 5-FU chemoradiation," J Gastrointest Oncol. 9(5):922-35 (2018).
	2	Clinical Trials Identifier NCT02551991: 2019-09-30 update, first posted 2015-09-16, "A Randomized, Open-label, Phase 2 Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens Versus Nab-Paclitaxel Plus Gemcitabine in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma." Retrieved from ClinicalTrials.gov archive, 5 printed pages.
	3	MAXWELL F, et al., "CA 19-9 levels in patients with metastatic pancreatic adenocarcinoma receiving first-line therapy with liposomal irinotecan plus 5-fluorouracil/leucovorin and oxaliplatin (NAPOX)," Poster presented at the American Association for Cancer Research (AACR) Special Conference on Pancreatic Cancer: Advances in Science and Clinical Care, September 6-9, 2019, Boston, MA, 7 pages.
	4	WAINBERG Z, et al., "A phase 1/2, open-label, dose-expansion study of liposomal irinotecan (nal-IRI) plus 5-fluorouracil/leucovorin (5-FU/LV) and oxaliplatin (OX) in patients with previously untreated metastatic pancreatic cancer (mPAC)." Presentation presented at the ESMO 21st World Congress on Gastrointestinal Cancer, Barcelona, Spain, July 3-6, 2019, 13 pages.
	5	WAINBERG Z, et al., Abstract SO-005: "A Phase 1/2, Open-Label, Dose-Expansion Study of Liposomal Irinotecan (Nal-IRI) Plus 5-Fluorouracil/Leucovorin (5-FU/LV) and Oxaliplatin (OX) in Patients with Previously Untreated Metastatic Pancreatic Cancer," Ann Oncol. 30(Suppl 4): doi:10.1093/annonc/mdz157 | iv123 (July 2019), 1 page.

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Name/Print	Mary R. Henninger	Registration Number	56992

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Can we downstage locally advanced pancreatic cancer to resectable? A phase I/II study of induction oxaliplatin and 5-FU chemoradiation

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Contributions: (I) Conception and design: T Ryan, HS Hochster, DJ Cohen, A Chandra, HL Pachter, E Newman, M Melis; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: T Ryan, HS Hochster, DJ Cohen, A Chandra, E Newman, M Melis, A Masi; (V) Data analysis and interpretation: E Newman, M Melis, A Masi, S Amodeo; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Background: Half of patients with pancreatic adenocarcinoma (PC) present with regionally advanced disease. This includes borderline resectable and locally advanced unresectable tumors as defined by current NCCN guidelines for resectability. Chemoradiation (CH-RT) is used in this setting in attempt to control local disease, and possibly downstage to resectable disease. We report a phase I/II trial of a combination of 5FU/Oxaliplatin with concurrent radiation in patients presenting with borderline resectable and locally advanced unresectable pancreatic cancer.

Methods: Patients with biopsy-proven borderline resectable or locally advanced unresectable PC were eligible. Chemotherapy included continuous infusion 5FU (200 mg/m^2) daily and oxaliplatin weekly for 5 weeks in dose escalation cohorts, ranging from 30 to 60 mg/m^2. Concurrent radiation therapy consisted of 4,500 cGy in 25 fractions (180 cGy/fx/d) followed by a comedown to the tumor and margins for an additional 540 cGy ×3 (total dose 5,040 cGy in 28 fractions). Following completion of CH-RT, patients deemed resectable underwent surgery; those who remained unresectable for cure but did not progress (SD, stable disease) received mFOLFOX6 ×6 cycles. Survival was calculated using Kaplan-Meier analysis. End-points of the phase II portion were resectability and overall survival.

Results: Overall, 24 subjects (15 men and 9 women, mean age 64.5 years) were enrolled between June 2004 and December 2009 and received CH-RT. Seventeen patients were enrolled in the Phase I component of the study, fifteen of whom completed neoadjuvant therapy. Reasons for not completing treatment included grade 3 toxicities (1 patient) and withdrawal of consent (1 patient). The highest dose of oxaliplatin (60 mg/m^2) was well tolerated and it was used as the recommended phase II dose. An additional 7 patients were treated in the phase II portion, 5 of whom completed CH-RT; the remaining 2 patients did not complete treatment because of grade 3 toxicities. Overall, 4/24 did not complete CH-RT. Grade 4 toxicities related to initial CH-RT were observed during phase I (n=2, pulmonary embolism and lymphopenia) and phase II (n=3, fatigue, leukopenia and thrombocytopenia). Following restaging after completion of CH-RT, 4 patients had progressed (PD); 9 patients had SD and received additional chemotherapy with mFOLFOX6 (one of them had a dramatic response after two cycles and underwent curative resection); the remaining 7 patients (29.2%) were noted to have a response and were explored: 2 had PD, 4 had SD, still unresectable, and 1 patient was resected for cure with negative margins. Overall 2 patients (8.3%) in the study received curative resection following neoadjuvant therapy. Median overall survival for the entire study population was 11.4 months. Overall survival for the two resected patients was 41.7 and 21.6 months.

Conclusions: Combined modality treatment for borderline resectable and locally advanced unresectable

pancreatic cancer with oxaliplatin, 5FU and radiation was reasonably well tolerated. The majority of patients remained unresectable. Survival data with this regimen were comparable to others for locally advanced pancreas cancer, suggesting the need for more novel approaches.

Keywords: Pancreas cancer; radiochemotherapy; neoadjuvant treatment; 5-FU

Submitted Jul 25, 2017. Accepted for publication Sep 22, 2017.
doi: 10.21037/jgo.2017.10.04
View this article at: http://dx.doi.org/10.21037/jgo.2017.10.04

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States (1). The 5-year survival rate of patients with newly diagnosed disease remains about 8% (1). The high incidence of metastatic disease at diagnosis and the relative chemo-resistance of this tumor contribute to this poor survival rate. Long term survival is only possible with curative resection. However, only 15% to 20% of patients present with disease confined to the pancreas at the time of diagnosis and as such deemed resectable (2): approximately 40% have distant metastases, and another 30% to 40% have tumors that extend outside the pancreas, in absence of distant metastases. Two terms are currently used by the National Comprehensive Cancer Network (NCCN) guidelines in order to identify this last category of patients: "borderline resectable" and "locally advanced unresectable disease". The difference between these groups relates to the degree of invasion of the regional vasculature (portal vein-superior mesenteric vein confluence, celiac axis and superior mesenteric artery) by tumor, and to the possibility of performing an adequate vascular resection and reconstruction during the operation (3,4). Upfront surgery in this category of patients is either not technically feasible or likely to lead to microscopically positive margins of resection, which does not seem to confer a survival benefit compared with no resection (5). Therefore, neoadjuvant therapy is the appropriate treatment strategy in this setting, with the purpose of controlling local disease and converting to resectable (4,6,7).

Around the end of the last century and the beginning of the new one, some data was published in favor of the use of induction chemotherapy and radiotherapy followed by surgery in the treatment of pancreatic cancer (8-13). Results, however, were not unanimous, with some studies still reporting no survival benefit and a significant higher toxicity for the combined treatment modality (14). Randomized trials, therefore, were being started to assess the new strategy, as the 2000-01 FFCD/SFRO study (15), evaluating the role of radiation together with 5-FU and cisplatin versus gemcitabine alone, or the intergroup study lead by ECOG, comparing radiation therapy plus gemcitabine with gemcitabine alone (16).

In 1997, NYU had undertaken a phase I/II evaluation of a novel combination of Gemcitabine/Cisplatin combined with radiation in patients with locally advanced unresectable disease. The tested regimen was well tolerated and yielded good tumor control, but was limited in its ability to render locally advanced disease resectable (17).

At that time, oxaliplatin had been identified as a promising new agent, with greater activity compared to cisplatin and a demonstrated *in vitro* cytotoxic effect against pancreatic cancer cell lines (18,19). Furthermore, a synergistic effect of oxaliplatin in combination with 5FU had already been demonstrated, preclinically (20), in metastatic colorectal cancer patients (21), and even in patients with metastatic pancreatic cancer (22). Such a combination, moreover, had been tested in association with radiotherapy in patients with recurrent or locally advanced rectal cancer, and the regimen was well tolerated (23).

Based on promising pre-clinical data and the need for more effective therapy in combination with radiation for loco-regionally advanced pancreatic cancer, we designed a phase I/II study to test the safety and efficacy of combined weekly infusional 5-FU and oxaliplatin with concurrent radiotherapy.

Methods

Eligibility and evaluation

Patients with locoregionally advanced pancreatic carcinoma were enrolled at New York University Medical Center and Bellevue Hospital Center. The protocol (NYU 03-64) was

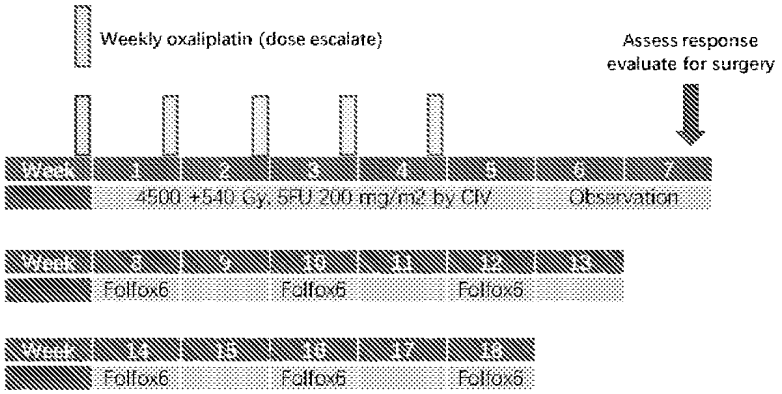


Figure 1 Protocol schema.

approved by the NYU School of Medicine Institutional Review Board (FWA 00004952), which oversees both participating institutions, and written informed consent was obtained for all patients before performing study-related procedures.

Eligibility criteria included pathologic diagnosis of pancreatic adenocarcinoma (PC), locoregionally advanced, non-metastatic disease on computed tomography (CT) imaging, Eastern Cooperative Oncology Group (ECOG) performance status ≤1, and adequate bone marrow, liver and renal function. When the study was initiated, the current NCCN guidelines to define resectability status had not been established yet. Borderline resectable and unresectable diseases were retrospectively defined according to the M.D. Anderson criteria for resectability, which were subsequently incorporated into the guidelines of National Comprehensive Cancer Network (NCCN) (4).

Exclusion criteria included histology other than adenocarcinoma, metastatic disease, prior chemotherapy and/or radiotherapy, active uncontrolled infection, inadequate respiratory, renal, cardiac, hepatic or hematologic organ function, and pregnancy.

Pre-study evaluation and staging included a complete history and physical examination, chest radiograph, blood analysis (complete blood cell count, basic metabolic profile, coagulation profile, and liver function tests), carcinoembryonic antigen and cancer antigen 19-9 levels, and abdomen/pelvis CT or magnetic resonance imaging. Staging laparoscopy was performed in selected cases.

Throughout and after various phases of treatment, patients were followed regularly, through regular reevaluations of ECOG performance status, physical examinations including body weight, and laboratory values. Serial CT or magnetic resonance imaging was used to reevaluate disease stage after administration of chemoradiation (CH-RT) treatment. When feasible, post-treatment follow-up was pursued every 3 months until patient death.

Study regimen and design

Radiation consisted of 4,500 cGy in 25 fractions (180 cGy/fx daily) over 5 weeks, followed by a comedown to the tumor and margins for an additional 540 cGy in 3 fractions, for a total dose of 5,040 cGy in 28 fractions over 5 and a half weeks.

Radiation was combined with 5FU 200 mg/m^2 daily by continuous infusion for 5 weeks and weekly oxaliplatin for 5 weeks in dose escalation cohorts as following: level I =30 mg/m^2; level II =40 mg/m^2; level III =50 mg/m^2; level IV =60 mg/m^2. Following the phase I portion of the trial, a phase II trial at the recommended dose continued.

Two weeks following completion of CRT, patients were re-staged with CT scan. Those considered resectable underwent surgery; those who remained unresectable for cure (stable disease, SD) but did not progress (PD) received a modified FOLFOX6 (Oxaliplatin 85 mg/m^2 administered at day 1 as a 2-hour IV infusion concurrently with Leucovorin 350 mg administered as a 2-hour IV infusion, followed by 5-FU 400 mg/m^2 as an IV bolus, followed by 5-FU 2,400 mg/m^2 as a 46-hour infusion) every 2 weeks for 6 cycles (*Figure 1*).

Throughout treatment, patients were evaluated at least weekly by history, physical examination, and laboratory values to monitor for toxicity.

Toxicity was graded according to the NCI Common Toxicity Criteria (NCI CTC), Version 2.0 (24). Neurosensory toxicity was graded according to the Neurologic Toxicity Scale for Oxaliplatin (25).

Treatment toxicity including gastrointestinal symptoms, fever, fatigue, neutropenia, thrombocytopenia, anemia,

Table 1 Patient characteristics at baseline (n=24)

Characteristics	Value
Sex, n (%)	
Male	15 (62.5)
Female	9 (37.5)
Age (years)	
Mean	64.5
Median	63
Range	46–76
Race, n (%)	
Caucasian	16 (66.7)
Hispanic	4 (16.7)
Asian	2 (8.3)
Black	2 (8.3)
Primary tumor site, n (%)	
Head/neck	16 (66.7)
Body/tail	7 (29.2)
N/A	1 (4.2)
Tumor size at presentation (cm)	
Mean	4.03
Median	3.75
Range	1.50–7.90

and high liver function tests was monitored. Appropriate chemotherapy and/or radiation dose modification was performed accordingly; the dose was held for absolute neutrophil count <500 cells/μL, platelet count <25,000/μL, grade 3–4 non-hematologic toxicity (except neurologic toxicity and grade 3 diarrhea). Treatment was resumed when absolute neutrophil count >1,000/μL, platelets >50,000/μL, resolution of non-hematologic toxicity to grade 2 or less. If toxicity required a dosing delay of more than three weeks from the last planned Oxaliplatin dose, study treatment was discontinued.

Dose-limiting toxicity was defined as: prolonged grade 4 neutropenia or complicated grade 3–4 neutropenia (fever >38.5 °C or sepsis); grade 4 thrombocytopenia or symptomatic grade 3–4 thrombocytopenia (hemorrhage); any other grade 4 toxicity of clinical relevance that is not reduced to grade 1 within 2 days of appropriate therapy.

Primary endpoint for the phase I portion of the study was to determine the safety and the MTD of the combined CIV5FU, oxaliplatin and radiation. Secondary endpoints were rates of R0 resectability and overall survival (OS), calculated from time since first treatment. Primary endpoint for the phase II portion was resectability rate, with secondary outcomes including overall survival and toxicity.

Resectability rate included the proportion of patients who successfully underwent complete surgical resection with microscopically negative margins as a function of all patients treated with CH-RT therapy. Survival was measured from the date of start of treatment to the time of death from any cause.

Statistical analyses

A standard 3+3 cohort dose-escalation design was utilized for the phase I portion of the trial. The recommended phase II dose was defined as the highest dose tested in which none or one patient experienced dose-limiting toxicity attributable to the study drug. At least six patients were treated at the recommended phase II dose.

Overall survival time was illustrated using Kaplan-Meier curves. Descriptive statistics were provided according to the nature of variables. All analyses were performed with SPSS statistical software, version 13.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics

Between June 2004 and December 2009, 15 men and 9 women were enrolled in the study. Demographics and tumor characteristics are reported in *Table 1*. The mean age was 64.5 (range, 46–76) years. A total of 16 cancers arose in the head/neck of the pancreas. The median tumor size at presentation as measured radiographically by computed tomography was 3.75 (range, 1.50–7.90) cm. Thirteen tumors were classified retrospectively as "borderline resectable" and eleven as "unresectable".

Maximum tolerated dose and toxicity

CH-RT

Seventeen patients were enrolled in the phase I portion of the study. They received radiation therapy combined with daily 5-FU and weekly oxaliplatin in 4 dose cohorts. Six patients were included in cohort 1 (oxaliplatin 30 mg/m^2), three patients in cohort 2 (oxaliplatin 40 mg/m^2), three

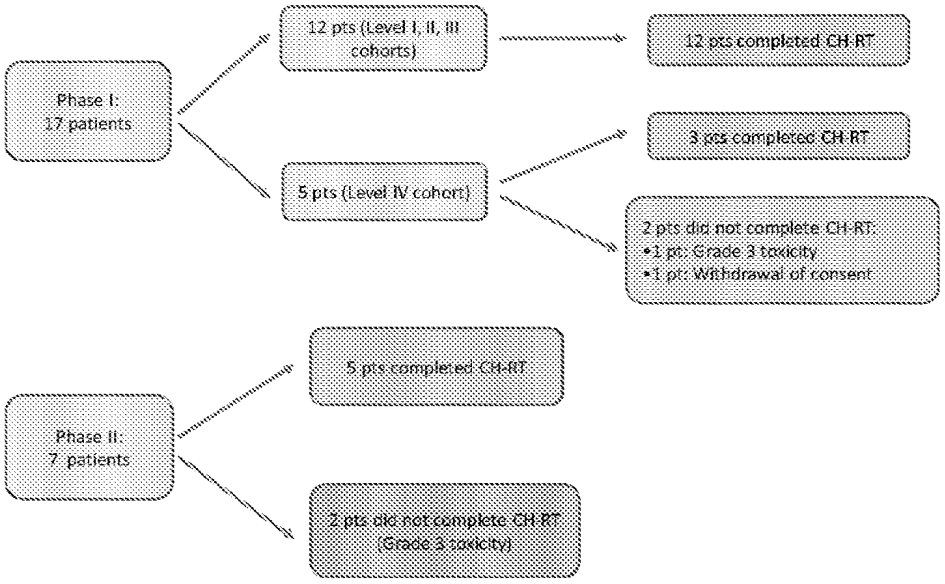


Figure 2 Enrollment and completion of chemo-radiation treatment (CH-RT). Pts, patients.

Table 2 Doses of chemotherapy received per cohort and number of toxicities during chemo-radiation treatment

Dose level	Oxaliplatin (mg/m^2)	n	No. of toxicities			
			Grade I	Grade II	Grade III	Grade IV
I	30	6	47	28	14	1
II	40	3	13	10	4	1
III	50	3	29	8	7	0
IV	60	12	73	40	27	3

patients in cohort 3 (oxaliplatin 50 mg/m^2) and five patients in cohort 4 (oxaliplatin 60 mg/m^2).

All of the 12 patients in the first three cohorts completed 5 weeks of treatment without need for dose reduction. Among the 5 patients in level 4 cohort, three patients completed the treatment, one patient developed grade 3 toxicity (gastritis and dehydration) that mandated interruption of treatment, and one patient withdrew consent for research. The highest dose (60 mg/m^2) of oxaliplatin, thus, was well tolerated and it was therefore carried forward in the phase II portion of the study.

Seven patients were enrolled in the phase II portion of the study and they all received Oxaliplatin at a dose of 60 mg/m^2. One patient developed grade 3 toxicities (mucositis, lymphopenia, fatigue) which did not allow for completion of the 5 weeks. Another patient had to interrupt the treatment because of grade 3 lymphopenia. Overall, 5 patients out of 7 in the phase II portion completed CH-RT.

A schematic of patients' enrollment and number of patients who completed CH-RT treatment is shown in *Figure 2*.

Overall number of toxicities within each cohort and specific grade 3/4 toxicities are summarized in *Table 2* and *Table 3*. Overall, grade 4 toxicities related to initial CH-RT were observed during phase I (n=2, pulmonary embolism and lymphopenia) and phase II (n=3, fatigue, leukopenia and thrombocytopenia).

Folfox6

Fourteen patients started additional chemotherapy with Folfox6: eleven from phase I (of the initial 17) and three from phase II (of the initial 7). As will be described in detail next section, these were patients who had stable, still unresectable disease after CH-RT, either at imaging or at exploratory laparotomy, plus one patient who had regression of disease and received Folfox6 after radical resection.

The eleven phase I patients included four out of

Table 3 Grade 3 and grade 4 toxicities during chemo-radiation treatment

Symptom/sign	Grade 3, n (%)	Grade 4, n (%)
Hematologic		
Lymphopenia	15 (62.5)	1 (4.2)
Neutropenia	3 (12.5)	0
Anemia	2 (8.3)	0
Leukopenia	0	1 (4.2)
Thrombocytopenia	0	1 (4.2)
Non-hematologic		
Dehydration	5 (20.8)	0
Anorexia	4 (16.7)	0
Fatigue	4 (16.7)	1 (4.2)
Hypokalemia	3 (12.5)	0
Nausea	2 (8.3)	0
Elevated serum alkaline phosphatase	2 (8.3)	0
Elevated serum AST	2 (8.3)	0
Cholecystitis	1 (4.2)	0
Diarrhea	1 (4.2)	0
Neuropathy	1 (4.2)	0
Vomiting	1 (4.2)	0
Gastritis	1 (4.2)	0
Hyponatremia	1 (4.2)	0
Hyperglycemia	1 (4.2)	0
Hypernatremia	1 (4.2)	0
Mucositis	1 (4.2)	0
Renal failure	1 (4.2)	0
Pulmonary embolism	0	1 (4.2)

six patients from cohort 1, two of three patients from cohort 2, all three patients from cohort 3 and two of five patients from cohort 4. Nine of these 11 patients were able to complete all 6 cycles. Treatment was stopped in 2 patients, one for clinical progression of disease and one for development of toxicity.

Of the three phase II patients that started Folfox6, none completed the treatment. One patient withdrew consent after 3 cycles. One patient experienced toxicity that precluded continuation of treatment after the second cycle.

Table 4 Grade 3/4 toxicities during treatment with Folfox6

Symptom/sign	Grade 3, n (%)	Grade 4, n (%)
Hematologic		
Neutropenia	4 (28.6)	1 (7.1)
Leukopenia	0	2 (14.3)
Lymphopenia	1 (7.1)	0
Thrombocytopenia	1 (7.1)	0
Non-hematologic		
Hyperglycemia	2 (14.3)	0
Ascites	1 (7.1)	0
Dizziness	1 (7.1)	0
Hiccoughs	1 (7.1)	0
Hypotension	1 (7.1)	0
Hypothermia	1 (7.1)	0
Vertigo	1 (7.1)	0
Hypernatremia	1 (7.1)	0
Hyponatremia	1 (7.1)	0
Elevated serum alkaline phosphatase	1 (7.1)	0
Hypokalemia	1 (7.1)	0
Sepsis	0	1 (7.1)

Finally, the last patient experienced a significant regression during Folfox6 after the second cycle, appeared to have become resectable, and was brought to the operating room for a pancreaticoduodenectomy.

Grade 3/4 toxicities during treatment with Folfox6 are reported in *Table 4*.

Overall, grade 4 toxicities related to additional treatment with Folfox6 were observed twice during phase I (one each leukopenia and sepsis) and twice in phase II (one each leukopenia and neutropenia).

Overall patient response

The overall response of patients to treatment as they progressed through the protocol is outlined in *Figure 3*.

Twenty-four patients began CH-RT treatment. Of those, 20 patients (15 from phase I and 5 from phase II) completed it. Reasons for stopping CH-RT were grade 3 toxicity (n=3) and withdrawal of consent (n=1).

Of the twenty patients who completed the oxaliplatin-

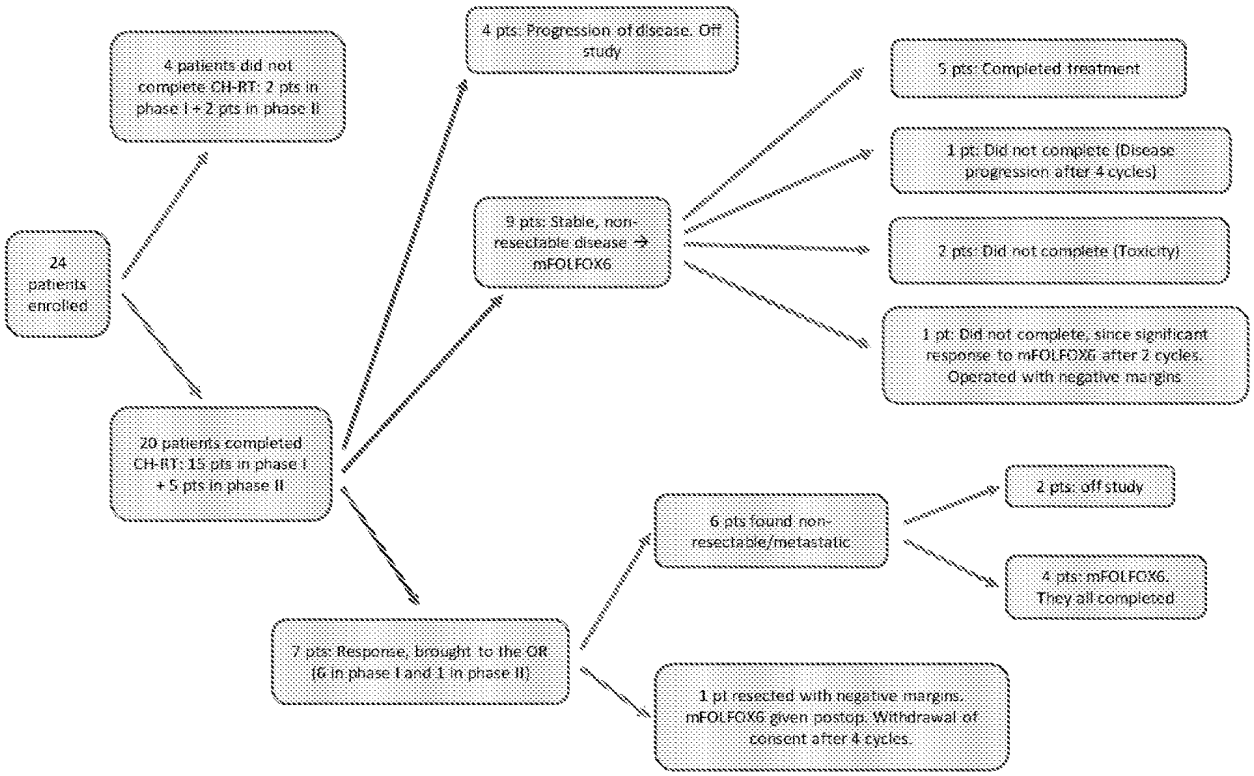


Figure 3 Patient response. CH-RT, chemo-radiation treatment; pts, patients; OR, operating room; mFOLFOX6, modified FOLFOX6.

based CH-RT, at re-staging after CH-RT, 4 patients had progressive disease.

Seven patients (29.2%) were noted to have a response following CH-RT, and were offered exploratory laparotomy and potential resection. Of these seven patients, 5 initially had borderline resectable disease, and 2 had locally advanced unresectable disease. Of these patients, however, 6 were found with non-resectable disease at the time of surgery: 2 with carcinomatosis and 4 with stable, but non-resectable disease. Only 1 patient was found to be resectable at the time of surgery, and received radical resection. This patient had originally presented with a cT2N0M0, 3.8-cm mass of the uncinate process, and was deemed borderline resectable on retrospective analysis, based on current NCCN guidelines (4). Postoperative pathology revealed a 2 cm mass, T1N1M0, resected with negative microscopic margins.

Nine patients had stable, non-resectable disease following CH-RT and received additional chemotherapy with Folfox6. Five completed all 6 cycles of Folfox6; one patient had disease progression after 4 cycles, and two patients stopped their treatment due to toxicity after 2 and 4 cycles, respectively; finally, one patient demonstrated a dramatic tumor response after the second Folfox6 treatment cycle and underwent curative intent resection at another institution. She had originally presented with a cT4N0M0 mass of the head of pancreas, retrospectively deemed borderline resectable. Outside postoperative pathology revealed a T3N0M0 adenocarcinoma, resected with negative microscopic margins.

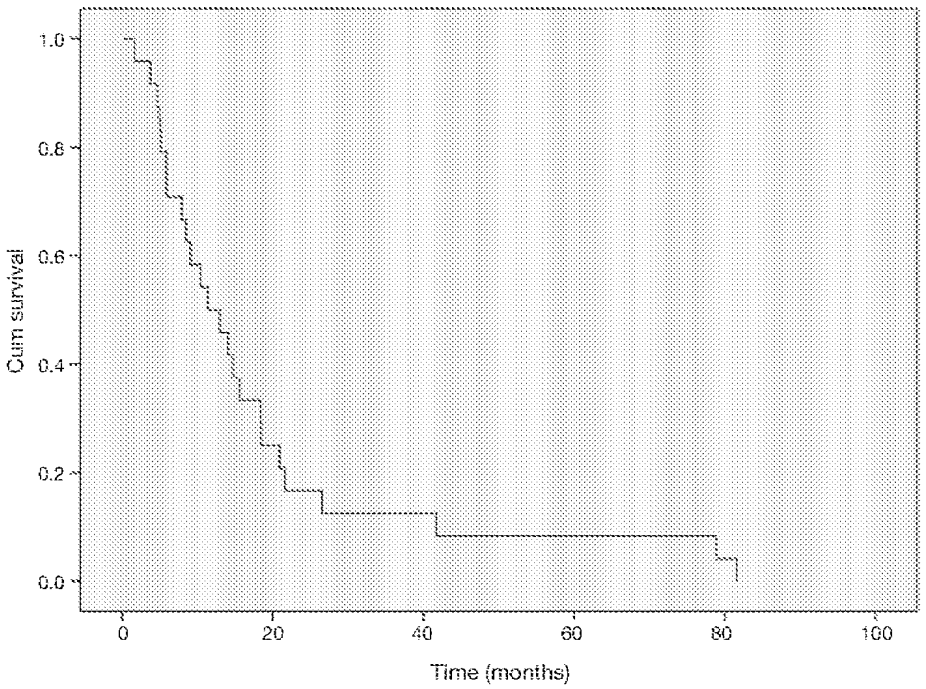
Overall, thus, two patients (8.3%) received curative resection.

Follow-up and survival

There is complete follow up on all patients and all 24 patients have died of disease.

The median survival for the entire study group was 11.4 (range, 1.7–81.6) months (*Figure 4*).

Among the 20 patients who completed CH-RT, median overall survival was 12.9 months. Fourteen of these twenty had stable disease after CH-RT: their median survival was 14.1 months. Six of the 20 who completed CH-RT showed immediate progression or were explored and found to have progression on the basis of carcinomatosis and their median survival was only 9.1 months. The two survival curves are shown in *Figure 5*.



Time (months)	0	5	10	15	20	25	30	35	40	50	60	70	80	90
No. at risk	24	20	14	9	6	4	3	3	3	2	2	2	1	0

Figure 4 Overall survival.

Overall survival for patients initially diagnosed with borderline resectable disease was 11.4 months, while it was 14.1 months for patients originally deemed to have locally advanced unresectable disease. The difference was not significant (*Figure 6*).

The two resected patients had a survival of 41.7 and 21.6 months, respectively. Overall survival in non-resected patients was 10.4 months (*Figure 7*).

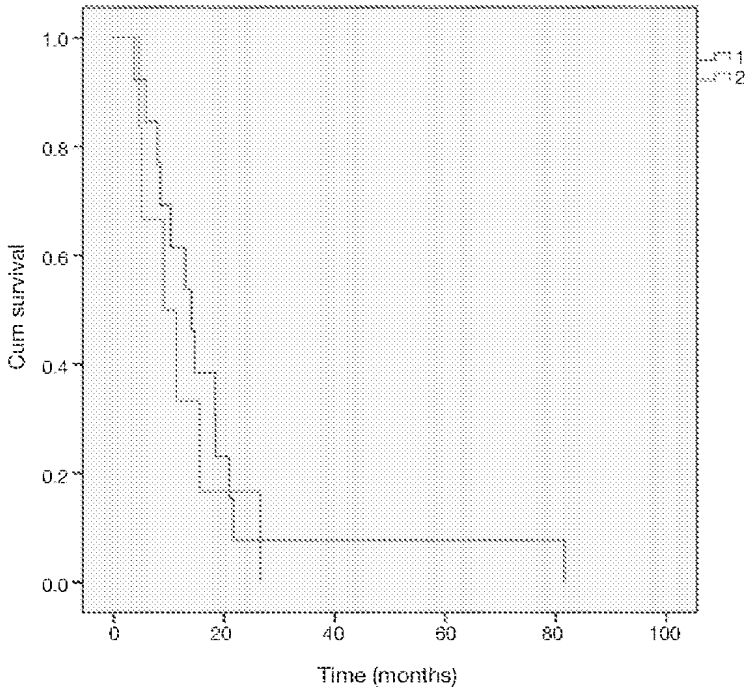
Discussion

The present report demonstrates that combined modality treatment for NCCN-designated borderline resectable and locally advanced unresectable pancreatic cancer with Oxaliplatin, 5-FU and radiation was reasonably well tolerated in our phase I/II study. The majority of patients in the study (91.7%), however, remained unresectable. Of note, two patients who were resected had negative margins on postoperative pathology. Survival data with the tested regimen were comparable to other studies for locally advanced pancreas cancer, with a better outcome, as expected, for those patients who had stable disease or were resected versus those who progressed on study.

At present, surgical resection is the main curative modality for the adenocarcinoma of the pancreas. However, in the setting of NCCN-defined borderline resectable or locally advanced unresectable disease, resection for cure (defined as both gross and microscopically negative margins) is generally not possible. In addition, an R1 surgical resection does not confer a survival benefit compared with no resection (5). The use of neoadjuvant therapy, therefore, is reasonable in for this patient population in order to control local disease, prevent development of metastases and to possibly downstage to a resectable status, thus maximizing the potential for an R0 resection (4).

At the time when our study was initiated, several protocols had already demonstrated the potential benefit for induction chemotherapy and radiation therapy followed by surgery in the treatment of pancreatic cancer (8-12,26,27).

In 1997, NYU undertook a phase I/II evaluation of a novel combination of gemcitabine/cisplatin before and combined with radiation in patients with locally advanced



1= stable disease
2= progression of disease

Time (months)	0	5	10	15	20	25	30	35	40	50	60	70	80	90
No. at risk (stable disease)	14	13	10	6	4	2	2	2	2	1	1	1	1	0
No. at risk (progression of disease)	6	4	3	2	1	2	0	0	0	0	0	0	0	0

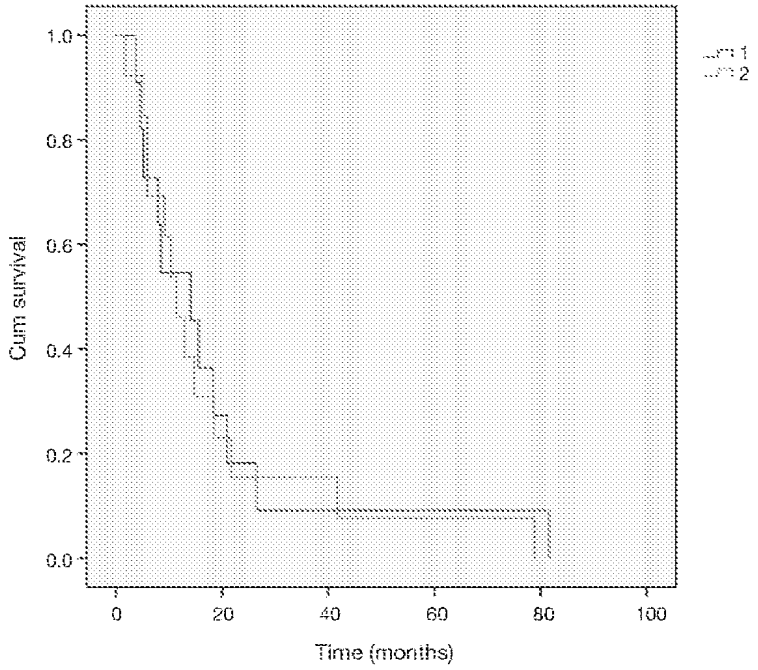
Figure 5 Overall survival (stable disease versus progression of disease).

unresectable pancreatic cancer. The regimen was well tolerated and allowed for good tumor control, although was limited in converting locally advanced disease to resectable (17).

The present trial was conceived with a similar design, but with different agents. Oxaliplatin was chosen for its superior pre-clinical efficacy in pancreatic cancer, its radiosensitizing properties, and its synergistic effect with 5-FU (18-23,28).

In terms of our primary endpoint of toxicity for this regimen, oxaliplatin fared quite well. One of the issues regarding the use of oxaliplatin is its potential neurotoxicity, which is considered its dose-limiting factor. The most common acute side effect is a transient peripheral neurotoxicity characterized by paresthesia and dysesthesia in hands, feet and the perioral area, triggered and/or enhanced by contact with cold. These symptoms, though, are dose-dependent, becoming observable usually at doses of 90 mg/m^2, and, interestingly, disappear within 12 weeks after stopping treatment in 50% of patients and in the majority after 30 weeks (29). The maximum dose used in our study, however, was 60 mg/m^2: only one patient developed grade 3 neuropathy (4.2%).

At doses higher than 45 mg/m^2, oxaliplatin induces nausea and vomiting with rapid onset in the great majority of patients, but this is usually controlled by the standard anti-emetic measures used for all platinum derivatives. We only had two cases of grade 3 nausea (8.3%), and one single case of grade 3 vomiting (4.2%) during induction CH-RT. Furthermore, as expected, no significant renal toxicity was associated with the use of oxaliplatin. The regimen was overall well tolerated, and the most frequent single hematologic toxicity was lymphopenia, with 15 grade 3 episodes (62.5%) and 1 grade 4 episode (4.2%) (overall



1= locally advanced unresectable disease
2= borderline resectable disease

Time (months)	0	5	10	15	20	25	30	35	40	50	60	70	80	90
No. at risk (locally advanced unresectable disease)	11	9	6	5	3	2	1	1	1	1	1	1	1	0
No. at risk (borderline resectable disease)	13	11	8	4	3	2	2	2	2	1	1	1	0	0

Figure 6 Overall survival (borderline resectable versus locally advanced unresectable disease).

grade 3–4: 66.7%). Among non-hematologic toxicities, dehydration, with 5 grade 3 episodes (20.8%), was most common. Other grade 3–4 toxicities are summarized in *Table 3*.

Overall, 20 patients out of 24 completed CH-RT treatment (83.3%).

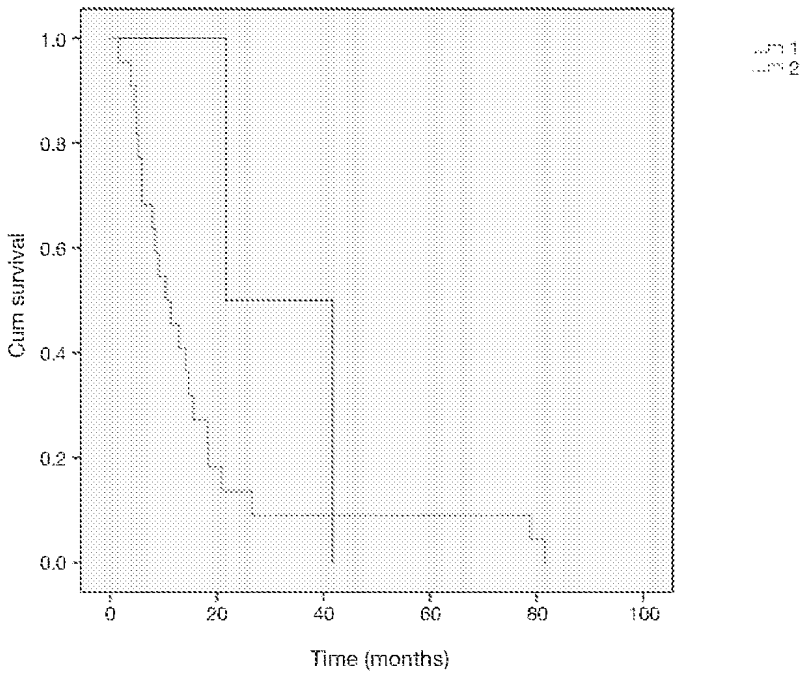
Other studies assessing combination regimens in advanced pancreas cancer with oxaliplatin have reported similar or higher rates of toxicity.

The phase III GERCOR/GISCAD Intergroup trial randomized 326 patients with locally advanced or metastatic unresectable pancreatic cancer to either gemcitabine alone or gemcitabine (1 g/m^2 every 2 weeks) + oxaliplatin (100 mg/m^2 every 2 weeks). It reported an overall good tolerance of the combination therapy, although with a higher incidence of grade 3 and 4 thrombocytopenia, vomiting, neurosensory symptoms, nausea and neutropenia in the Gem/Ox arm (30).

A prospective, phase II clinical trial by Sahora *et al.* evaluated gemcitabine (900 mg/m^2) and oxaliplatin (60 mg/m^2) weekly for patients with locally advanced, non-metastatic pancreatic cancer: the most common toxicities observed were neutropenia/leukopenia (25%) and peripheral neuropathy (18%); diarrhea was described in 4% of patients and vomiting in 14% (31).

Other combination regimens have been reported in advanced diseases that were not oxaliplatin based. Grade 3/4 toxicities were again similar or higher (32,33).

Finally, the use of gemcitabine plus nab-paclitaxel is in the current NCCN guidelines as a first-line therapy option for locally advanced pancreatic cancer (4), although no data have been published to date in this group of patients: all available



1= resected
2= non-resected

Time (months)	0	5	10	15	20	25	30	35	40	50	60	70	80	90
No. at risk resected)	2	2	2	2	2	1	1	1	1	0	0	0	0	0
No. at risk non-resected)	22	11	12	7	4	3	2	2	2	2	2	2	1	0

Figure 7 Overall survival (resected versus non-resected patients).

evidence is derived from patients with metastatic disease. The phase III multinational randomized trial in metastatic disease reported grade 3 or 4 toxicity in the gemcitabine/nab-paclitaxel arm of neutropenia (38%), thrombocytopenia (13%), febrile neutropenia (3%), fatigue (17%), diarrhea (6%), and neuropathy (17%) (34). These data show an overall higher rate of adverse events than our regimen, although data regarding survival are not comparable because of the different type of studied population. A modified regimen of gemcitabine + nab-paclitaxel has recently been proposed for metastatic cancer, and a preliminary report presented at 2015 ASCO Gastrointestinal Cancer Symposium showed similar survival rates with a better grade 3–4 toxicity profile: neutropenia 10%, fatigue 6%, neuropathy 2%, thrombocytopenia 4%, diarrhea 0% (35). However, even with a better toxicity profile, the use of gemcitabine/nab-paclitaxel is costly, currently the highest among the most common regimens (36).

Our survival results are comparable with those of other regimens for treatment of locally advanced pancreatic cancer.

Among those studies which evaluated the use of Gemcitabine in mixed populations of patients with both locally advanced and metastatic disease (30,32,37), the GERCOR/GISCAD trial showed combined therapy to be superior to monotherapy only in terms of response rate (26.8% *vs.* 17.3%), progression-free survival (5.8 *vs.* 3.7 months), but not for median overall survival, which was 9.0 and 7.1 months, respectively (30).

A phase II study by Ishii *et al.*, instead, focusing exclusively on locally advanced pancreatic carcinoma, reported a median overall survival of 15.0 months for 50 patients treated with gemcitabine alone (38).

Thirteen patients (39%) had a curative resection after neoadjuvant therapy with gemcitabine and oxaliplatin in the study by Sahora *et al.*: median overall survival of patients undergoing resection was 22 months, as opposed to 12 months for those without resection (31).

In the last few years, combination chemotherapy with

FOLFIRINOX has become a standard regimen in metastatic disease based on randomized data (39), for patients with a good performance status, a favorable comorbidity profile, and a support system to permit aggressive medical therapy. Although it appears that objective response rates in the primary tumor are at least as good as they are in metastatic disease, few data and no randomized studies are today available for locally advanced unresectable disease (40-46).

However, a systematic review and meta-analysis of studies for locally advanced pancreatic cancer patients treated with FOLFIRINOX has recently been published in which 490 patients across ten studies were included: the median overall survival was of 24.2 (range, 10.0–32.7) months; the proportion of patients who underwent surgical resection ranged from 0% to 43%, with a pooled proportion of 25.9%. The pooled proportion of patients who had R0 resection of those who underwent resection was 78.4% (47).

With respect to borderline resectable disease, no randomized data exist. The use of neoadjuvant therapy in patients with borderline resectable disease has been evaluated only in small single institution series and small phase II trials. The largest single institution series included 160 patients; of these, 125 (78%) completed preoperative therapy and restaging, and 66 (41%) underwent pancreatectomy. Sixty-two patients (94%) had a margin-negative postoperative pathology; at a median follow-up of 27 months, the median survival durations for the unresectable and resectable cohorts were 13 and 40 months, respectively (48). Interestingly, a 2011 meta-analysis of phase II trials testing a variety of neoadjuvant strategies concluded that approximately one third of tumors initially considered marginal for resection were able to be ultimately resected after neoadjuvant treatment and that the median survival in this group was 22.3 (range, 18–26) months (49). In general, current recommendations from NCCN in the setting of borderline resectable disease are for an initial attempt at neoadjuvant therapy, followed by restaging and surgical exploration in the absence of metastatic disease, rather than upfront surgery (4). This is particularly appropriate, when considering that borderline resectable tumors are usually more likely to have a curative resection as opposed to a truly defined locally advanced unresectable disease (17). In fact, both patients who underwent curative resection in our study were originally classified as borderline resectable.

Overall, despite its limited size, our phase I/II trial has demonstrated that the addition of 5-FU and oxaliplatin to radiation therapy in the neoadjuvant setting for patients with locally advanced unresectable and borderline resectable pancreatic tumor is feasible and well tolerated. However, the greater majority of patients remained unresectable after treatment, and survival rates were comparable to those shown by other reports about different therapy combinations. Thus, more novel approaches will have to be tested in order to truly improve outcomes in patients with locally advanced unresectable and borderline resectable disease.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The protocol (NYU 03-64) was approved by the NYU School of Medicine Institutional Review Board (FWA 00004952), which oversees both participating institutions, and written informed consent was obtained for all patients before performing study-related procedures.

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Cite this article as: Amodeo S, Masi A, Melis M, Ryan T, Hochster HS, Cohen DJ, Chandra A, Pachter HL, Newman E. Can we downstage locally advanced pancreatic cancer to resectable? A phase I/II study of induction oxaliplatin and 5-FU chemoradiation. J Gastrointest Oncol 2018;9(5):922-935. doi: 10.21037/jgo.2017.10.04

Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens Versus Nab-paclitaxel Plus Gemcitabine in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma

ClinicalTrials.gov Identifier: NCT02551991

Recruitment Status : Active, not recruiting
First Posted : September 16, 2015
Last Update Posted : September 30, 2019

Sponsor:
Ipsen

Information provided by (Responsible Party):
Ipsen

Study Description

Go to

Brief Summary:

This is an open-label, phase 2 comparative study to assess the safety, tolerability, and preliminary efficacy of nal-IRI in combination with other anticancer therapies in patients not previously treated for metastatic pancreatic adenocarcinoma. This study will assess the following regimen:

• nal-IRI + 5-fluorouracil (5-FU)/leucovorin (LV) + oxaliplatin

The study will be conducted in two parts:

1. Part 1a: a safety run-in as initial dose exploration
2. Part 1b: dose expansion of the nal-IRI + 5FU/LV + oxaliplatin regimen

Condition or disease	Intervention/treatment	Phase
Pancreatic Cancer	Drug: nal-IRI Drug: 5 fluorouracil Drug: Leucovorin Drug: Oxaliplatin	Phase 1 Phase 2

Study Design

Go to

Study Type : Interventional (Clinical Trial)

Actual Enrollment :	56 participants
Intervention Model:	Single Group Assignment
Masking:	None (Open Label)
Primary Purpose:	Treatment
Official Title:	A Randomized, Open-label Phase 2 Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens Versus Nab-Paclitaxel Plus Gemcitabine in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma
Study Start Date :	September 2015
Estimated Primary Completion Date :	February 28, 2020
Estimated Study Completion Date :	February 28, 2020

Resource links provided by the National Library of Medicine NLM

Genetic and Rare Diseases Information Center resources: Pancreatic Cancer

U.S. FDA Resources

Arms and Interventions

Go to

Arm	Intervention/treatment
Experimental: nal-IRI + 5-FU/LV + oxaliplatin	Drug: nal-IRI Other Name: MM-398 Drug: 5 fluorouracil Other Name: 5-FU Drug: Leucovorin Drug: Oxaliplatin

Outcome Measures

Go to

Primary Outcome Measures :

1. Safety by reporting the adverse events and serious adverse events [Time Frame: Up to 18 months]
2. Determine dose limiting toxicities (DLT) [Time Frame: Up to 28 Days post first treatment]

   For nal-IRI administered in combination with 5-FU/LV and oxaliplatin, the following adverse events will be considered as dose limiting toxicities (DLTs) if the following adverse events occur within 28 days of Cycle 1 or 14 days after Cycle 2 of treatment and are deemed related to the study treatment regimen:

Secondary Outcome Measures :

1. Pharmacokinetic Cmax of total irinotecan, SN-38 and oxaliplatin [Time Frame: Day 1 Predose, Day 1 at 1.5 hours (irinotecan and SN-38 only), Day 1 at 5.5 hours, Day 3, Day 8, Day 15, Day 30 Post last dose]

   Cmax (Observed maximal (peak) concentration)

2. Pharmacokinetic Cavg of total irinotecan, SN-38 and oxaliplatin [Time Frame: Day 1 Predose, Day 1 at 1.5 hours (irinotecan and SN-38 only), Day 1 at 5.5 hours, Day 3, Day 8, Day 15, Day 30 Post last dose]

   Cavg (Average plasma concentration)

3. Pharmacokinetic Cmin of total irinotecan, SN-38 and oxaliplatin [Time Frame: Day 1 Predose, Day 1 at 1.5 hours (irinotecan and SN-38 only), Day 1 at 5.5 hours, Day 3, Day 8, Day 15, Day 30 Post last dose]

   Cmin (Minimum plasma concentration)

4. Pharmacokinetic AUCt of total irinotecan, SN-38 and oxaliplatin [Time Frame: Day 1 Predose, Day 1 at 1.5 hours (irinotecan and SN-38 only), Day 1 at 5.5 hours, Day 3, Day 8, Day 15, Day 30 Post last dose]

   AUCt (Area under the plasma concentration time curve within a dosage interval (0 to last measurable concentration))

5. Pharmacokinetic CL of total irinotecan, SN-38 and oxaliplatin [Time Frame: Day 1 Predose, Day 1 at 1.5 hours (irinotecan and SN-38 only), Day 1 at 5.5 hours, Day 3, Day 8, Day 15, Day 30 Post last dose]

   CL (apparent total body clearance of the drug from plasma after administration)

6. Pharmacokinetic Vd of total irinotecan, SN-38 and oxaliplatin [Time Frame: Day 1 Predose, Day 1 at 1.5 hours (irinotecan and SN-38 only), Day 1 at 5.5 hours, Day 3, Day 8, Day 15, Day 30 Post last dose]

   Vd (apparent volume distribution after administration)

7. Progression Free Survival (PFS) [Time Frame: up to 16 weeks post first treatment]
8. Overall Survival (OS) [Time Frame: up to 16 weeks post first treatment]

   Duration from the date of enrolment/randomization to the time of death.

9. Overall Response Rate (ORR) [Time Frame: up to 16 weeks post first treatment]

   Proportion of patients with Best overall response (BOR) characterized as either a Complete or Partial Response (CR or PR) relative to the total number of evaluable patients.

10. Disease Control Rate (DCR) [Time Frame: up to 16 weeks post first treatment]
11. Safety and adverse event profile [Time Frame: up to 18 months]

    The incidence of adverse events will be summarized by type of adverse event and severity. All patients who have received at least one dose of irinotecan liposome injection will be included in the safety analysis according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI CTCAE)

Eligibility Criteria

Go to

Information from the National Library of Medicine NLM

Choosing to participate in a study is an important personal decision. Talk with your doctor and family members or friends about deciding to join a study. To learn more about this study, you or your doctor may contact the study research staff using the contacts provided below. For general information, Learn About Clinical Studies.

Ages Eligible for Study:	18 Years and older (Adult, Older Adult)
Sexes Eligible for Study:	All
Accepts Healthy Volunteers:	No

Criteria

Inclusion Criteria:

- Histologically or cytologically confirmed adenocarcinoma of the pancreas that has not been previously treated in the metastatic setting

- Unresectable, locally advanced or metastatic disease; diagnosed within 6 weeks prior to screening
- At least one tumor lesion measurable by CT or MRI scan (according to RECIST v1.1)
- ECOG performance status of 0 or 1 at screening and within 72 hours prior to first dose if first dose occurs more than 72 hours post-screening
- Adequate hematological, hepatic, renal and cardiac function
- Recovered from the effects of any prior surgery or radiotherapy
- Patient has a Karnofsky performance status (KPS) ≥ 70 at Screening, and within 72 hours prior to date of first dose if first dose occurs more than 72 hours after screening (Part 1B only)

Exclusion Criteria:

- Prior treatment of pancreatic cancer in the metastatic setting (or locally advanced setting) with surgery (placement of stent is allowed), radiotherapy, chemotherapy or investigational therapy
- Prior treatment of pancreatic cancer with chemotherapy in adjuvant setting, except those where at least 12 months have elapsed since completion of the last dose and no persistent treatment-related toxicities present
- Uncontrolled Central Nervous System (CNS) metastases
- Clinically significant gastrointestinal disorder
- History of any second malignancy in the last 3 years. Patients with prior history of in-situ cancer or basal or squamous cell skin cancer are eligible
- Presence of any contraindications for nal-IRI, irinotecan, 5-FU, leucovorin, oxaliplatin
- Use of strong CYP3A4 or inducers or presence of any other contra indications for irinotecan
- Pregnant or breast feeding
- Neuroendocrine or acinar pancreatic carcinoma
- Serum albumin < 3 g/dL at screening visit and within 72 hours prior to first dose if first dose occurs more than 72 hours post screening
- Patients with symptoms and signs of clinically unacceptable deterioration of primary disease at time of screening
- Previous treatment with irinotecan-based, nab-paclitaxel-based or gemcitabine-based resulting in disease progression

Contacts and Locations

Go to

Information from the National Library of Medicine NLM

To learn more about this study, you or your doctor may contact the study research staff using the contact information provided by the sponsor.

Please refer to this study by its ClinicalTrials.gov identifier (NCT number): ***NCT02551991***

Show 24 Study Locations

Sponsors and Collaborators

Ipsen

Investigators

Study Director: Ipsen Medical Director Ipsen

More Information

Go to

Publications automatically indexed to this study by ClinicalTrials.gov Identifier (NCT Number):

Liu X, Jiang J, Chan R, Ji Y, Lu J, Liao YP, Okene M, Lin J, Lin P, Chang CH, Wang X, Tang I, Zheng E, Qiu W, Wainberg ZA, Nel AE, Meng H. Improved Efficacy and Reduced Toxicity Using a Custom-Designed Irinotecan-Delivering Silicasome for Orthotopic Colon Cancer. ACS

Nano. 2019 Jan 22;13(1):38-53. doi: 10.1021/acsnano.8b06164. Epub 2018 Dec 11.

Responsible Party:	Ipsen
ClinicalTrials.gov Identifier:	NCT02551991 History of Changes
Other Study ID Numbers:	MM-398-07-02-03
First Posted:	September 16, 2015 Key Record Dates
Last Update Posted:	September 30, 2019
Last Verified:	September 2019

Keywords provided by Ipsen:

- Pancreatic cancer
- MM-398
- Metastatic pancreatic cancer
- First line pancreatic cancer treatment

Additional relevant MeSH terms:

- Adenocarcinoma
- Pancreatic Neoplasms
- Carcinoma
- Neoplasms, Glandular and Epithelial
- Neoplasms by Histologic Type
- Neoplasms
- Digestive System Neoplasms
- Neoplasms by Site
- Endocrine Gland Neoplasms
- Digestive System Diseases
- Pancreatic Diseases
- Endocrine System Diseases
- Gemcitabine
- Paclitaxel
- Albumin-Bound Paclitaxel
- Oxaliplatin
- Irinotecan
- Fluorouracil
- Antineoplastic Agents, Phytogenic
- Antineoplastic Agents
- Tubulin Modulators
- Antimitotic Agents
- Mitosis Modulators
- Molecular Mechanisms of Pharmacological Action
- Antimetabolites, Antineoplastic
- Antimetabolites
- Antiviral Agents
- Anti-Infective Agents
- Enzyme Inhibitors
- Immunosuppressive Agents

CA 19-9 levels in patients with metastatic pancreatic adenocarcinoma receiving first-line therapy with liposomal irinotecan plus 5-fluorouracil/leucovorin and oxaliplatin (NAPOX)

Fiona Maxwell,[1] Fanthei Dayyani,[2] Patrick M. Boland,[3] Andrew Dean,[4] Christopher H. Lieu,[5] Teresa Macarulla,[6] Bin Zhang,[7] Bruce Bellinger,[7] Yan Moore,[7] Tiffany Wang,[7] Zev A. Wainberg[8]

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BACKGROUND

- Pancreatic cancer has an extremely poor prognosis; the 5-year survival rate is 8% and, for patients with metastatic pancreatic adenocarcinoma (mPAC), the median survival rate is less than 1 year.[1,2]
- In the USA, in 2018, pancreatic cancer was the fourth leading cause of cancer death after lung, colon and prostate/breast cancer.[1]
  - New treatment options are needed, as well as research into novel and predictive markers to help to manage the disease.[2]
- Liposomal irinotecan (nal-IRI) is a liposomal encapsulation of the topoisomerase 1 inhibitor irinotecan.
  - The half-life of total irinotecan after administration of nal-IRI is 25.8 hours.
  - 95% of irinotecan remains within the liposome during circulation.[4]
  - The ratio between total and encapsulated forms does not change in the 169.5 hours after the dose is given.
  - A five fold higher level of drug is found in tumors than in plasma at 72 hours, suggesting metabolic activation of irinotecan.
- nal-IRI+5-fluorouracil/leucovorin (5-FU/LV) is approved in the USA for the treatment of mPAC after disease progression following gemcitabine-based therapy,[3] based on findings from the NAPOLI-1 trial.
  - In the NAPOLI-1 trial, the combination of nal-IRI+5-FU/LV significantly prolonged overall survival compared with 5-FU/LV treatment alone in patients with mPAC.[4]
- The combination of nal-IRI+5-FU/LV and oxaliplatin (NAPOX) is being investigated as first-line treatment for patients with mPAC in a phase 1/2 dose-exploration and dose-expansion study (NCT02551991) to determine the most appropriate dose for phase 3 studies.[5]

OBJECTIVE

- The aim of this exploratory analysis of NCT02551991 was to investigate changes in the levels of carbohydrate antigen 19-9 (CA 19-9), a pancreatic cancer biomarker,[6] and their association with response rates in patients with mPAC receiving NAPOX.

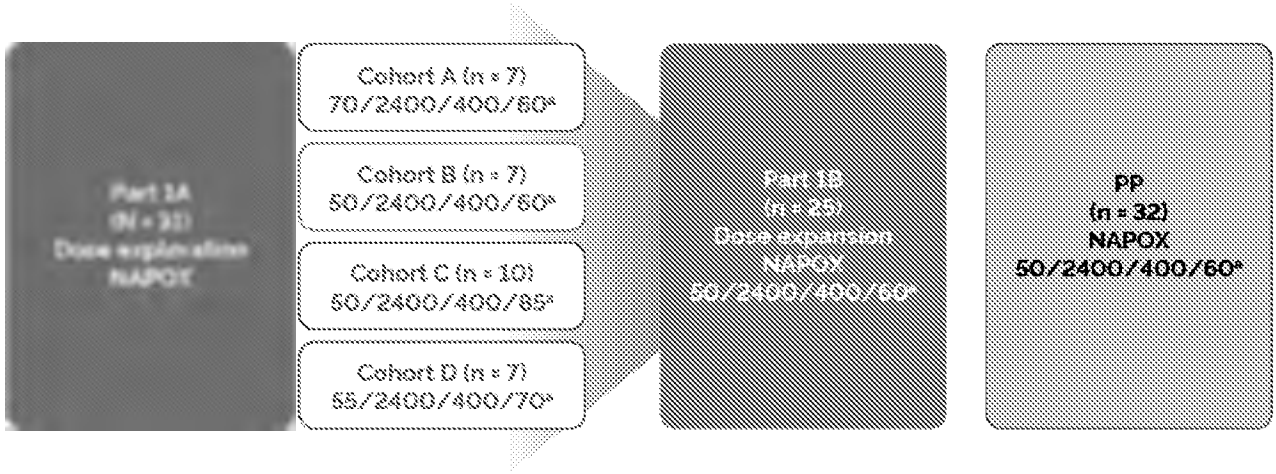
METHODS

Study design

- NCT02551991 is an open-label comparative study that is being conducted in 15 sites across the USA (10), Spain (4) and Australia (1) (Figure 1).
- A dose-exploration (part 1A) safety run-in (traditional 3+3 design) was performed to identify an appropriate dosing regimen for NAPOX in the dose-expansion phase.
- The regimen selected for the dose-expansion phase (cohort B) was nal-IRI 50 mg/m² (free-base equivalent), 5-FU 2400 mg/m², LV 400 mg/m², and oxaliplatin 60 mg/m², administered on days 1 and 15 of each 28-day cycle.
  - Overall, 7 patients were receiving NAPOX according to the selected regimen during the exploration phase (cohort B) and 25 were enrolled into the dose-expansion phase, giving a total of 32 patients receiving treatment according to the selected regimen (pooled population [PP]).

Presented at the American Association for Cancer Research (AACR) Special Conference on Pancreatic Cancer: Advances in Science and Clinical Care, September 6–9, 2019, Boston, MA, USA

Figure 1. Study design and numbers of patient cohorts



ªDoses of nal-IRI/5-FU/LV/oxaliplatin, respectively, in mg/m².
5-FU, 5-fluorouracil; LV, leucovorin; nal-IRI, liposomal irinotecan; NAPOX, nal-IRI+5-FU/LV and oxaliplatin; PP, pooled population.

- Treatment is continued until disease progression or intolerable toxicity, or at the treating physician's discretion. Final safety assessments will be completed 30 days after the last dose of study treatment.

Key eligibility criteria

- Adults (≥ 18 years of age).
- Pathologically confirmed adenocarcinoma of the pancreas not previously treated in the metastatic setting.
- Unresectable, locally advanced or metastatic disease diagnosed in the 6 weeks prior to enrollment.
- At least one tumor lesion measurable by computed tomography or magnetic resonance imaging scan (according to Response Evaluation Criteria in Solid Tumors [RECIST] v1.1 criteria).
- Eastern Cooperative Oncology Group performance status of 0 or 1.

Dosing cohort B and the dose-expansion cohort

- All patients were premedicated with dexamethasone and a 5-hydroxytryptamine (5-HT_3) antagonist (e.g. ondansetron or granisetron) or equivalent.
- NAPOX was administered on days 1 and 15 of each 28-day cycle according to the following regimen:
  - nal-IRI, dose range of 50 mg/m² to 85 mg/m² intravenously (i.v.) over 90 minutes (± 10 minutes)
  - 5-FU, fixed dose of 2400 mg/m² i.v. over 46 hours (± 60 minutes)
  - LV (L + D racemic form), fixed dose of 400 mg/m² i.v. over 30 minutes (± 5 minutes)
  - Oxaliplatin, intended dose levels of 60 mg/m² to 85 mg/m² i.v. over 120 minutes (± 10 minutes).

Present analysis

- The current study is based on an interim analysis, which was performed after all patients in the dose-expansion cohort completed their second scheduled tumor evaluation at week 16 (with a data cut-off of February 19, 2019).
- The study includes patients in the PP (cohort B/dose-expansion cohort).
- CA 19-9 levels (assessed at baseline and every 8 weeks) are reported up to week 16 of treatment.
- Clinical responses (based on tumor evaluations by RECIST v1.1; assessed every 8 weeks) reported in this analysis include:
  - disease control rate by week 16 (DCR16)
  - overall response rate (ORR; complete response or partial response over entire follow-up period).

RESULTS

Baseline characteristics

- All patients had a baseline diagnosis of stage III or IV mPAC at study entry (Table 1).

Table 1. Clinical characteristics at baseline

	All patients (PP) (n = 32)
Median age, years (range)	62 (39–76)
< 65 years, n (%)	23 (71.9)
Male, n (%)	14 (43.8)
White, n (%)	28 (87.5)
Baseline stage at diagnosis, n (%)[a]	
III	3 (9.4)
IV	29 (90.6)
Baseline metastatic location, n (%)[b]	
Liver	20 (62.5)
Lung	4 (12.5)
Neck nodes	1 (3.1)
Pancreas	2 (6.3)
Other	13 (59.4)
Baseline ECOG performance status, n (%)	
Fully active (score = 0)	14 (43.8)
Restricted activity (score = 1)	18 (56.3)

[a]One patient in the dose-expansion cohort received a diagnosis of stage IIA at baseline but entered the treatment phase as stage IV.
[b]Patients could have metastatic lesions in multiple locations.
ECOG, Eastern Cooperative Oncology Group; PP, pooled population.

CA 19-9 levels at baseline and through week 16

- Overall, 30 of 32 patients in the PP had CA 19-9 data available at baseline. The median CA 19-9 level at baseline was 316 U/mL, and 23 patients (77% of patients with CA 19-9 data available) had baseline CA 19-9 levels above the normal level (over 37 U/mL) (Table 2).
- Among patients with baseline CA 19-9 levels over 37 U/mL and measurements up to week 16 (n = 17), the median best reduction from baseline in CA 19-9 was 35.9% (Table 2).

Clinical response

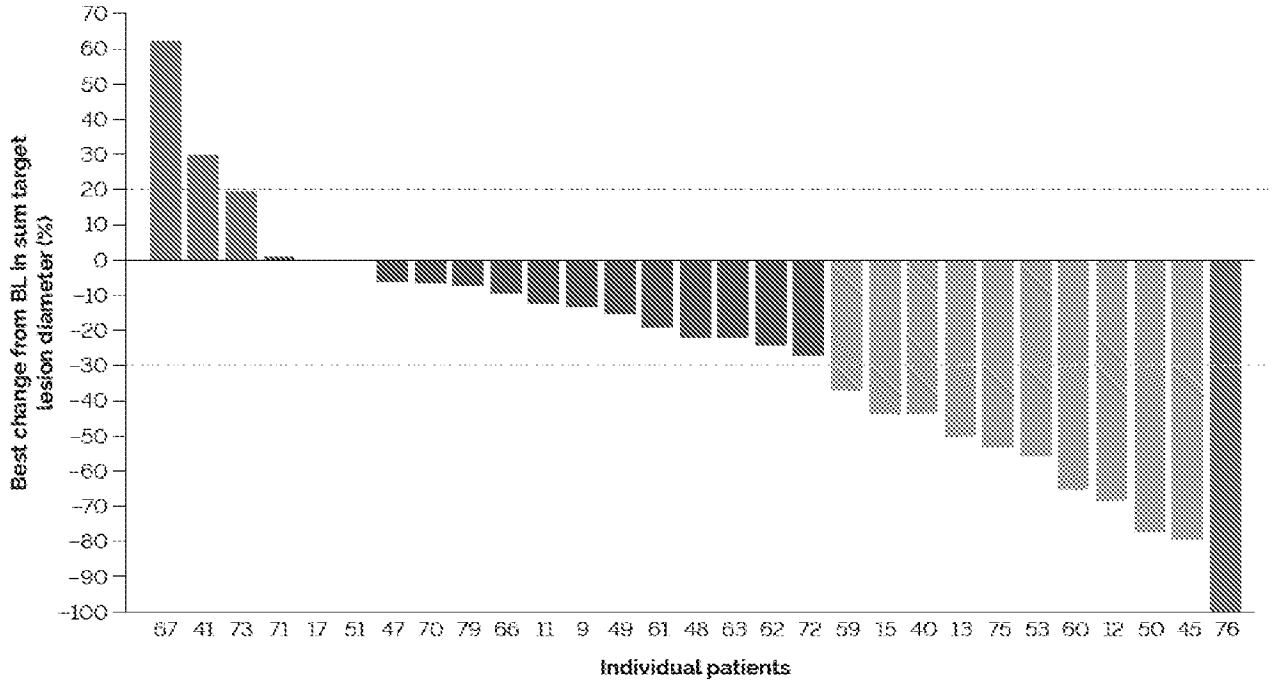
- A total of 72% of patients (23/32) achieved DCR16, and the ORR was 34% (11/32) (Figures 2 and 3).
- In all patients with any reduction in CA 19-9 by week 16, the DCR16 rate was 88% (14/16) and the ORR was 63% (10/16) (Figure 3).
- Among patients with baseline CA 19-9 levels over 37 U/mL and any CA 19-9 reduction by week 16, the DCR16 rate was 83% (10/12) and the ORR was 67% (8/12).
- Among patients with baseline CA 19-9 levels over 37 U/mL, the rates of DCR16 and ORR increased with week 16 CA 19-9 reductions, reaching 100% and 75%, respectively, among those achieving a CA 19-9 decrease of 70% or more (Figure 3).

Table 2. Measures of CA 19-9 at baseline and up to week 16 in all patients and in patients with CA 19-9 levels above normal

	All patients (PP) (n = 32)	Patients with above normal BL CA 19-9 (n = 23)
Patients with CA 19-9 value at baseline, n (%)	30 (94)	23 (100)
Baseline CA 19-9 U/mL, median (min, max)	313.5 (2, 127 115)	1265.0 (38, 127 115)
Patients with CA 19-9 value at baseline and up to week 16, n (%)	23 (77)	17 (74)
Best change in CA 19-9 from baseline, %, median (min, max)	-48.7 (-100[a], 362)	-35.9 (-100[a], 275)
Change in CA 19-9 category, n (%[b])		
No change or increase	7 (30)	5 (29)
Any decrease	16 (70)	12 (71)
≥ 20% decrease	14 (61)	10 (59)
≥ 50% decrease	11 (48)	7 (41)
≥ 70% decrease	6 (26)	4 (24)

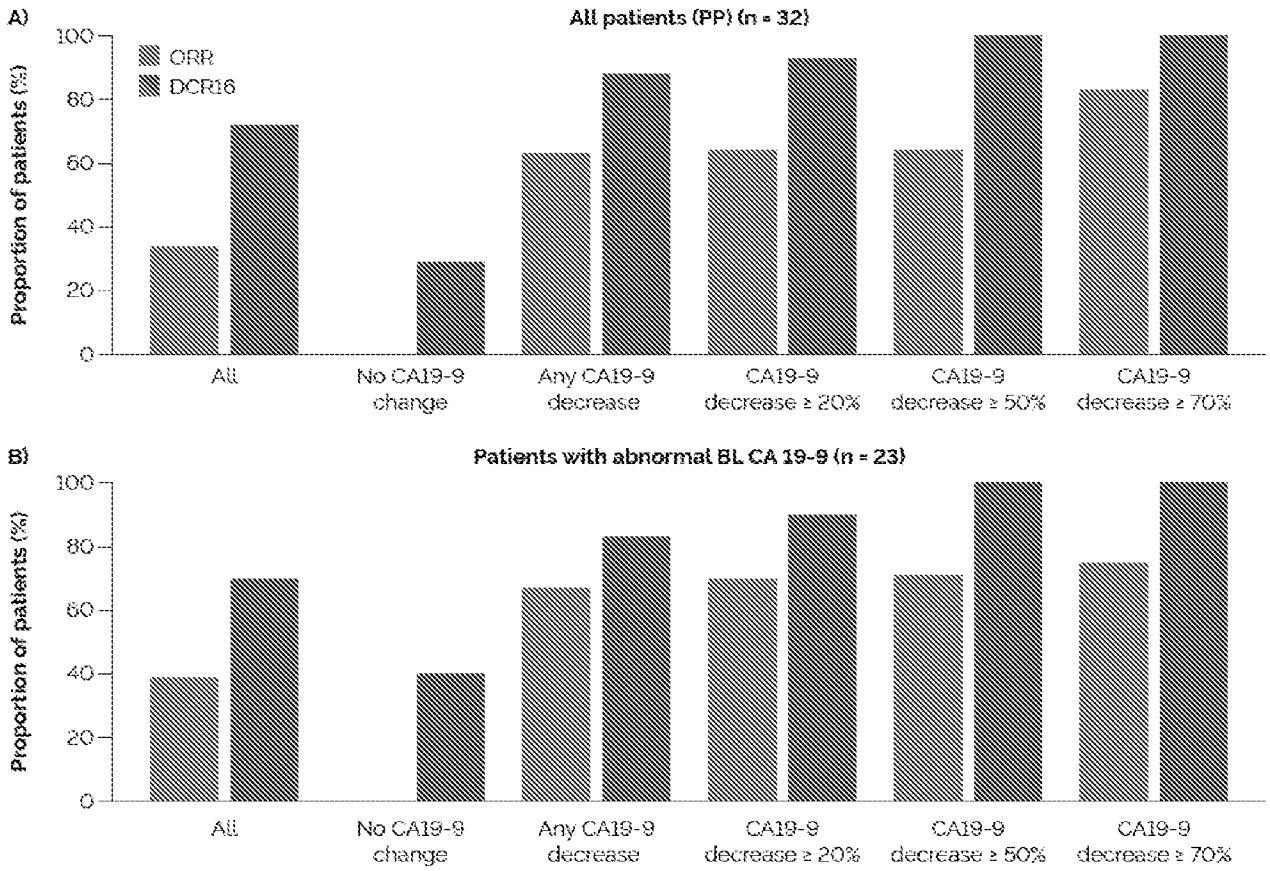
[a]Rounded from -99.8% (based on one patient who had a reduction from 50 392 U/mL to 56 U/mL).
[b]Based on number of patients with CA 19-9 values at baseline and up to week 16.
BL, baseline; CA 19-9, carbohydrate antigen 19-9; PP, pooled population.

Figure 2. Best change from baseline in sum target lesion diameter (evaluable patients in PP; n = 29)



BL, baseline; PP, pooled population.

Figure 3. Treatment responses in A) all patients and B) patients with CA 19-9 levels above normal at baseline



BL, baseline; CA 19-9, carbohydrate antigen 19-9; DCR16, disease control rate by week 16; ORR, overall response rate; PP, pooled population.

CONCLUSIONS

- In patients with mPAC, first-line NAPOX therapy (with a dosing schedule of nal-IRI 50 mg/m² (free-base equivalent), 5-FU 2400 mg/m², LV 400 mg/m², and oxaliplatin 60 mg/m² administered on days 1 and 15 of each 28-day cycle) resulted in substantial reductions in CA 19-9 levels from baseline, indicative of clinically meaningful antitumor activity.
- Response rates were higher in patients with large CA 19-9 reductions than in those with small CA19-9 reductions. Further assessment of CA19-9 levels and response rates over a longer follow-up period is ongoing.

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Disclosures

FM, BZ, BB, YM and TW are employees of Ipsen.
FD has acted as a consultant to Array, Eisai and Genentech, and has taken part in a speaker's bureau for Amgen, Eisai, Ipsen and Sirtex (now Genentech).
PMB has received research funding (to institute) from Boehringer Ingelheim, Boston Biomedical, Clinical Genomics, Ipsen, Isofol, Kinex (now Athenex), Merck, Seattle Genetics and Taiho Pharma.
AD has acted as a non-paid consultant to Shire and Specialised Therapeutics Australia, and has received a travel grant from Amgen.
CHL has nothing to disclose.
TM has received honoraria for consultancy from Baxter, Baxalta, Bayer, Celgene, Genzyme, Genzyme Europe, Incyte, QED Therapeutics, Roche, Sanofi, Shire and Tesaro, and has received support for travel or accommodation from Bayer, HG Biomedicine, Merck and Sanofi.
ZAW has acted as a consultant to Bayer, Bristol-Myers Squibb, Ipsen, Lilly, Merck and Novartis.

Acknowledgments

The authors thank all patients involved in the study, as well as their caregivers, care team, investigators and research staff in participating institutions.

The study was supported by Ipsen. The authors thank Oxford PharmaGenesis, Oxford, UK, for providing medical writing support, which was sponsored by Ipsen in accordance with Good Publication Practice guidelines.

B14

A phase 1/2, open-label, dose-expansion study of liposomal irinotecan (nal-IRI) plus 5-fluorouracil/leucovorin (5-FU/LV) and oxaliplatin (OX) in patients with previously untreated metastatic pancreatic cancer (mPAC)

Zev Wainberg,[1] Patrick Boland,[2] Christopher H. Lieu,[3] Farshid Dayyani,[4] Teresa Macarulla,[5] Bin Zhang,[6] Bruce Belanger,[6] Yan Moore,[6] Tiffany Wang,[6] Fiona Maxwell,[7] Andrew Dean[8]

[1] University of California Los Angeles, Los Angeles, California, USA; [2] Roswell Park Cancer Institute, Buffalo, New York, USA; [3] University of Colorado, Denver, Colorado, USA; [4] University of California, Irvine, Irvine, California, USA; [5] Hospital Universitari Vall d'Hebron, Barcelona, Spain; [6] Ipsen Biopharma, Cambridge, MA, USA; [7] Ipsen Biopharmaceuticals, Slough, UK; [8] Department of Oncology, Mt Saint Antoine Hospital, Nedlands, WA, Australia

This study is funded by Ipsen

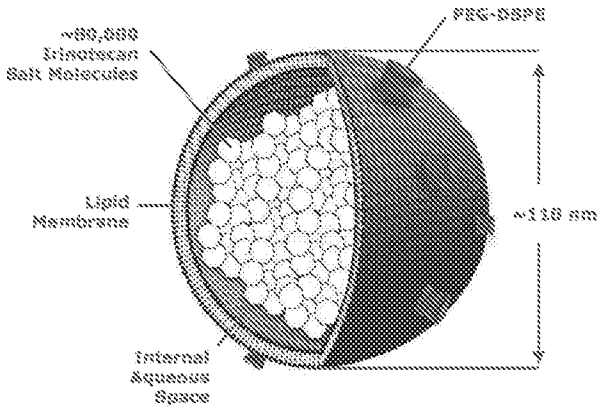
ClinicalTrials.gov: NCT02551991

Presented at the ESMO 21st World Congress on Gastrointestinal Cancer; Barcelona, Spain; July 3–6, 2019

Liposomal Irinotecan

Liposomal irinotecan (nal-IRI) is a long-acting, liposomal encapsulation of irinotecan

- The half-life ($t_{½}$) of total irinotecan following administration of nal-IRI is 25.8 hours
- 95% of irinotecan remains contained within the liposome during circulation
- The ratio between total and encapsulated forms did not change from 0 to 169.5 hours post-dose
- ~5-fold higher levels of drug are found in tumors compared with plasma at 72 hours, suggesting local metabolic activation of irinotecan



Study Objectives

To assess the safety and efficacy of nal-IRI, in combination with 5-FU/LV and OX, in patients with previously untreated mPAC

Primary objectives:

- Evaluate the safety and tolerability of nal-IRI+5-FU/LV+OX
- Characterize dose-limiting toxicities (DLTs) associated with nal-IRI+5-FU/LV+OX
- Determine the triplet combination dose of nal-IRI+5-FU/LV+OX for future studies

Secondary objectives:

- Interim analysis of clinical efficacy
  - Overall response rate (ORR)
  - Disease control rate (DCR)
  - Best overall response (BOR)
- Premature evaluation
  - Progression-free survival (PFS)
  - Overall survival (OS)

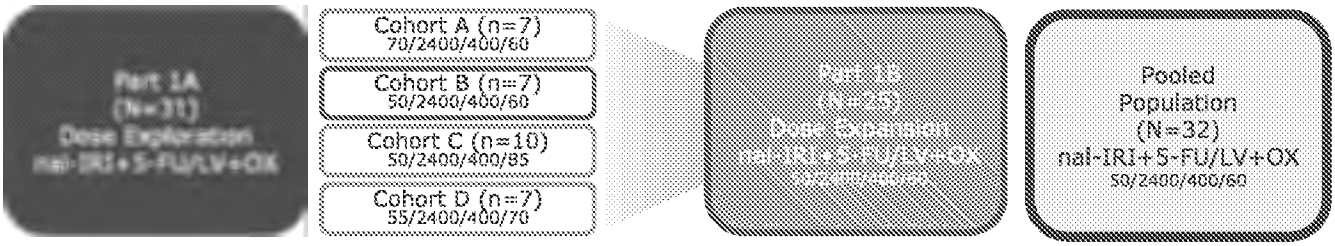
Inclusion criteria:

- Adults aged ≥18 years
- Pathologically confirmed, measurable/non-measurable mPAC* (RECIST v1.1), not previously treated in the metastatic setting**
- Eastern Cooperative Oncology Group (ECOG) performance 0 or 1
- Controlled central nervous system metastases
- No history of any second malignancy in the last 3 years

* Per RECIST, Response Evaluation Criteria In Solid Tumors v1.1.;
**Unresectable, locally advanced, or metastatic disease allowed, diagnosed ≤6 weeks prior to enrollment

Study Methods

This is an open-label, comparative study conducted at 15 sites across the United States (n=10), Spain (n=4), and Australia (n=1)



A Dose Exploration safety run-in (traditional 3+3 design) was performed to confirm an appropriate dose regimen for nal-IRI+5-FU/LV+OX in the Dose Expansion phase

- An interim data analysis was performed February 19th, 2019*
  - N=31 patients treated as part of Dose Exploration safety run-in
  - N=25 patients treated as part of Dose Expansion phase
  - N=32 patients included in the Pooled Population analysis (50/60 PP)
    - All patients in 50/60 PP treated with nal-IRI 50 mg/m^2 [FBE], LV 400 mg/m^2, 5-FU 2400 mg/m^2, and OX 60 mg/m^2

* Interim analysis conducted after all patients in the Dose Exploration cohorts had completed their second scheduled tumor evaluation at 16 weeks

Demographics, Characteristics, and Disposition

	Dose-Exploration Cohort				Dose-Expansion Cohort	50mg/60mg Pooled Population
	Cohort A N = 7	Cohort B N = 7	Cohort C N = 10	Cohort D N = 7	N = 25	N = 32
Age (years)						
Median (Range)	64 (58-78)	57 (44-74)	66.5 (57-73)	61 (54-73)	58 (39-76)	58 (39-76)
Age Group, n (%)						
<65 Years	4 (57.1)	4 (57.1)	3 (30.0)	4 (57.1)	19 (76.0)	23 (71.9)
Gender, n (%)						
Male	1 (14.3)	3 (42.9)	8 (80.0)	5 (71.4)	11 (44.0)	14 (43.8)
Race, n (%)						
White	6 (85.7)	7 (100)	9 (90.0)	7 (100)	21 (84.0)	28 (87.5)
Baseline Stage at Diagnosis*						
III	3 (42.9)	1 (14.3)	2 (20.0)	2 (28.6)	2 (8.0)	3 (9.4)
IV	4 (57.1)	6 (85.7)	8 (80.0)	6 (71.4)	23 (92.0)	29 (90.6)
Baseline ECOG Performance Status						
Fully Active (ECOG 0)	1 (14.3)	6 (85.7)	6 (60.0)	5 (71.4)	8 (32.0)	14 (43.8)
Restricted Activity (ECOG 1)	6 (85.7)	1 (14.3)	4 (40.0)	2 (28.6)	17 (68.0)	18 (56.3)
Disposition						
Treated, n (%)	7 (100)	7 (100)	10 (100)	7 (100)	25 (100)	32 (100)
Discontinued, n (%)	7 (100)	6 (85.7)	10 (100)	7 (100)	11 (44.0)	17 (53.1)

* One patient in Dose Expension Cohort was diagnosed as Stage IIA, but entered the treatment phase as Stage IV

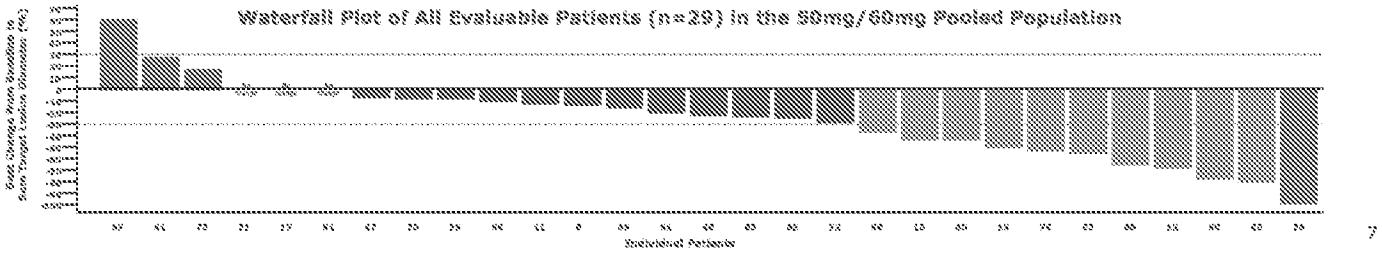
Dose-Limiting Toxicity and Treatment-related TEAEs Grade ≥3

	Dose-Exploration Cohort				Dose-Expansion Cohort	50mg/60mg Pooled Population
	Cohort A N = 7	Cohort B N = 7	Cohort C N = 10	Cohort D N = 7	N = 25	N = 32
Any DLT Event	2 (28.6)	1 (14.3)	2 (20.0)	0		
Diarrhea	0	0	2 (20.0)	0		
Vomiting	0	0	1 (10.0)	0		
Anal Fissure	0	0	1 (10.0)	0		
Anal Inflammation	0	0	1 (10.0)	0		
Proctalgia	0	0	1 (10.0)	0		
Neutropenia Infection	1 (14.3)	0	0	0		
Neutropenic Sepsis	1 (14.3)	0	0	0		
Febrile Neutropenia	0	1 (14.3)	0	0		
Grade ≥3 Treatment-related TEAE	6 (85.7)	4 (57.1)	8 (80.0)	5 (71.4)	16 (64.0)	20 (62.5)
Neutropenia	1 (14.3)	2 (28.6)	3 (30.0)	1 (14.3)	7 (28.0)	9 (28.1)
Febrile Neutropenia	0	1 (14.3)	0	0	3 (12.0)	4 (12.5)
Anemia	0	1 (14.3)	0	0	1 (4.0)	2 (6.3)
Diarrhea	3 (42.9)	1 (14.3)	4 (40.0)	1 (14.3)	2 (8.0)	3 (9.4)
Vomiting	1 (14.3)	0	3 (30.0)	0	2 (8.0)	2 (6.3)
Nausea	0	0	2 (20.0)	0	3 (12.0)	3 (9.4)
Colitis	0	0	0	2 (28.6)	1 (4.0)	1 (3.1)
Hypokalemia	1 (14.3)	2 (28.6)	2 (20.0)	2 (28.6)	2 (8.0)	4 (12.5)
Decreased Appetite	2 (28.6)	0	0	1 (14.3)	1 (4.0)	1 (3.1)
Peripheral Neuropathy	0	0	1 (10.0)	0	0	0
Fatigue	1 (14.3)	0	0	0	0	0

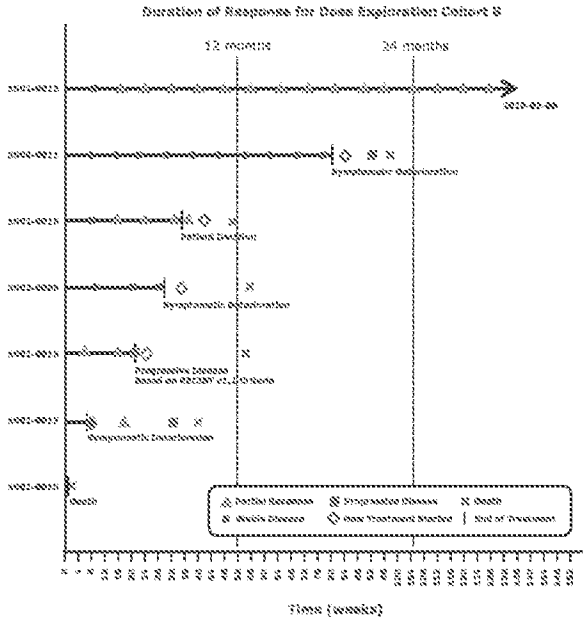
Clinical Response

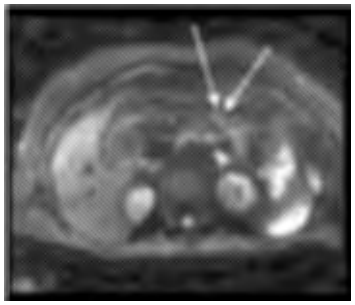
	Dose-Exploration Cohort				Dose-Expansion Cohort	50mg/60mg Pooled Population
	Cohort A N = 7	Cohort B N = 7	Cohort C N = 10	Cohort D N = 7	N = 25	N = 32
Best Overall Response at anytime						
Complete Response (CR)	0	0	0	0	1 (4.0)*	1 (3.1)*
Partial Response (PR)	0	3 (42.9)	3 (30.0)	1 (14.3)	7 (28.0)	10 (31.3)
Stable Disease (SD)	2 (28.6)	3 (42.9)	1 (10.0)	3 (42.9)	12 (48.0)	15 (46.9)
Best CR + PR + SD	2 (28.6)	6 (85.7)	4 (40.0)	4 (57.1)	20 (80.0)	26 (81.3)
Disease Control Rate						
Disease Control Rate at Week 16 (DCR_{16wk}, 95% CI)	42.9 (9.9, 81.6)	71.4 (29.0, 96.3)	40.0 (12.2, 73.8)	28.6 (3.7, 71.0)	72.0 (50.6, 87.9)	71.9 (53.3, 86.3)
Complete Response (CR)	0	0	0	0	1 (4.0)*	1 (3.1)*
Partial Response (PR)	0	3 (42.9)	2 (20.0)	1 (14.3)	5 (20.0)	8 (25.0)
Stable Disease (SD)	2 (28.6)	2 (28.6)	2 (20.0)	1 (14.3)	12 (48.0)	14 (43.8)

*Patient diagnosed with locally-advanced Stage III disease

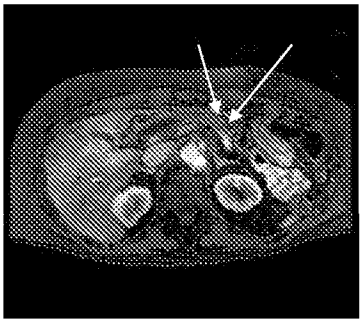


Duration of Response





Diagnostic scan outside hospital
9/24/2018.
A 2 cm focus of diffusion restriction
in the pancreatic body with
corresponding upstream dilatation



Most recent examination
6/2/2019
No residual mass or diffusion
restriction in the pancreatic body.
Decreased upstream dilatation

Conclusions

- The safety profile of NAPOX is manageable for the first-line treatment for patients with mPAC.
  - nal-IRI 50 mg/m^2 [FBE], LV 400 mg/m^2, 5-FU 2400 mg/m^2, and OX 60 mg/m^2
- Promising anti-tumor activity was identified
  - DCR_{16wk} of 71.9%
- Best overall response was
  - Complete Response in 1 patient
  - Partial Response in 10 patients
  - Stable Disease in 15 of 32 patients
  - CR+PR+SD: 81.3%
- OS and PFS not yet mature for evaluation
- At the time of data cut-off, 15 of 32 patients remain on treatment

A phase 1/2, open-label, dose-expansion study of liposomal irinotecan (nal-IRI) plus 5-fluorouracil/leucovorin (5-FU/LV) and oxaliplatin (OX) in patients with previously untreated metastatic pancreatic cancer (mPAC)

Zev Wainberg,[1] Patrick Boland,[2] Christopher H. Lieu,[3] Farshid Dayyani,[4] Teresa Macarulla,[5] Bin Zhang,[6] Bruce Belanger,[6] Yan Moore,[6] Tiffany Wang,[6] Fiona Maxwell,[7] Andrew Dean[8]

[1] University of California Los Angeles, Los Angeles, California, USA; [2] Roswell Park Cancer Institute, Buffalo, New York, USA; [3] University of Colorado, Denver, Colorado, USA; [4] University of California, Irvine, Irvine, California, USA; [5] Hospital Universitari Vall d'Hebron, Barcelona, Spain; [6] Ipsen Biopharma, Cambridge, MA, USA; [7] Ipsen Biopharmaceuticals, Slough, UK; [8] Department of Oncology, St John of God Subiaco Hospital, Subiaco, WA, Australia

This study is funded by Ipsen

ClinicalTrials.gov: NCT02551991

Presented at the ESMO 21st World Congress on Gastrointestinal Cancer; Barcelona, Spain; July 3–6, 2019

Disclosures

Author	Disclosure(s)
Zev Wainberg	Consultation: Eli Lilly, Merck, Bristol Myers Squibb, Bayer, Novartis, Ipsen
Patrick Boland	Research/Grant Funding (Institution): Merck, Genentech, Boehringer Ingelheim, Hemispherx, Boston Biomedical, Isofol Medical, Ipsen, Athenex, Bayer, Clinical Genomics; Travel & Accommodation Support: Ipsen.
Christopher H. Lieu	Consultation: Ipsen
Farshid Dayyani	Consultation: Array, Eisai, Genentech; Speaker's Bureau: Amgen, Eisai, Ipsen, Genetech, Sirtex
Teresa Macarulla	Consultation: Baxalta, Baxter, Bayer, Celgene, Genzyme, Incyte, QED Therapeutics, Shire Pharmaceuticals, Roche, Tesaro, Sanofi; Travel & Accommodation Support: Bayer, H3 Biomedicine, Merck, Sanofi.
Bin Zhang, Bruce Belanger, Yan Moore, Tiffany Wang, Fiona Maxwell	Employees of Ipsen
Andrew Dean	Non-paid Consultation: Shire, Specialised Therapeutics Australia; Travel & Accommodation Support: Amgen

Overview of Serious Adverse Events

	Dose-Exploration Cohort				Dose-Expansion Cohort	50mg/60mg Pooled Population
	Cohort A N = 7	Cohort B N = 7	Cohort C N = 10	Cohort D N = 7	N = 25	N = 32
≥ 1 SAE	6 (85.7)	2 (28.6)	7 (70.0)	4 (57.1)	12 (48.0)	14 (43.8)
≥1 treatment-related SAE	4 (57.1)	1 (14.3)	5 (50.0)	4 (57.1)	9 (36.0)	10 (31.3)
≥1 AE leading to discontinuation	5 (71.4)	1 (14.3)	3 (30.0)	3 (42.9)	3 (12.0)	4 (12.5)
≥1 AE leading to dose adjustment	2 (28.6)	4 (57.1)	7 (70.0)	4 (57.1)	19 (76.0)	23 (71.9)
≥1 AE leading to death	0	1 (14.3)	1 (10.0)	2 (28.6)	0	1 (3.1)
Any Treatment-related SAE	4 (57.1)	1 (14.3)	5 (50.0)	4 (57.1)	9 (36.0)	10 (31.3)
Diarrhea	2 (28.6)	1 (14.3)	1 (10.0)	1 (14.3)	1 (4.0)	2 (6.3)
Vomiting	1 (14.3)	0	2 (20.0)	0	2 (8.0)	2 (6.3)
Nausea	0	0	1 (10.0)	0	3 (12.0)	3 (9.4)
Colitis	0	0	0	2 (28.6)	1 (4.0)	1 (3.1)
Abdominal Pain	0	0	0	2 (28.6)	0	0
Enterocolitis	0	0	1 (10.0)	0	1 (4.0)	1 (3.1)
Stomatitis	0	0	0	0	1 (4.0)	1 (3.1)
Febrile Neutropenia	0	1 (14.3)	0	0	2 (8.0)	3 (9.4)
Anemia	0	0	0	0	1 (4.0)	1 (3.1)
Dehydration	1 (14.3)	0	1 (10.0)	0	0	0
Hypokalaemia	0	0	0	1 (14.3)	0	0
Neutropenic Sepsis	1 (14.3)	0	0	0	0	0
Pneumonia	0	0	0	0	1 (4.0)	1 (3.1)
Arteriospasm Coronary	0	0	1 (10.0)	0	0	0
Pyrexia	0	0	0	0	1 (4.0)	1 (3.1)
Hypotension	0	0	1 (10.0)	0	0	0

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Acknowledgements

Medical writing support

- The authors thank Julie O'Grady and Philip Sjostedt of The Medicine Group, New Hope, PA, USA for providing medical writing / editorial support, which was sponsored by Ipsen in accordance with Good Publication Practice guidelines

Acknowledgements

- The authors thank all patients involved in the study, as well as their caregivers, care team, investigators, and research staff in participating institutions

Funding

- This study was sponsored by Ipsen

phase 1/2, open-label trial to assess the safety, tolerability, and dose-limiting toxicities (DLTs) of nal-IRI+5-FU/LV+OX (NAPOX) for the first-line treatment of patients with mPAC and to determine phase 3 dosing.

Methods: Following 4 dose exploration cohorts (Part 1A), a recommended dose for dose expansion (Part 1B) was selected based on DLTs and cumulative safety (nal-IRI 50 mg/m2 [free-base equivalent; FBE], OX 60 mg/m^2, LV 400 mg/m^2, 5-FU 2400 mg/m^2 on days 1 & 15 of each 28-day cycle). The expansion phase enrolled 25 patients at the selected dose level, with 32 subjects treated at the selected dose level (pooled population; PP 50/60). Patients were age ≥18 yrs with previously untreated locally advanced or mPAC, ECOG performance status ≤1, and adequate organ function. The primary endpoint was safety and tolerability, with secondary assessments based on 19/Feb/2019 data cut-off when all patients had completed their second scheduled tumor assessment after 16 weeks of treatment.

Results: 56 patients were enrolled and treated, with 32 patients from (n = 7) and; Dose Expansion Cohort (n = 25) included in the PP 50/60 analysis (n = 29 mPDAC; n=3 locally advanced PDAC). 9 DLTs were reported by 5 patients across the 4 dose exploration cohorts (diarrhea, n = 2; vomiting, anal fissure, anal inflammation, proctalgia, neutropenic infection, neutropenic sepsis, and febrile neutropenia, all n = 1), including 1 patient in (febrile neutropenia). Treatment-related TEAEs Grade 3 or higher were reported by 39 of 56 patients (50/60 PP, n = 20/32: neutropenia, n = 9; febrile neutropenia, hypokalemia, both n = 4; diarrhea, nausea, both n=3; anemia, vomiting, both n = 2), with no reported Grade 3 or higher fatigue or peripheral neuropathy. Serious adverse events (SAEs) were reported by 31 of 56 patients (50/60 PP, n = 14/32), with n = 23 patients reporting treatment-related SAEs (50/60 PP, n = 10/32 patients: nausea, febrile neutropenia, both n = 3; diarrhea, vomiting, both n = 2; colitis, enterocolitis, stomatitis, anemia, pneumonia, and pyrexia, all n = 1). 15 patients reported TEAEs leading to discontinuation (50/60 PP, n = 4/32), with 36 patients requiring dose adjustment due to AEs (50/60 PP, n = 23/32). 23 of 32 patients (71.9%) in the 50/60 PP achieved disease control at 16 weeks (DCR16wk). Best overall response in the 50/60 PP was complete response (CR) in 1 patient (diagnosed with locally advanced Stage III disease), partial response (PR) in 10 patients, and stable disease (SD) in 15/32 patients (sum of CR+PR+SD: 81.3%), with an overall response rate (ORR) of 34%. At data cut-off, 15/32 patients in the PP 50/60 remain on treatment. Preliminary analysis of median progression-free survival and median overall survival are not yet mature for evaluation.

Conclusion: In the first-line treatment of patients with mPAC, NAPOX (nal-IRI 50 mg/m^2 (FBE), OX 60 mg/m^2, LV 400 mg/m^2, and 5-FU 2400 mg/m^2) appears manageable, with promising anti-tumor activity (DCR16wk of 71.9%, sum of CR+PR+SD: 81.3%, and ORR of 34%) warranting further clinical assessment. This study is ongoing, with additional analyses planned.

SO – 005 **A phase 1/2, open-label, dose-expansion study of liposomal irinotecan (nal-IRI) plus 5-fluorouracil/leucovorin (5-FU/LV) and oxaliplatin (OX) in patients with previously untreated metastatic pancreatic cancer**

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[1]*Ronald Reagan UCLA Medical Center, Los Angeles, California, USA,* [2]*Roswell Park Cancer Institute, Buffalo, New York, USA,* [3]*University of Colorado, Aurora, Colorado, USA,* [4]*University of California, Orange, California, USA,* [5]*Hospital Vall D'Hebron, Barcelona, Spain,* [6]*Ipsen Bioscience, Cambridge, Massachusetts, USA,* [7]*Ipsen Bioinnovation, Milton, UK,* [8]*St John of God Hospital Subiaco, Subiaco, Australia*

Introduction: nal-IRI+5-FU/LV is approved for patients with mPAC after disease progression following gemcitabine-based therapy. The current study (NCT02551991) is a

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	15809815
Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

	1	EP2861210: Opposition filed February 5, 2018, D11 (Hoskins J, et al., "UGT1A1*28 Genotype and Irinotecan-Induced Neutropenia: Dose Matters," J Natl Cancer Inst. 99(17):1290-95 (2007)).
	2	EP2861210: Opposition dated February 5, 2018, D12 (Tsai C, et al., "Nanovector-Based Therapies in Advanced Pancreatic Cancer," J Gastroint Oncol 2(3):185-94 (2011)).
	3	EP2861210: Opposition dated February 5, 2018, D13 (Ko A, et al., "A Multinational Phase II Study of Liposome Irinotecan (PEP02) for Patients with Gemcitabine-Refractory Metastatic Pancreatic Cancer," J Clin Oncol. 29:2011 (Suppl; Abstract 237). 2011 ASCO Annual Meeting (2011), 2 printed pages).
	4	EP2861210: Opposition dated February 5, 2018, D15 (Clinical Trials Identifier NCT01494506: 2013-01-25 version, "A Randomized, Open Label Phase 3 Study of MM-398, With or Without 5-Fluorouracil and Leucovorin, Versus 5 Fluorouracil and Leucovorin in Patients With Metastatic Pancreatic Cancer Who Have Failed Prior Gemcitabine-based Therapy," Retrieved from ClinicalTrials.gov archive, 1 printed page).
	5	EP2861210: Response to EP Opposition Proceedings filed August 24, 2018, 22 pages.
	6	EP2861210: Response to EP Opposition Proceedings filed August 24, 2018, D15a (Clinical Trials Identifier NCT01494506: 2011-12-16 version, "A Randomized, Open Label Phase 3 Study of MM-398 Versus 5-Fluorouracil and Leucovorin in Patients With Metastatic Pancreatic Cancer." Retrieved from ClinicalTrials.gov archive, 3 printed pages).
	7	EP2861210: Response to EP Opposition Proceedings filed August 24, 2018, D17 (European Commission Implementing Decision granting marketing authorisation for Onivyde, October 14, 2016), 39 pages.
	8	EP2861210: Response to EP Opposition Proceedings filed August 24, 2018, D18 (FDA News Release, "FDA Approves New Treatment for Advanced Pancreatic Cancer." Retrieved from http://ww.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm468654.htm, October 22, 2015, 3 printed pages).
	9	EP2861210: Response to EP Opposition Proceedings filed August 24, 2018, D19 (Wang-Gillam A, et al., 'Nanoliposomal Irinotecan with Flourouracil and Folinic Acid in Metastatic Pancreatic Cancer After Previous Gemcitabine-Based Therapy (NAPOLI-1): A Global, Randomised, Open-Label, Phase 3 Trial," Lancet, 387 (10018):545-57 (2016). Epub doi: 10.1016/S0140-6736(15)00986-1, pages 1-13 (2015)).
	10	EP2861210: Response to EP Opposition Proceedings filed August 24, 2018, D20 (MHRA Public Assessment Report for 5-Fluorouracil, 2006, 60 pages).
	11	EP2861210: Summons to attend oral proceedings including preliminary opinion of the Opposition Division dated January 30, 2019, 12 pages.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15809815
	Filing Date	2017-11-10
	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

	12	EP2861210: Opponent submission in opposition proceedings made following summons to attend oral proceedings, dated May 10, 2019, 20 pages.
	13	EP2861210: Opponent submission in opposition proceedings made following summons to attend oral proceedings, dated May 10, 2019, D1b (Leucovorin calcium injection product label, November 2011, 2 pages).
	14	EP2861210: Opponent submission in opposition proceedings made following summons to attend oral proceedings, dated May 10, 2019, D22 (Chen L, et al., "Phase I Study of Liposome Irinotecan (PEP02) in Combination with Weekly Infusion of 5-FU/LV in Advanced Solid Tumors," J Clin Oncol., 2010 ASCO Annual Meeting Abstracts, 28(15_suppl) (May 20 Suppl):e13024 (2010), 1 page).
	15	EP2861210: Proprietor's Auxiliary Requests in Opposition Proceedings filed June 28, 2019, including cover letter and clean and marked-up AR1, AR2, and AR3, 12 pages.
	16	EP2861210: Minutes of the oral proceedings before the Opposition Division, dated August 28, 2019, 9 pages.
	17	EP2861210: Opposition Division's decision to revoke patent, dated August 28, 2019, 27 pages.
	18	FDA News Release, "FDA Approves New Treatment for Advanced Pancreatic Cancer." Retrieved from http://ww.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm468654.htm, October 22, 2015, 3 printed pages.
	19	FUCHS C, et al., "Phase III Comparison of Two Irinotecan Dosing Regimens in Second-Line Therapy of Metastatic Colorectal Cancer," J Clin Oncol. 21(5):807-14 (2003).
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	22	HONG K, et al., "Anti-HER2 Immunoliposomes for Targeted Drug Delivery," Ann N Y Acad Sci. 886:293-6 (1999).

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	Filing Date	2017-11-10
	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

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Signature	/Mary R. Henninger/	Date (YYYY-MM-DD)	2020-01-07
Name/Print	Mary R. Henninger	Registration Number	56992

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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	1	VERREAULT M, et al., "Vascular Normalization in Orthotopic Glioblastoma Following Intravenous Treatment with Lipid-Based Nanoparticulate Formulations of Irinotecan (Irinophore C™), Doxorubicin (Caelyx®) or Vincristine," BMC Cancer. 11:124, pages 1-18 (2011).
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	4	YEH B, et al., "Structural Basis for Activation of Fibroblast Growth Factor Signaling by Sucrose Octasulfate," Mol Cell Biol. 22(20):7184-92 (2002).

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Electronic Patent Application Fee Transmittal

Application Number:	15809815
Filing Date:	10-Nov-2017
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Filer:	Mary Rucker Henninger/Richard King
Attorney Docket Number:	263266-421428

Filed as Large Entity

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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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Pages:				
Claims:				
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EFS ID:	38229719
Application Number:	15809815
International Application Number:	
Confirmation Number:	5137
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Customer Number:	153749
Filer:	Mary Rucker Henninger/Richard King
Filer Authorized By:	Mary Rucker Henninger
Attorney Docket Number:	263266-421428
Receipt Date:	07-JAN-2020
Filing Date:	10-NOV-2017
Time Stamp:	18:45:47
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13	Fee Worksheet (SB06)	fee-info.pdf	32801 b425fc6ca37dcb1e713c534a6724d9bbf1624912	no	2

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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REMARKS

I. Status of Claims

Following entry of this amendment, claims 1, 4-15, 18, 19, and 21-23 are pending in the application. Claims 2, 3, 16, 17, and 20 were previously canceled without prejudice or disclaimer. Applicant expressly reserves the right to pursue the subject matter of those claims in the future. Claims 1, 11, 12, 14, and 19 were amended to even more clearly recite the subject matter being claimed. Support for the amendments can be found throughout the specification and originally filed claims. The amendments add no new matter.

II. Rejections Under 35 U.S.C. § 103

Rejection of claims 1, 5-8, 10 and 19

Claims 1, 5-8, 10 and 19 are rejected under 35 U.S.C. 103 as allegedly being unpatentable over WO 2013/188586 ("Bayever"), in view of Conroy et al., N Engl J Med., 364(19):1816-25, 2011) ("Conroy"), and further in view of Melis et al., The Society for Surgery of the Alimentary Tract, 2011; http://meetings.ssat.com/abstracts/11ddw/P57.cgi) ("Melis"). Office Action at p. 2. The Examiner asserted that Bayever discloses treatment of metastatic pancreatic cancer comprising "co-administering to the patient active agents, at a dose of 60 mg/m^2 (e.g., liposomal irinotecan)," a dose of 2400 mg/m^2 5-fluorouracil, and a dose of 200 mg/m^2 1 form or 400 mg/m^2 l+d form leucovorin for at least one cycle of two weeks. *Id.* at pp. 2-3. The Examiner also alleged that Conroy discloses treatment of metastatic pancreatic cancer with oxaliplatin, irinotecan, leucovorin, and fluorouracil. *Id.* at p. 3. Furthermore, the Examiner alleged that "it would have been prima facie obvious to one of ordinary skill in the art to include oxaliplatin within Bayever's methods of treatment" and that "[a]n ordinarily skilled artisan would have been motivated because oxaliplatin has clinical activity against pancreatic cancer when combined with fluorouracil, and because oxaliplatin and irinotecan have synergistic activity *in vitro*, as taught by Conroy… ." *Id.*

Regarding the 60 mg/m^2 oxaliplatin dose recited in claims 1 and 19, the Examiner alleged that Conroy taught 85 mg/m^2 oxaliplatin[1], but not 60 mg/m^2 oxaliplatin. *Id.* at p. 4. The Examiner then pointed to Melis for allegedly teaching "that a dosage of 60 mg/m^2 oxaliplatin was well tolerated in advanced pancreatic adenocarcinoma patients." *Id.* The Examiner alleged that the dosage of oxaliplatin is "recognized to be result effective" and that "it would have been prima facie obvious to optimize the dosage of the oxaliplatin present in the combined composition of Bayever and Conroy, as taught by Melis." *Id.*

Applicant respectfully traverses. Bayever discloses treatment of pancreatic cancer by administering a combination of liposomal irinotecan (e.g., 60 or 80 mg/m^2), in combination with leucovorin (e.g., 400 mg/m^2 l+d form) and 5-fluorouracil (e.g., 2400 mg/m^2) to a patient once every two weeks. Conroy discloses treatment of patients with first-line metastatic pancreatic cancer by administering a different combination of therapeutic agents in different doses: Conroy administers a combination of 85 mg/m^2 oxaliplatin, 180 mg/m^2 non-liposomal irinotecan, 400 mg/m^2 leucovorin, 400 mg/m^2 fluorouracil as a bolus injection followed by 2400 mg/m^2 fluorouracil as a continuous infusion once every two weeks. Melis is an abstract summarizing a phase I/II chemo-radiation (CRT) study of continuous infusion of 200 mg/m^2 5-fluorouracil and escalating doses of oxaliplatin (30 mg/m^2 in 10 mg intervals up to 60 mg/m^2) weekly for 5 weeks with concurrent radiation in patients with regionally advanced pancreatic cancer. A detailed analysis of the Melis study appears to be reported in Amodeo et al, "Can we downstage locally advanced pancreatic cancer to resectable? A phase I/II study of induction oxaliplatin and 5-FU chemoradiation," J Gastrointest Oncol, 9(5):922-935, 2018 ("Amodeo") (cited in the accompanying IDS)[2]. However, neither Bayever, Conroy, nor Melis teaches or suggests (solely or in combination) the claimed methods of treating a patient with metastatic adenocarcinoma of

[1] Applicant assumes that the Examiner's statement that Bayever teaches 85 mg/m^2 oxaliplatin was meant to refer to Conroy. *See* Office Action at p. 4. Applicant responds accordingly.

[2] Amodeo lists the same authorship as Melis (aside from the addition of Amodeo) and also describes treatment of patients with locally advanced pancreatic cancer using the same chemoradiation therapy study design as Melis: "Radiation was combined with 5FU 200 mg/m^2 daily by continuous infusion for 5 weeks and weekly oxaliplatin for 5 weeks in dose escalation cohorts as follows: level I =30 mg/m^2; level II =40 mg/m^2; level III =50 mg/m^2; level IV =60 mg/m^2. Following the phase I portion of the trial, a phase II trial at the recommended dose continued." Amodeo at p. 924. "The highest dose (60 mg/m^2) of oxaliplatin, thus, was well tolerated and it was therefore carried forward in the phase II portion of the study." *Id.* at p. 926.

the pancreas who has not previously been treated with an antineoplastic agent (claim 1) or gemcitabine (claim 19) comprising co-administering to the patient 60 mg/m^2 liposomal irinotecan, 60 mg/m^2 oxaliplatin, leucovorin (200 mg/m^2 l-form or 400 mg/m^2 l+d form), and 2,400 mg/m^2 5-fluorouracil once every two weeks.

The objectives of the Melis Study, as described in the Abstract, were to "control regional disease" and "downstage to resectable disease" in patients with borderline resectable or locally advanced unresectable pancreatic adenocarcinoma. Patients with metastatic disease were excluded. *See* Amodeo at p. 924. "Following completion of CRT, patients deemed resectable underwent surgery; those who remained unresectable for cure but did not progress (PD) received mFOLFOX6 x6 cycles." Unfortunately, only 2 patients were resected and 22 of the 24 patients (91.7%) remained unresectable. Amodeo, at page 924, explains that the modified FOLFOX6 therapy involved administration of a higher 85 mg/m^2 oxaliplatin dose at day 1 (as a 2-hour IV infusion) concurrently with 350 mg leucovorin (as a 2-hour IV infusion), followed by 400 mg/m^2 5-fluorouracil (as an IV bolus), followed by 2,400 mg/m^2 5-fluorouracil (as a 46-hour infusion) every 2 weeks for 6 cycles. Even though the investigators of the Melis study concluded that the CRT regime was well tolerated, the majority of patients remained unresectable with some patients requiring the modified FOLFOX6 regime with a higher 85 mg/m^2 oxaliplatin dose, and survival data were comparable to other combination therapies for locally advanced pancreatic cancer. *See* Melis Abstract and Amodeo at p. 933.

The Examiner has failed to establish a *prima facie* case of obviousness of the claimed methods. A person of ordinary skill in the art would not have been motivated to select and combine the weekly 60 mg/m^2 dose of oxaliplatin referenced in Melis with the teachings of Bayever and Conroy to reach the claimed methods with a reasonable expectation of success for numerous reasons. First, the Melis Study involved patients with locally advanced pancreatic cancer and ***excluded*** patients with metastatic disease. Patients with locally advanced pancreatic cancer have a better prognosis than patients diagnosed with metastatic adenocarcinoma of the pancreas, as recited in the pending claims. Second, in contrast to the "once every two weeks" coadministration schedule recited in the pending claims, the Melis Study involved ***weekly*** administration of 60 mg/m^2 oxaliplatin. Third, the Melis study included ***continuous infusion*** of 200 mg/m^2 5-fluorouracil compared to the claimed coadministration of 2,400 mg/m^2 5-fluorouracil once every two weeks. Fourth, the Melis treatment regime did not result in

improved outcomes compared to other combination therapies for locally advanced pancreatic cancer. Specifically, the investigators of the Melis study concluded that while the CRT used was "reasonably well tolerated," the "majority of patients [22 out of 24] remained unresectable" and "[s]urvival data with this regimen are comparable to others for locally advanced pancreas cancer." Fifth, "those who remained unresectable for cure but did not progress received mFOLFOX6 x6 cycles"—a treatment regime involving a higher 85 mg/m^2 oxaliplatin dose every two weeks. *See* Melis Abstract and Amodeo at p 924.

Only by impermissible hindsight did the Examiner pick Melis from the literature to piece with the disclosures of Bayever and Conroy. The Examiner alleged that "it would have been prima facie obvious to use oxaliplatin at 60 mg/m^2 because the said dosage is well tolerated in advanced pancreatic adenocarcinoma patients." Office Action at p. 10, see also *id.* at p. 4. However, the Examiner's reasoning fails to account for the many factors, such as patient population, disease severity, drug combination, dose, dosing schedule, drug-drug interactions, and overlapping toxicities, that each affect ***tolerability and efficacy*** of a particular cancer treatment method. For example, even though a particular drug, drug combination, dose, or dosing schedule may be well tolerated in one patient population, it is not necessarily well tolerated and efficacious in a different patient population.

The Examiner did not, and cannot, explain why one of ordinary skill in the art would have been motivated to combine a CRT study in patients with locally advanced adenocarcinoma with the teachings of Bayever and Conroy to achieve the claimed methods with a reasonable expectation of success, in view of only a favorable tolerability profile. The tolerability profile of the Melis CRT regime cannot be separated from Melis' concurrent teaching that (1) metastatic patients were excluded from the study, (2) the 60 mg/m^2 oxaliplatin dose was administered weekly, (3) the majority of the patients having locally advanced pancreatic cancer treated in the study remained unresectable, (4) survival data was comparable to other combination treatments for the same patient population, and (5) patients who remained unresectable for cure but did not progress continued on a modified FOLFOX6 regime involving a higher dose of oxaliplatin. If anything, the teachings of Melis would have discouraged treatments of metastatic adenocarcinoma of the pancreas involving a 60 mg/m^2 dose of oxaliplatin once every two weeks.

"A patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *Kinetic Concepts, Inc. v.*

Smith & Nephew, Inc., 688 F.3d 1342, 1369 (Fed. Cir. 2012) (quoting *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 418 (2007)). The Supreme Court has held that "it can be important to identify a reason that would have prompted a person of ordinary skill in the art to combine the elements as the new invention does." *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 401 (2007) (emphasis added). Further, "[a] factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon *ex post* reasoning." *Id.* at 421 (emphasis added).

The requirement that the content of the prior art is determined at the time the invention was made is to avoid impermissible hindsight. MPEP § 2141.01 III. Furthermore, a "prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." MPEP § 2141.02 VI., citing *W.L. Gore & Assoc., Inc. v. Garlock, Inc.* 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984). "It is impermissible to use the claimed invention as an instruction manual or 'template' to piece together the teachings of the prior art so that the claimed invention is rendered obvious." *In re Fritch*, 972 F.2d 1260, 1266 (Fed. Cir. 1992) (followed e.g., by *Ex parte Alagappan*, 2017-005866, 2018 WL 3004459 (BPAI May 29, 2018). Thus, the Office must provide an objective reason why one of ordinary skill in the art *would have*, not merely *could have*, combined or modified the teachings of the cited art.

The Examiner's piecing together of drugs and doses from the prior art without providing an objective reason as to why one of ordinary skill in the art would have been motivated to combine Melis with Bayever and Conroy in view of the complete teachings of Melis is impermissible hindsight. Furthermore, even if a prima facie case of obviousness were to be established regarding any of the pending claims, which Applicant fervently traverses, one or more objective indicia of nonobvious would support a finding of nonobviousness. "Applicant can rebut a presumption of obviousness based on a claimed invention that falls within a prior art range by showing '(1) [t]hat the prior art taught away from the claimed invention… or (2) that there are new and unexpected results relative to the prior art.' *Iron Grip Barbell Co., Inc. v. USA Sports, Inc.*, 392 F.3d 1317, 1322, 73 USPQ2d 1225, 1228 (Fed. Cir. 2004)." MPEP § 2144.05 III B. Other objective evidence of nonobviousness includes evidence of criticality, commercial success, long-felt but unsolved needs, failure of others, skepticism of experts, etc. *See* MPEP §§ 716.01(a) and 2145.

Melis is an example of how the prior art would have discouraged the claimed co-administration of 60 mg/m^2 oxaliplatin once every two weeks for the treatment of metastatic adenocarcinoma of the pancreas. As discussed above, Melis' exclusion of patients with metastatic disease, weekly administration of 60 mg/m^2 oxaliplatin, subsequent increased oxaliplatin dose, and results comparable to other combination treatments for the same patient population would have directed against co-administration of 60 mg/m^2 oxaliplatin at a less frequent interval for treatment of a more severe patient population. While the right to present additional objective evidence of nonobviousness is reserved, Applicant respectfully asserts that the evidence presented above negates any *prima facie* case of obviousness.

In sum, the Examiner has failed to establish a *prima facie* case of obviousness at least with respect to the claimed treatment of metastatic adenocarcinoma of the pancreas comprising, in-part, co-administration of a dose of 60 mg/m^2 oxaliplatin once every two weeks by engaging in impermissible hindsight by cherry picking a drug and dose from the art. The Examiner did not, and cannot, explain why one of ordinary skill in the art would have been motivated to combine a CRT study in patients with locally advanced adenocarcinoma and no metastatic disease involving administration of a weekly 60 mg/m^2 dose of oxaliplatin with the teachings of Bayever and Conroy to achieve the claimed methods with a reasonable expectation of success, when that weekly regime was no more effective than other combination treatments for that patient population and some patients required a higher dose of oxaliplatin.

Accordingly, the pending claims, which, in part, recite or otherwise incorporate treatment of metastatic adenocarcinoma of the pancreas comprising co-administration of 60 mg/m^2 liposomal irinotecan, 60 mg/m^2 oxaliplatin, leucovorin (200 mg/m^2 l-form or 400 mg/m^2 l+d form), and 2,400 mg/m^2 5-fluorouracil once every two weeks are nonobvious over Bayever, Conroy, and/or Melis. Applicant respectfully requests reconsideration and withdrawal of the rejection of claims 1, 5-8, 10 and 19 under 35 U.S.C. § 103 over Bayever in view of Conroy, and further in view of Melis.

Rejection of claims 4, 9, 18, and 23

The Examiner rejected claims 4, 9, 18, and 23 under 35 U.S.C. § 103 as allegedly being obvious over Bayever in view of Conroy and further in view of Melis and Fleming et al. found at http://www.oncologynurseadvisor.com/advisor-forum/importance-of-sequence-in-chemotherapy-

administration/article/378072/ ("Fleming"). Office Action at p. 5. The Examiner alleged that Fleming discloses at the last sentence of the first paragraph that "the sequence of various chemotherapy drugs in general does not matter, as the half-life of each drug makes it impossible to determine what drug is at what level at any particular time, based on individual patient pharmacodynamics." *Id.* at p. 6. The Examiner alleged that in view of Fleming, one of ordinary skill in the art would have been motivated to vary the order of administration of the combined methods of Bayever and Conroy. *Id.*

Applicant respectfully traverses for at least the reasons discussed above with respect to claims 1 and 19, from which claims 4, 9, 18, and 23 depend. As discussed, the Examiner has failed to establish a *prima facie* case of obviousness at least with respect to the claimed treatment of metastatic adenocarcinoma of the pancreas comprising, in-part, co-administration of a dose of 60 mg/m^2 oxaliplatin once every two weeks by engaging in impermissible hindsight by cherry picking a drug and dose from the art. The Examiner did not, and cannot, explain why one of ordinary skill in the art would have been motivated to select and combine a CRT study in patients with locally advanced adenocarcinoma and no metastatic disease involving administration of a weekly 60 mg/m^2 dose of oxaliplatin with the teachings of Bayever and Conroy to achieve the claimed methods with a reasonable expectation of success, when the Melis regime was no more effective than other combination treatments for that patient population and some patients required a higher dose of oxaliplatin. If anything, the teachings of Melis would have discouraged treatments of metastatic adenocarcinoma of the pancreas involving a 60 mg/m^2 dose of oxaliplatin once every two weeks.

Accordingly, claims 4, 9, 18, and 23, which, in part, incorporate treatment of metastatic adenocarcinoma of the pancreas comprising co-administration of 60 mg/m^2 liposomal irinotecan, 60 mg/m^2 oxaliplatin, leucovorin (200 mg/m^2 l-form or 400 mg/m^2 l+d form), and 2,400 mg/m^2 5-fluorouracil once every two weeks are nonobvious over Bayever, Conroy, Melis, and/or Fleming. Applicant respectfully requests reconsideration and withdrawal of the rejection of claims 4, 9, 18, and 23 under 35 U.S.C. § 103 over Bayever in view of Conroy, and further in view of Melis and Fleming.

Rejection of claims 11-15 and 21-22

The Examiner rejected claims 11-15 and 21-22 under 35 U.S.C. § 103 as allegedly being obvious over Bayever in view of Conroy, further in view of Melis, and as evidenced by WO 2016/094402 ("Bayever II"). *Id.* at pp. 8-9. The Examiner alleged that while "Bayever was not specific as to the ingredients of the liposome, as recited in claims 11-12 and 21-22," Bayever II "evidenced that MM-398 contained irinotecan sucrose octasulfate, DSPC, cholesterol and MPEG-2000-DSPE." The Examiner also alleged that claims 13-15 and 21-22 are rendered obvious because of the administration durations and cycles disclosed in Bayever. *Id.* at pp. 7-8.

Applicant respectfully traverses for at least the reasons discussed above with respect to claims 1 and 19, from which claims 11-15 and 21-22 depend. As discussed, the Examiner has failed to establish a *prima facie* case of obviousness at least with respect to the claimed treatment of metastatic adenocarcinoma of the pancreas comprising, in-part, co-administration of a dose of 60 mg/m^2 oxaliplatin once every two weeks by engaging in impermissible hindsight by cherry picking a drug and dose from the art. The Examiner did not, and cannot, explain why one of ordinary skill in the art would have been motivated to combine a CRT study in patients with locally advanced adenocarcinoma involving administration of a weekly 60 mg/m^2 dose of oxaliplatin with the teachings of Bayever and Conroy to achieve the claimed methods with a reasonable expectation of success, when the Melis regime was no more effective than other combination treatments for that treatment population and some patients required a higher dose of oxaliplatin. If anything, the teachings of Melis would have discouraged treatments of metastatic adenocarcinoma of the pancreas involving a 60 mg/m^2 dose of oxaliplatin once every two weeks.

Accordingly, claims 11-15 and 21-22, which, in part, incorporate treatment of metastatic adenocarcinoma of the pancreas comprising co-administration of 60 mg/m^2 liposomal irinotecan, 60 mg/m^2 oxaliplatin, leucovorin (200 mg/m^2 l-form or 400 mg/m^2 l+d form), and 2,400 mg/m^2 5-fluorouracil once every two weeks are nonobvious over Bayever, Conroy, Melis, and/or Bayever II. Applicant respectfully requests reconsideration and withdrawal of the rejection of claims 11-15 and 21-22 under 35 U.S.C. § 103 over Bayever in view of Conroy, further in view of Melis, and as evidenced by Bayever II.

III. Nonstatutory Double Patenting

The Examiner rejected claims 1, 4-15, 18-19, and 21-23 on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 1-18 of U.S. Patent No. 9,492,442 ("the '442 Patent") in view of Conroy, and further in view of Melis. *Id.* at pp. 9-10. The Examiner alleged that the "issued claims recite all of the features instantly recited for the method of treatment except for the administration of oxaliplatin." *Id.* at p. 10. The Examiner further alleged that "it would have been prima facie obvious to use oxaliplatin in the issued method, because oxaliplatin has clinical activity against pancreatic cancer only when combined with fluorouracil, and because oxaliplatin and irinotecan have been shown to have synergistic activity *in vitro*." *Id.* The Examiner argued that Melis allegedly teaches that "a dosage of 60 mg/m^2 oxaliplatin was well tolerated in advanced pancreatic adenocarcinoma patients" and that "[i]t would have been prima facie obvious to use oxaliplatin at 60 mg/m^2 because the said dosage is well tolerated in advanced pancreatic adenocarcinoma patients." *Id.* at p. 10.

Applicant respectfully traverses. Coadministration of a dose of 60 mg/m^2 oxaliplatin once every two weeks for the treatment of metastatic adenocarcinoma of the pancreas would not have been an obvious variation of any of claims 1-18 of the '442 Patent for at least the reasons discussed above. The Examiner did not, and cannot, explain why one of ordinary skill in the art would have been motivated to combine a CRT study in patients with locally advanced adenocarcinoma involving administration of a weekly dose of 60 mg/m^2 of oxaliplatin with the teachings of Bayever and Conroy to achieve the claimed methods with a reasonable expectation of success, when the Melis regime was no more effective than other combination treatments for that patient population and some patients required a higher dose of oxaliplatin. If anything, the teachings of Melis would have discouraged treatments of metastatic adenocarcinoma of the pancreas involving a 60 mg/m^2 dose of oxaliplatin once every two weeks. Accordingly, the pending claims are not obvious variations of issued claims 1-18 of the '442 Patent.

Applicant respectfully requests reconsideration and withdrawal of the nonstatutory double patenting rejection over claims 1-18 of the '442 Patent, in view of Conroy, and further in view of Melis.

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account 506488.

Respectfully submitted,

MCNEILL BAUR PLLC.

Dated: January 7, 2020

By: /Mary R. Henninger/
Mary R. Henninger, PhD
Reg. No. 56,992
404-891-1400

AMENDMENTS TO THE CLAIMS:

This listing of claims will replace all prior versions and listings of claims in the application. Please amend the claims as follows:

1. (Currently Amended) A method of treating metastatic adenocarcinoma of the pancreas in a human patient who has not previously received an antineoplastic agent to treat the metastatic adenocarcinoma of the pancreas, the method comprising administering an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of administering to the patient a total of:
   a. 60 mg/m^2 of liposomal irinotecan,
   b. 60 mg/m^2 oxaliplatin,
   c. 200 mg/m^2 of the (l)-form of leucovorin or 400 mg/m^2 of the (l+d) racemic form of leucovorin, and
   d. 2,400 mg/m^2 5-fluorouracil;

   to treat the metastatic adenocarcinoma of the pancreas in the human patient.

2. (Canceled)

3. (Canceled)

4. (Original) The method of claim 1, wherein each administration of the oxaliplatin begins 2 hours after completing each administration of the liposomal irinotecan.

5. (Original) The method of claim 1, wherein the 5-fluorouracil is administered as an infusion over 46 hours.

6. (Original) The method of claim 1, wherein the leucovorin is administered immediately prior to the 5-fluorouracil.

7. (Original) The method of claim 1, wherein the liposomal irinotecan, oxaliplatin and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle.

8. (Original) The method of claim 1, wherein the liposomal irinotecan is administered as an infusion over a total of about 90 minutes.

9. (Original) The method of claim 1, wherein the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil.

10. (Original) The method of claim 1, wherein the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes.

11. (Currently Amended) The method of claim 1, wherein the liposomal irinotecan comprises irinotecan encapsulated in liposomes ~~composed of~~ comprising 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).

12. (Currently Amended) The method of claim 1, wherein the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes ~~composed of~~ comprising 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).

13. (Previously Presented) The method of claim 12, wherein the liposomal irinotecan, oxaliplatin, leucovorin, and 5-fluorouracil are administered beginning on days 1 and 15 of a 28-day treatment cycle; each administration of the liposomal irinotecan is administered prior to each administration of the leucovorin; each administration of the leucovorin is administered immediately prior to each administration of the 5-fluorouracil; and each administration of the 5-fluorouracil is administered as an infusion over 46 hours.

14. (Currently Amended) The method of claim 19, wherein the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes ~~composed of~~ comprising 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).

15. (Previously Presented) The method of claim 14, wherein the liposomal irinotecan, oxaliplatin, leucovorin, and 5-fluorouracil are administered beginning on days 1 and 15 of a

28-day treatment cycle; each administration of the liposomal irinotecan is administered prior to each administration of the leucovorin; each administration of the leucovorin is administered immediately prior to each administration of the 5-fluorouracil; and each administration of the 5-fluorouracil is administered as an infusion over 46 hours.

16. (Canceled)

17. (Canceled)

18. (Previously Presented) The method of claim 19, wherein each administration of the oxaliplatin begins after completing each administration of the liposomal irinotecan, and the method further comprises administering a corticosteroid and an anti-emetic to the patient prior to the antineoplastic therapy.

19. (Currently Amended) A method of treating metastatic adenocarcinoma of the pancreas in a human patient who has not previously received gemcitabine to treat the metastatic adenocarcinoma of the pancreas, the method comprising administering an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of administering to the patient a total of:
    a. 60 mg/m^2 of liposomal irinotecan,
    b. 60 mg/m^2 oxaliplatin,
    c. 200 mg/m^2 of <u>the</u> (l)-form of leucovorin or 400 mg/m^2 of the (l+d) racemic form of leucovorin, and
    d. 2,400 mg/m^2 5-fluorouracil;

    to treat the metastatic adenocarcinoma of the pancreas in the human patient.

20. (Canceled)

21. (Previously Presented) The method of claim 1, wherein the liposomal irinotecan, oxaliplatin, leucovorin, and 5-fluorouracil are administered beginning on days 1 and 15 of a 28-day treatment cycle; each administration of the liposomal irinotecan is administered prior to each administration of the leucovorin; each administration of the leucovorin is

administered prior to each administration of the 5-fluorouracil; and each administration of the 5-fluorouracil is administered as an infusion over 46 hours.

22. (Previously Presented) The method of claim 19, wherein the liposomal irinotecan, oxaliplatin, leucovorin, and 5-fluorouracil are administered beginning on days 1 and 15 of a 28-day treatment cycle; each administration of the liposomal irinotecan is administered prior to each administration of the leucovorin; each administration of the leucovorin is administered prior to each administration of the 5-fluorouracil; and each administration of the 5-fluorouracil is administered as an infusion over 46 hours.

23. (Previously Presented) The method of claim 1, wherein each administration of the oxaliplatin begins after completing each administration of the liposomal irinotecan, and the method further comprises administering a corticosteroid and an anti-emetic to the patient prior to the antineoplastic therapy.

BMC Cancer

RESEARCH ARTICLE Open Access

Vascular normalization in orthotopic glioblastoma following intravenous treatment with lipid-based nanoparticulate formulations of irinotecan (Irinophore C™), doxorubicin (Caelyx®) or vincristine

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Abstract

Background: Chemotherapy for glioblastoma (GBM) patients is compromised in part by poor perfusion in the tumor. The present study evaluates how treatment with liposomal formulation of irinotecan (Irinophore C™), and other liposomal anticancer drugs, influence the tumor vasculature of GBM models grown either orthotopically or subcutaneously.

Methods: Liposomal vincristine (2 mg/kg), doxorubicin (Caelyx®; 15 mg/kg) and irinotecan (Irinophore C™; 25 mg/kg) were injected intravenously (i.v.; once weekly for 3 weeks) in Rag2M mice bearing U251MG tumors. Tumor blood vessel function was assessed using the marker Hoechst 33342 and by magnetic resonance imaging-measured changes in vascular permeability/flow (K_{trans}). Changes in CD31 staining density, basement membrane integrity, pericyte coverage, blood vessel diameter were also assessed.

Results: The three liposomal drugs inhibited tumor growth significantly compared to untreated control ($p < 0.05$-0.001). The effects on the tumor vasculature were determined 7 days following the last drug dose. There was a 2-3 fold increase in the delivery of Hoechst 33342 observed in subcutaneous tumors ($p < 0.001$). In contrast there was a 5-10 fold lower level of Hoechst 33342 delivery in the orthotopic model ($p < 0.01$), with the greatest effect observed following treatment with Irinophore C. Following treatment with Irinophore C, there was a significant reduction in K_{trans} in the orthotopic tumors ($p < 0.05$).

Conclusion: The results are consistent with a partial restoration of the blood-brain barrier following treatment. Further, treatment with the selected liposomal drugs gave rise to blood vessels that were morphologically more mature and a vascular network that was more evenly distributed. Taken together the results suggest that treatment can lead to normalization of GBM blood vessel the structure and function. An *in vitro* assay designed to assess the effects of extended drug exposure on endothelial cells showed that selective cytotoxic activity against proliferating endothelial cells could explain the effects of liposomal formulations on the angiogenic tumor vasculature.

Keywords: glioblastoma multiforme vasculature normalization, liposomal drugs, endothelial cells

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Background

Glioblastoma (GBM) tumors are largely refractory to systemic treatments; the median survival time for patients with GBM is 10 months and the 2-year survival rate is less than 10%. Chemotherapy for GBM is compromised in part by the blood-brain barrier limiting drug access to the malignant cells. In addition, pre-clinical models showed that GBM tumors are poorly perfused [1,2] due to factors such as reduced blood flow rates, elevated hematocrit and interstitial fluid pressure, and an increase in geometric resistance [3-6], all of which impede drug delivery to the tumor tissue. Strategies which improve vascular function in GBM tumors should improve the delivery of other drugs capable of crossing the blood brain barrier and this should be associated with an increase in therapeutic activity.

Our laboratory has previously characterized and described the effects of a liposomal formulation of irinotecan (Irinophore C™) [7,8]. Encapsulation of irinotecan into liposomes improved the pharmacokinetic profile of the drug and its active metabolite, SN-38. More specifically, administration of Irinophore C™ resulted in a 1000-fold increase in the area-under-the-curve of plasma irinotecan concentration when compared to free drug (Camptosar). In addition, following irinophore C™ injection, the plasma levels of SN-38 were maintained at concentrations that were up to 40-fold higher than that achieved following injection of free drug [7]. Following irinophore C™ treatment, the s.c. (subcutaneous) colorectal tumors (HT-29) exhibited more functional tumor blood vessels, reduced hypoxia, and increased tumor perfusion. Importantly, these changes in tumor vasculature were associated with increased tumor uptake of doxorubicin and 5-FU given intravenously [8]. The latter data were consistent with the idea that the tumor vasculature in the treated tumors acquires a more "normal-like" function; an effect of anti-angiogenic therapies described as 'normalization' [9,10].

The primary goal of the studies reported here was to determine whether Irinophore C™ is efficacious in models of GBM, and whether treatment with this drug formulation would also result in normalization of GBM vasculature. The effects of Irinophore C™ on the growth rates and vascular function of the HT-29 colorectal cancer model was attributed to significant increases in the drug circulation lifetime and plasma concentration when encapsulated in liposomes [7,8]. We further reasoned that liposomal formulations of other drugs with known activity against proliferating endothelial cells should have preferential cytotoxicity towards angiogenic tumor vessels and could potentially also 'normalize' the chaotic and erratic vasculature of tumors. Thus, part of these studies assessed the effects of liposomal vincristine [11] and doxorubicin (Caelyx®) on tumor vasculature. Vincristine has previously been shown to be active against proliferating endothelial cells [12]. Liposomal formulations of doxorubicin have also been shown to have direct effects on tumor associated vasculature [13-15].

The data reported here assess the effects of Irinophore C™, Caelyx® (a commercially available and FDA-approved liposomal formulation of doxorubicin), and liposomal vincristine on tumor vasculature in subcutaneous and orthotopic models of GBM. The results indicate that Irinophore C™ was the most active formulation when using treatment endpoints based on changes in tumor size as well as tumor vascular morphology and function in GBM grown subcutaneously and orthotopically. The effects were consistent with the idea that following treatment, there was normalization of tumor vasculature. In the subcutaneous tumors, vascular 'normalization' was associated with increased tumor uptake of Hoechst 33342, while in the orthotopic glioma tumors, treatment-induced vascular 'normalization' was associated with decreased tumor uptake of Hoechst 33342.

Methods

Cell culture

Adult dermal human microvascular endothelial cells (d-HMVEC; Cambrex Bio Science, Walkersville, MD), Human brain microvascular endothelial cells (HBMEC; ScienCell Research Laboratories, San Diego, California) and U251MG glioblastoma cells (American Type Culture Collection, Manassas, VA) were characterized and authenticated by the cell banks using immunofluorescent methods and used for a maximum of eight passages for the endothelial cells and fifteen passages for U251MG. Stock cells lines were maintained in the absence of penicillin and streptomycin and screened for mycoplasma prior to preparing a stock of cells that were frozen for use in experiments. D-HMVEC cells were maintained in Endothelial Cell Basal Medium-2 (Clonetics®, Lonza, Basel, Switzerland) supplemented with 5 ng/mL Fibroblast Growth Factor, 20 ng/mL Vascular Endothelial Growth Factor, 10 ng/mL Epidermal Growth Factor (Clonetics®, Lonza), 10 unit/mL Heparin (Pharmaceutical Partners of Canada) 1% L-glutamine, 1% penicillin/streptomycin (Stem Cell Technologies, Vancouver, BC, Canada) and 10% Fetal Bovine Serum (FBS; Hyclone, Logan, UT), and plated in 1% gelatin (Sigma, Oakville, ON, Canada) pre-coated dish. HBMEC cells were maintained in Endothelial Cell Medium supplemented with Endothelial Cell Growth Supplement (ScienCell Research Laboratories) containing 5 μg/mL Insulin, 10 ng/mL Epidermal Growth Factor, 2 ng/mL Fibroblast Growth Factor, 2 ng/mL Insulin-like Growth Factor-1, 2 ng/mL Vascular Endothelial Growth Factor,

1 μg/mL hydrocortisone, 5% FBS and 1% penicillin/streptomycin, and plated in 15 μg/mL fibronectin (Sigma) pre-coated dish. U251MG cells were maintained in DMEM medium supplemented with 1% L-glutamine, 1% penicillin/streptomycin (Stem Cell Technologies, Vancouver, BC, Canada) and 10% FBS (Hyclone, Logan, UT). All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2, and used during exponential growth phase unless otherwise stated.

GBM animal model s.c. and orthotopic

All protocols involving work with live animals were reviewed and approved by the University of British Columbia Animal Care Committee (certificate of approval # A07-0423). For the subcutaneous GBM model, U251MG cells (5×10^6) were implanted subcutaneously into the backs of Rag2M mice (7-10 weeks old females, n = 9). To generate orthotopic GBM tumors, U251MG (7.5×10^4) cells were implanted into the right caudate nucleus-putamen (ML -1.5 mm; AP +1 mm; DV -3.5 mm) of mice (n = 5-6) using a stereotaxic injection frame (Stoelting Company, Wood Dale, IL). Animals were treated with 25 mg/kg Irinophore C™, 2 mg/kg liposomal vincristine or 15 mg/kg doxorubicin liposome (Caelyx®, Schering-Plough, QC, Canada) i.v. on day 21, 28 and 35 after inoculation. Dosing of liposomal vincristine and Caelyx® resulted in less than 5% body weight loss, while Irinophore C™ treatment did not cause any change in body weight. Previous tests in our laboratory have shown that the maximum tolerated single doses for Irinophore C™, Caelyx® and liposomal vincristine are >120 mg/kg, 17 mg/kg and 3 mg/kg, respectively. Irinophore C™ [16] and liposomal vincristine [17] were prepared as described previously. S.c. tumor size was measured throughout the study by caliper and tumor weights were extrapolated from the measurements using the following formula: mg = (tumor width^2 × tumor length)/2 [18]. Mice were injected with Hoechst 33342 (1.2 mg/mouse; Sigma) twelve (s.c. model) or twenty (orthotopic model) minutes prior to sacrifice on day 42. This timing was chosen based on previous study [8] and tests (not shown) aimed at determining the optimal timing for Hoechst 33342 injection without saturation of the tissue and before any decrease in Hoechst 33342 staining could be observed due to possible metabolic elimination. All animals were terminated by CO_2 asphyxiation and s.c. tumors or brains were harvested and cryopreserved in OCT (Sakura Finetek, CA) on dry ice and stored at -80°C.

Hoechst 33342, Ki67, CD31, VEGFR2, EF5, Collagen IV, NG2 and nuclei density staining and quantification

Optimal Cutting Temperature compound (OCT)-preserved s.c. tumors were cryosectioned using a Leica CM1850 Cryostat (Leica, ON, Canada) and 10 μm sections were collected in the middle of each tumor. OCT preserved brains were cryosectioned and 10 μm sections were collected from the Bregma +1.0 location. Sections were fixed in a 1:1 mixture of acetone:methanol for 15 minutes at room temperature, then blocked with blocking buffer (Odyssey blocking buffer, Rockland, PA) for 1 hour at room temperature. Sections were stained with rat anti-mouse CD31 antibody (1:100 dilution, PharMingen #550274, BD Biosciences), rabbit anti-human Ki-67 (Invitrogen #18-0191z; 1:100), rabbit anti-human/mouse vascular endothelial growth factor receptor 2 antibody (VEGFR2; 1:100; Cell Signaling technology #2479, NEB, Pickering, ON, Canada), rabbit anti-Collagen IV antibody (1:400, Abcam # ab19808, Cambridge, MA) and mouse anti-NG2 chondroitin sulfate proteoglycan antibody (1:100, Millipore # MAB5384, Billerica, MA). Primary antibodies were incubated on sections overnight at 4°C. Secondary antibodies (Alexa 488 goat anti-rat #A11006, Alexa 546 goat anti-rabbit #A-11035 and Alexa 633 goat anti-mouse #A-21126, 1:200, Invitrogen) were incubated for 1 hr at room temperature. Nuclei were stained with Draq5 (Biostatus, Leicestershire, UK; 1:200) for 30 min at 37°C. Slides were mounted with PBS and imaged for Alexa 488 (L5 filter), Hoechst 33342 (A4 filter), Alexa 546 (Cy3 filter), Cy5 (Cy5 filter) and Draq5 (Cy5 filter) using a robotic fluorescence microscope (Leica DM6000B, Leica, ON, Canada) and a composite color image of these markers was produced (Surveyor software, Objective Imaging Ltd.). Thresholds for each marker were set using Photoshop; the threshold level was set using a scale from 1 to 255 units, and was defined at 2 units higher than the minimal level necessary to obtain a negative signal for non-specific staining, and was kept the same for all sections. Acquired images were quantified for positive pixels or colocalization (double-positive pixels) using an in-house segmentation algorithm, normalized to the number of pixels in the tumor area and expressed as positive fraction (positive pixels divided by non-necrotic tumor area; MATLAB, The Mathworks, Natick, MA). Non-necrotic tumor areas were defined by cropping out necrotic and non-tumor tissue on the basis of positive Ki-67 and Draq5 co-stained sections and were quantified using the same in-house algorithm. Colocalization was considered positive when two positive pixels from one stain of interest were located within a 3 pixels radius from one pixel of the other stain of interest. Of note, one cell nucleus measures between 3 and 6 pixels. Blood vessel diameter was defined by taking 10 measurements/tumor section in a 15 × 15 cm box at 200% magnification using Photoshop, and was expressed in pixels. For differential analysis between the tumor's center and periphery, the boundary between the tumor center and periphery area was established at 20% of tumor diameter

distance from tumor margin. Another set of sections was stained with hematoxylin and eosin for histopathology analysis. The fraction of collagen IV-free blood vessels was defined as Collagen IV negative/CD31 positive pixels over total CD31 pixels. The fraction of NG2-free blood vessels was defined as NG2 negative/CD31 positive pixels over total CD31 pixels. The amount of basement membrane empty sleeves was defined as CD31 negative pixels/collagen IV positive pixels divided by the total non-necrotic tumor area.

Magnetic Resonance Imaging and K_{trans} measurement in U251MG orthotopic tumors

All magnetic resonance experiments were carried out using a 7.0 Tesla MR scanner (Bruker, Ettlingen Germany). A Bruker (Ettlingen, Germany) volume coil (inner diameter of 7 cm) and rectangular surface coil (1.7 × 1.4 cm) was used for signal transmission and reception respectively. The coil was tuned to the hydrogen proton frequency (300.3 MHz). The K_{trans} values were obtained from serial images acquired to monitor changes in the concentration of a MR-visible contrast agent (GD-DTPA; Bayer Schering Pharma) within each pixel, during the initial uptake and subsequent washout of the agent in the tumor. The MRI scans follow the protocol reported by Lyng et al. [19]; briefly, mice were anaesthetized with isofluorane (5% induction, 2% maintenance), a catheter inserted into the lateral tail vein and the animal was placed supine with its head above the surface coil. A proton-density weighted scan was first acquired to serve as a baseline for conversion of pixel intensity to absolute concentration values of the contrast agent. A volume equivalent to 10 uL per gram body weight of the contrast agent (0.03 M Gd-DTPA in saline) was injected via the tail vein catheter in a period of 10-15 seconds. The contrast series consisted of a 3D RF-spoiled Fast Low Angle Shot (FLASH) sequence with timing and resolution parameters as follows: echo time/repetition time = 2.8/9.2 ms, Field of view = 1.92 × 1.92 × 1.6 cm, Matrix size = 128 × 128 × 16 cm, acquisition time per image = 9.45 seconds. Twenty baseline scans were acquired before contrast agent injection and 250 scans were acquired afterwards, resulting in a total acquisition time of 43 minutes. The concentration-time curve for each pixel was fit to a two-compartment Kety model [20] which describes the pharmacokinetics of the contrast agent using three parameters: ve (volume of extracellular extravascular space), K_{trans} (volume transfer constant between the vasculature and tissue compartment) and Vp (fractional volume of the vascular compartment).

In vitro endothelial cell exposure and nuclei count

For proliferative conditions, Dermal Human MicroVascular Endothelial Cells (d-HMVEC; 600 cells/well) and Human Brain Microvascular Endothelial Cells (HBMEC; 5000 cells/well) were plated in black 96-well plates (Optilux™, BD Biosciences, Mississauga, ON, Canada) and drugs were added the day after. For non-proliferative conditions, d-HMVEC cells (5000 cells/well) and HBMEC (50000 cells/well) were plated in black 96 well plates and drugs were added four days after. Irinotecan (Sandoz, QC, Canada), SN-38 (LKT Laboratories, MN, USA), vincristine (Novopharm, ON, Canada), docetaxel, paclitaxel (Taxol®; Bristol Myers Squibb Canada, QC, Canada) and doxorubicin (Adriamycin™/MC, Pfizer, QC, Canada) were added in concentrations ranging from 1-100,000 picoMolar on cells and replaced daily for 7 days. At the end of drug treatment, cells were fixed with 3.5% paraformaldehyde (Electron Microscopy Sciences, PA) for 15 minutes at -20°C, permeabilized with 0.1% Triton (Perkin-Elmer, MA) in PBS for 10 minutes at room temperature, blocked for 1 hr at 4°C (Odyssey blocking buffer, Rockland, PA) and incubated overnight with Ki67 antibody (Invitrogen #18-0191z; 1:100 dilution in blocking buffer). Cells were then incubated with Anti-rabbit Alexa 488 secondary antibody (Molecular Probe #A11034, Invitrogen; 1:200 in blocking buffer) for 1 hr at room temperature. Nuclei were stained with Draq5 dye (Biostatus, Leicestershire, UK; 1:200 in PBS) for 30 min at 37°C. Twenty fluorescent photographs/well (Alexa 488 emission: 475 nm, excitation: 535 nm; Draq5 emission: 620 nm, excitation: 700 nm) were taken at 10 × magnification using an InCell Analyzer 1000 (Amersham Bioscience) and the total nuclei count (Draq5 stained nuclei) as well as Ki67 expressing nuclei count (Draq5 and Alexa 488 double stained nuclei) were quantified using InCell Developer Toolbox software (Amersham Bioscience, GE Healthcare, Baie d'Urfe, QC, Canada). Dose-response curves generated from total nuclei count were used to calculate drug concentrations causing a decrease in endothelial cell nuclei count by 20% (fraction affected: Fa = 0.2), 50% (Fa = 0.5), 75% (Fa = 0.75) and 90% (Fa = 0.9) and compared for both proliferative and non-proliferative cells. All data points represent the average of 3 independent experiments in triplicate +/- S.E.M.

Statistical analysis

All statistical data was collected using GraphPad Prism (San Diego, CA). Because all treatment drugs were chosen based on previous rationale justifying their inclusion in the study, the experimental design should not be regarded as a screening assay and statistical analysis was done using the single comparison non-parametric two-tailed Mann Whitney test and no correction was made for multiple comparisons. All data are expressed +/- S.E.M.

Results

Irinophore C™, Caelyx® and liposomal vincristine inhibit tumor growth and increase Hoechst 33342 delivery in subcutaneous GBM tumors

Rag2M mice bearing s.c. U251MG tumors (n = 9) were treated i.v. weekly for 3 weeks with 25 mg/kg Irinophore C™, 15 mg/kg Caelyx® and 2 mg/kg liposomal vincristine. Tumor growth was monitored during the entire treatment period, and tumors were harvested 7 days after the last treatment. As noted in Figure 1a, the three drugs inhibited tumor growth significantly compared to untreated control ($p < 0.05$-0.001). At the end of the study (day 42), the weight of treated tumors ranged from 34 to 80 mg compared to an average of 502 mg for untreated control animals. A representative tumor section (H&E) derived from each treatment group is also provided in Figure 1a. The total non-necrotic tumor area (excluding necrotic and non-tumor area) measured in number of image pixels for each treated group is summarized in Figure 1b. The measurements of area of viable tumor tissue correlated with the tumor weight measurement and was significantly reduced for all treatment groups (compared to untreated tumors; $p < 0.0001$). The proliferation marker Ki67 was used to estimate the fraction of viable cells undergoing active proliferation within the tumor (positive Ki67 staining divided by total viable tissue, expressed as Ki67 positive fraction). Liposomal vincristine had no apparent effect on the Ki67 staining compared to control tumors. Treatment with Irinophore C™ caused a 2-fold decrease in Ki67 staining ($p < 0.01$). In contrast, a significant ($p < 0.01$) increase in Ki67 staining was observed in tumors from animals treated with Caelyx® (Figure 1b). It should be noted that Caelyx® treatment was also associated with enlarged tumor cell nuclei (see arrow heads in insert H&E image Figure 1a) and this may suggest that the treatment promoted cell cycle arrest [21]. This observation is in accordance with previously published findings on the effects of doxorubicin on cell cycle [22-24] and the fact that cellular Ki67 antigen has been shown to accumulate in some types of cell cycle arrest [25]. Finally, a decrease in number of cell nuclei per area (nuclei density) with a concomitant increase in connective tissue was observed by examination of the H&E stained sections in tumors from mice treated with Irinophore C™.

The effects of the selected liposomal drugs on tumor blood vessels were also evaluated. As summarized in Figure 1c, the CD31 staining (positive CD31 fraction) did not change significantly when comparing tumors from control animals to those from treated animals. Prior to sacrifice, animals were injected with Hoechst 33342, a marker for tumor perfusion that was previously validated by correlation with K_{trans} measurements [8]. Total Hoechst 33342 staining in viable tissue (positive Hoechst 33342 fraction) was increased in the tumors obtained from treated animals ($p < 0.01$-0.001; Figure 1c). CD31 and Hoechst 33342 co-staining was measured to provide an indication of changes in functional blood vessels [8]. The results, summarized in Figure 1c, indicate that the number of functional blood vessels increased significantly ($p < 0.05$) in Caelyx® treated tumors while there were no significant changes observed in tumors from Irinophore C™ and liposomal vincristine treated animals.

Irinophore C™, Caelyx® and liposomal vincristine inhibit tumor growth and decrease Hoechst33342 delivery in orthotopic GBM tumors

Rag2M mice (n = 5 or 6) were inoculated with U251MG cells orthotopically (see Methods) and 21 days later the animals were treated i.v. (once weekly for 3 weeks) with 25 mg/kg Irinophore C™, 15 mg/kg Caelyx® and 2 mg/kg liposomal vincristine. Forty-two days after cell inoculation, animals were sacrificed and their brains harvested. A representative tissue section (Hematoxylin and Eosin; H&E) showing the site of tumor growth (dark blue) within the brain of treated animals is provided for each treatment group in Figure 2a. Insert images have been included to show that following treatments, the tumor nuclear density drops slightly when compared to untreated controls. The average total non-necrotic tumor tissue in the tumor area for each treatment group was quantified to provide a measure of efficacy (Figure 2b). There was a significant reduction in tumor area for all treatment groups when compared to controls ($p < 0.0001$). In contrast to the results obtained with the s.c. glioma model, there was no significant changes in Ki67 staining observed following treatment (Figure 2b).

Prior to sacrifice, animals were also injected with Hoechst 33342. In tumors from untreated control mice, Hoechst 33342 staining was significantly greater in tumor tissue compared to matched regions of normal brain tissue (0.398 +/- 0.083 and 0.023 +/- 0.015 pixels/unit area, respectively; $p < 0.01$; data not shown). This staining pattern has been described elsewhere [26,27] and is consistent with the fact that Hoechst 33342 does not cross the blood-brain barrier. Interestingly, the data summarized in Figure 2c show that Hoechst 33342 staining in the orthotopic tumor tissue from animals treated with the liposomal drugs was significantly reduced ($p < 0.01$) when compared to tumors from control animals. The decrease in Hoechst 33342 staining in orthotopic tumors from treated animals was in marked contrast to treatment-induced increases in

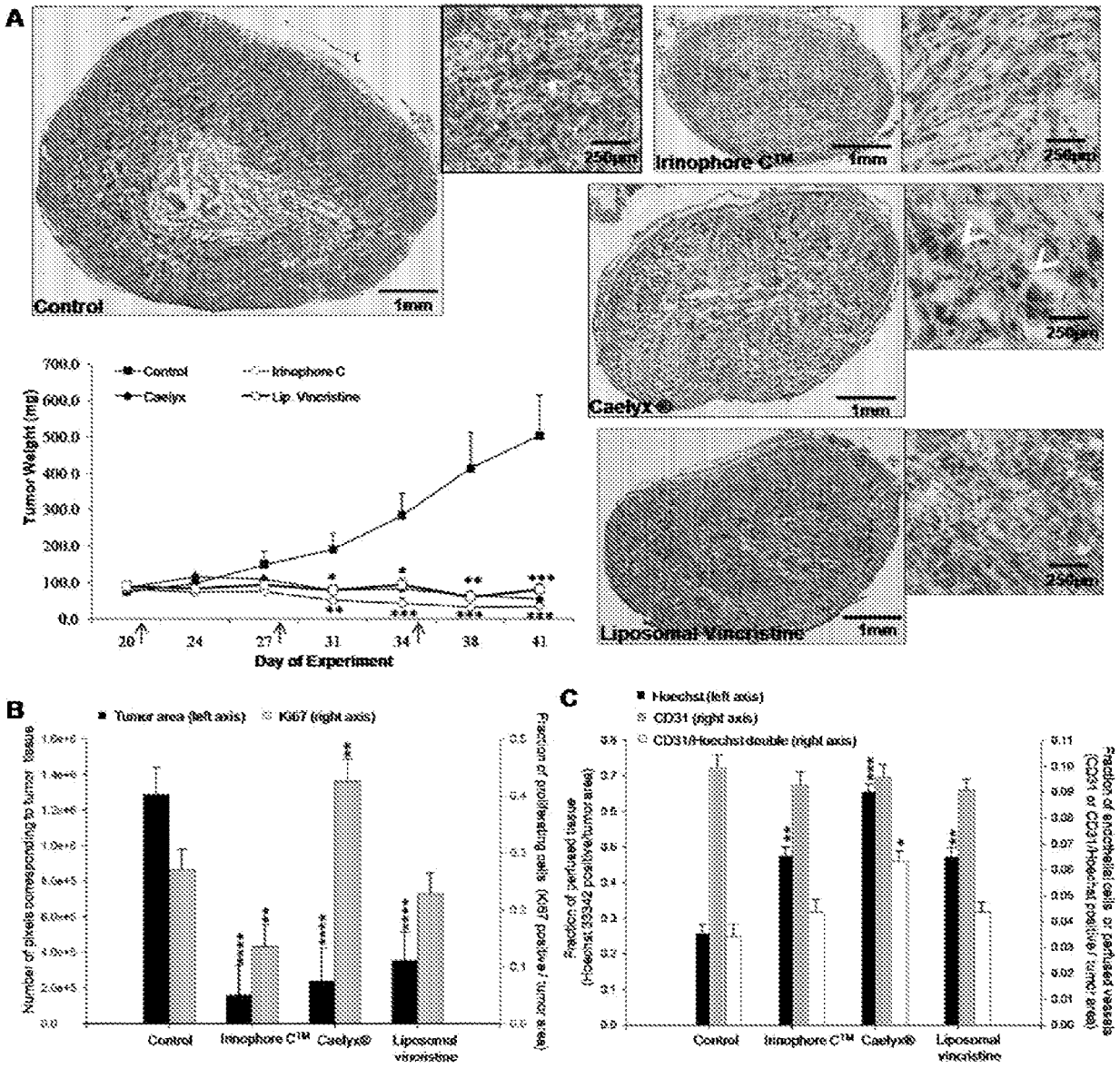

Figure 1 Irinophore C™, liposomal vincristine and Caelyx® significantly inhibits tumor growth, decreases proliferation and increases tumor perfusion in subcutaneous GBM tumors. a) Representative H&E sections of tumors from each treatment group show the efficacy of the treatments in controlling tumor growth. Arrow heads indicate enlarged nuclei associated with Caelyx® treatment. Tumor weights were calculated on the basis of caliper measurements; arrows indicate the treatment days. The Irinophore C™ statistical significance is indicated by bottom stars, while Caelyx® and liposomal vincristine statistical significances are indicated by top stars (*p-value ≤ 0.05; **p-value ≤ 0.01; ***p-value ≤ 0.001) b) The area of viable tissue in tumor sections following treatment was expressed in number of pixels and correlates well with tumor volumes (■, left axis). The fraction of viable, actively proliferating cells (■, right axis) in the tumors was significantly decreased by Irinophore C™. Ki67 staining was also increased in Caelyx®-treated tumors. c) Hoechst 33342 perfusion in the tumors was increased significantly by Irinophore C™ and Caelyx® treatment (■, left axis). The number of endothelial cells per unit area of viable tissue was unchanged by the treatments (■, right axis); however, the fraction of endothelial cells that were perfused (CD31 and Hoechst 33342 positive; □, right axis) was increased by treatment with Caelyx®. Statistical significances are indicated (*p-value ≤ 0.05; **p-value ≤ 0.01; ***p-value ≤ 0.001).

Hoechst 33342 staining noted for tumors derived from the same cell line (U251MG) and grown subcutaneously (Figure 1c).

No significant changes in overall CD31 staining (pixels/unit area) (Figure 2c) were noted in the orthotopic tumors obtained from treated animals (compared to controls). However, CD31/Hoechst 33342 co-staining was significantly reduced ($p < 0.01$-0.05) in tumors from treated animals when compared to control animals (Figure 2c). Moreover, treatment of orthotopic tumor

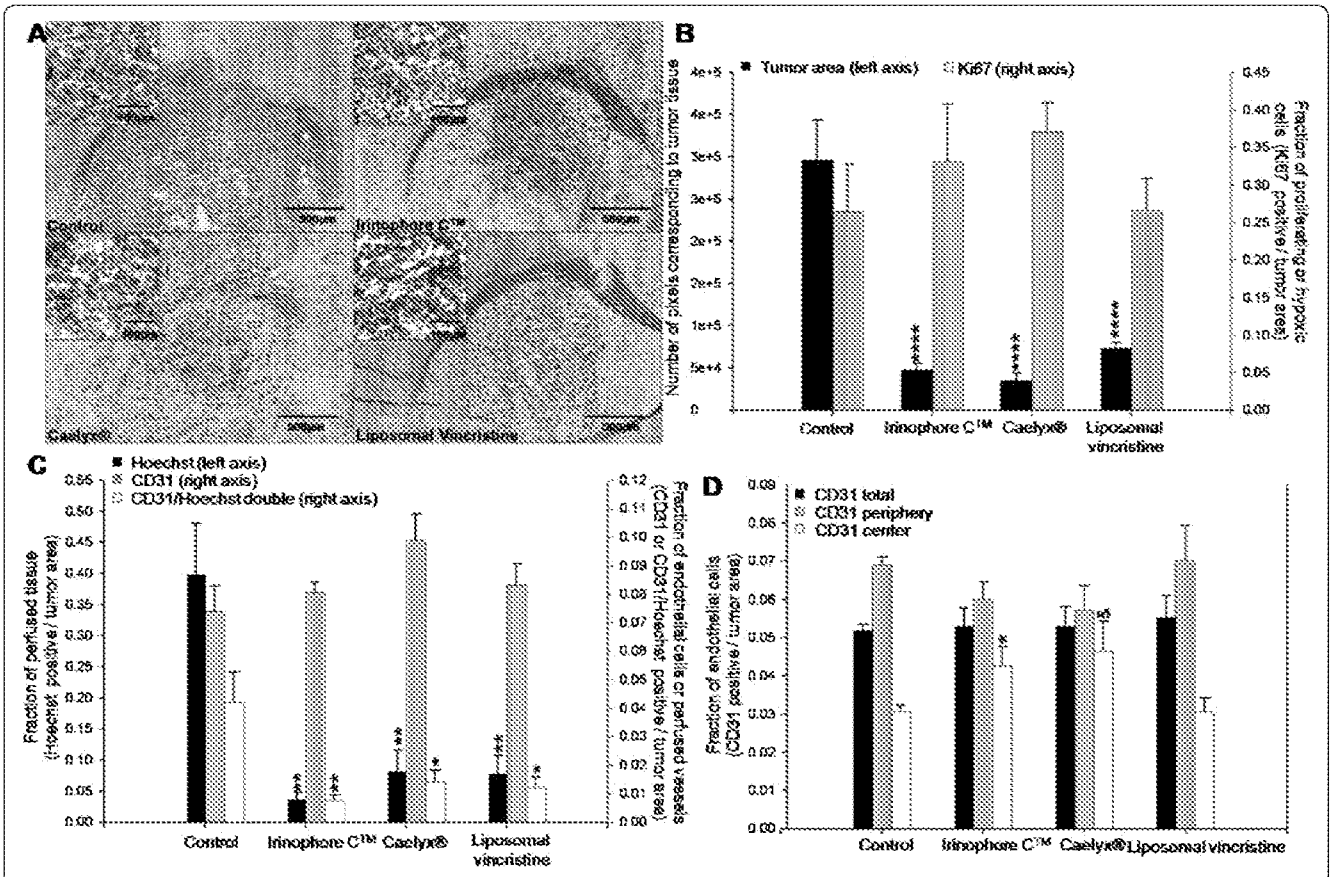

Figure 2 Orthotopic GBM tumors treated with Irinophore C™, liposomal vincristine and Caelyx® are significantly smaller than untreated controls. a) Representative H&E images of brain sections from mice in each treatment group show that the area of tumor tissue (dark blue) from treated animals are smaller than untreated controls. b) Tumor areas were quantified in number of pixels, and used as a measure of treatment induced reduction of the tumor mass (■, left axis). No significant changes in proliferative activity (■, right axis) were observed. c) Hoechst 33342 staining was reduced significantly following treatments with the three liposomal treatments (■, left axis). The total number of endothelial cells per unit area of viable tissue (■, right axis) was unchanged across all groups, but the fraction of endothelial cells that were co-stained with Hoechst 33342 was significantly reduced (□, right axis). d) The density of endothelial cells (positive CD31 pixels divided by periphery or center tumor area pixels) in the center of tumors treated with Irinophore C™ was significantly higher compared to control tumors (□). No changes in endothelial cell density were seen in the total tumor area (■) or the periphery of tumors (■). Statistical significances are indicated (*p-value ≤ 0.05; **p-value ≤ 0.01; ***p-value ≤ 0.001). Non-significant trends are indicated (&p-value = 0.067).

bearing animals with Irinophore C™ was associated with a significant ($p < 0.05$) increase in CD31 staining in the center of tumors when compared to untreated tumors (Figure 2d; $p < 0.05$).

Assessing vascular normalization in GBM tumors from animals treated with Irinophore C™, Caelyx® or liposomal vincristine

Several structural determinants, described as indicators of vascular normalization [28-30], were assessed in the orthotopic and s.c. GBM tumor models following treatment and these data were compared to tumors from untreated control animals. The parameters evaluated included: (i) the extent of discontinuous basement membrane (collagen IV-free CD31 pixels) in the tumor tissue, (ii) the fraction of pericyte-uncovered blood vessels (NG2-free CD31 pixels) in the tumor tissue and (iii) the blood vessel diameter. Furthermore, the proportion of empty basement membrane sleeves (CD31-free collagen IV pixels) was evaluated as an indication of regression of pre-existing blood vessels [9]. Treatment-induced changes in these factors are summarized in Figures 3 (s.c. tumors) and 4 (orthotopic tumors).

In s.c. GBM tumors, the fraction of NG2-free blood vessels was reduced by 25% in tumors from animals treated with Irinophore C™ ($p < 0.05$; Figure 3a). Decreases in NG2-free blood vessels were also noted in tumors from animals treated with Caelyx® ($p = 0.071$) or liposomal vincristine ($p = 0.121$); but the effects were not considered significant. The number of collagen IV-free blood vessels was decreased in s.c. tumors from animals treated with Irinophore C™ or Caelyx® (41-75%

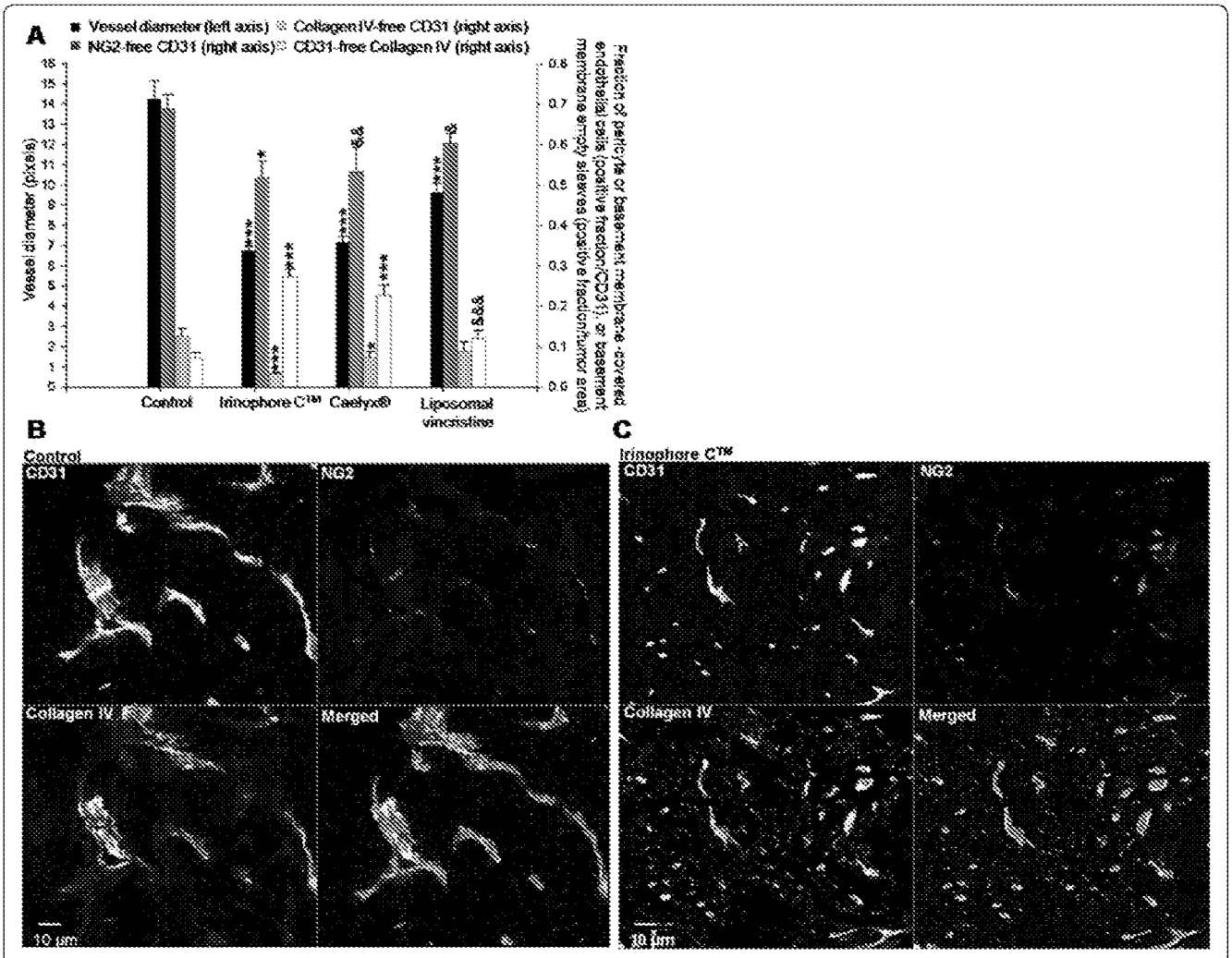

Figure 3 Irinophore C™, liposomal vincristine and Caelyx® treatments are associated with vascular normalization of the tumor vasculature in subcutaneous GBM tumors. a) The diameters of tumor blood vessels were reduced significantly by all three treatments compared to control tumors (■, left axis). The fraction of NG2-free CD31 pixels (■, right axis), collagen IV-free CD31 pixels (■, right axis) were reduced in Irinophore C™ treated tumors indicating that fewer immature vessels are present following treatments. The proportion of empty basement membranes (CD31 free-collagen IV staining; □, right axis) in the viable tissue was also reduced by all liposomal treatments. b and c) Representative and merged images of CD31, Collagen IV and NG2 staining for control tumors (b) and tumors from Irinophore C™ treated mice (c). A reduction in blood vessel diameter (CD31; green) and an increase in basement membrane coverage of blood vessels (collagen IV; yellow) following treatment can be seen. Following Irinophore C™ treatment, more pericytes are present (NG2; red) and more endothelial cells are associated with the pericytes (merged image, green and red). Treatment with Irinophore C™ also results in an increase in empty basement membrane sleeves (i.e. CD31 free-collagen IV; yellow). The entire image represents non-necrotic and viable tissue. Statistical significances are indicated (*p-value ≤ 0.05; **p-value ≤ 0.01; ***p-value ≤ 0.001; ****p-value ≤ 0.0001). Non-significant trends are indicated (&p-value = 0.121; &&p-value = 0.071; &&&p-value = 0.054).

decrease; $p < 0.05$-0.001; Figure 3a). Blood vessel diameter was also reduced (32%-51%; $p < 0.001$) in s.c. tumors from all treatments groups. Finally, the number of empty basement membrane sleeves in tumors from Irinophore C™ and Caelyx® treated animals was increased 3.4- to 3.8-fold following treatment ($p < 0.0001$). A similar effect was noted for tumors from animals treated with liposomal vincristine, but the effect was not considered significant ($p = 0.054$). Representative immunofluorescence micrographs highlighting the effects of Irinophore C™ treatment on the tumor vasculature of s.c. U251MG tumors (Figure 3c) compared to untreated tumor (Figure 3b) are provided to support the results summarized in Figure 3a.

Similar results were obtained when evaluating the orthotopic U251MG tumors from treated animals compared to controls. In addition, histological assessments of brain tissue surrounding the tumor allowed

comparisons between vessels in the tumor vs. normal brain tissue. The fraction of collagen IV-free blood vessels in normal brain (0.049 +/- 0.015) was 69% lower ($p < 0.05$) than that observed in tumor tissue from control untreated animals (0.160 +/- 0.033), indicating that the organization of the basement membrane architecture is decreased in the tumor compared to normal tissue (data not shown). Tumors from animals treated with Irinophore C™ showed a significant 71% ($p < 0.05$) decrease in the fraction of collagen IV-free blood vessels when compared to tumors from control animals (Figure 4a). A similar effect was observed in tumors from animals treated with Caelyx®, but the effect was not considered significant ($p = 0.064$). In normal brain tissue, blood vessel diameters were 54% smaller (4.9 +/- 0.5 pixels; $p < 0.0011$) than blood vessel diameters observed in orthotopic tumor tissue obtained from untreated animals (10.9 +/- 0.6 pixels; data not shown). Orthotopic tumors from animals treated with Irinophore C™ or Caelyx® exhibited a reduction in blood vessels diameters of 39% ($p < 0.01$; Figure 4a) when compared to control tumors. In contrast to results obtained with the s.c. tumors of treated animals, the level of empty basement membrane sleeves (Collagen IV-free CD31 staining) in the orthotopic tumors did not change following treatment (Figure 4a). It should be noted that the level of empty basement membrane sleeves in the normal brain tissue (0.035 +/- 0.009) was found to be similar to that measured in orthotopic tumor tissue from untreated animals (0.047 +/- 0.009) (data not shown). Treatments did not induce significant changes in fraction of NG2-free blood vessels (Figure 4a). The fraction of NG2-free vessels in the normal brain could not be evaluated as NG2 proteoglycan was found at the surface of polydendrocytes, a subpopulation of glial cells found in the brain [31]. Representative immunofluorescence micrographs illustrating the effects of Irinophore C™ treatment on the orthotopic tumor vasculature are provided in Figure 4b. Normal brain tissue sections are shown in Figure 4c for comparison.

Magnetic resonance imaging-measured changes in vascular permeability/flow (K_{trans})

The results summarized thus far are consistent with the idea that following treatment of animals bearing GBM tumors with lipid-based nanopharmaceutical formulations of vincristine, doxorubicin and irinotecan, there is a "normalization" of blood vessel structure. When considering these effects along with the antitumor activity, the greatest effects were observed following treatment with Irinophore C™. In order to confirm the idea of a Irinophore C™-induced vascular normalization, non-invasive magnetic resonance imaging was used to assess K_{trans}, a volume transfer constant of a solute between the blood vessels and extra-cellular tissue compartment, in orthotopic tumors grown in untreated and Irinophore C™treated mice. The median values of K_{trans} for the tumors within the control and treated groups have been summarized Figure 5. The results demonstrate that the median K_{trans} value in untreated tumors was ~7 times greater than in treated tumors (0.0232 and 0.0034 ml/g/min, respectively, $p < 0.05$). It should be noted that the values for K_{trans} in tumors from untreated animals were more variable when compared to the tumors from treated mice (s.e.m ± 0.010 and ± 0.0003, respectively).

In vitro studies on endothelial cells mimicking the extended drug exposure achieved when using liposomal drug delivery formulations

In an attempt to better understand the effects of liposomal formulations used here on tumor vasculature, an *in vitro* endothelial cell assay was used to assess the impact of extended drug exposure. It is well established that these liposomal formulations engender significant increases in plasma drug concentrations over extended time periods following intravenous administration [7,11]. Thus, an extended drug exposure protocol was used to assess the effects of drugs in a model representative of the endothelial cells forming vessels in the subcutaneous or brain microenvironment. Dermal Human MicroVascular Endothelial Cells (d-HMVEC) and Human Brain Microvascular Endothelial Cells (HBMEC) were cultured under proliferative or non-proliferative conditions and exposed to the indicated drugs for 7 days. As illustrated in Figure 6a, the total nuclei count and the number of nuclei expressing the Ki67 proliferation marker were quantified using high content screening (Incell analyzer 1000) to discriminate between cytotoxic (reduction in total number of nuclei) and cell proliferation inhibitory effects (reduction in Ki67 expressing fraction). Under proliferative conditions, the nuclei count for the endothelial cell lines used increased up to 3-fold over the 7 day time period. The Ki67 expressing nuclei fraction ranged from 42 to 68% over this time frame (Figure 6a and 6b). Under non-proliferative conditions, the nuclei count for cell lines (d-HMVEC and HBMEC) remained unchanged from day 1 to day 7, and the Ki67 expressing nuclei fraction ranged from 7 to 31% (Figure 6a and 6b).

The activity of the drugs against the cells maintained under the two conditions was compared to assess the selectivity of the drugs for proliferating endothelial cells compared to non-proliferating endothelial cells. For all drugs used in this study, the dose-response curves for Ki67 expressing nuclei of proliferating cells matched the ones for the total nuclei count, suggesting that the drugs tested were cytotoxic rather than

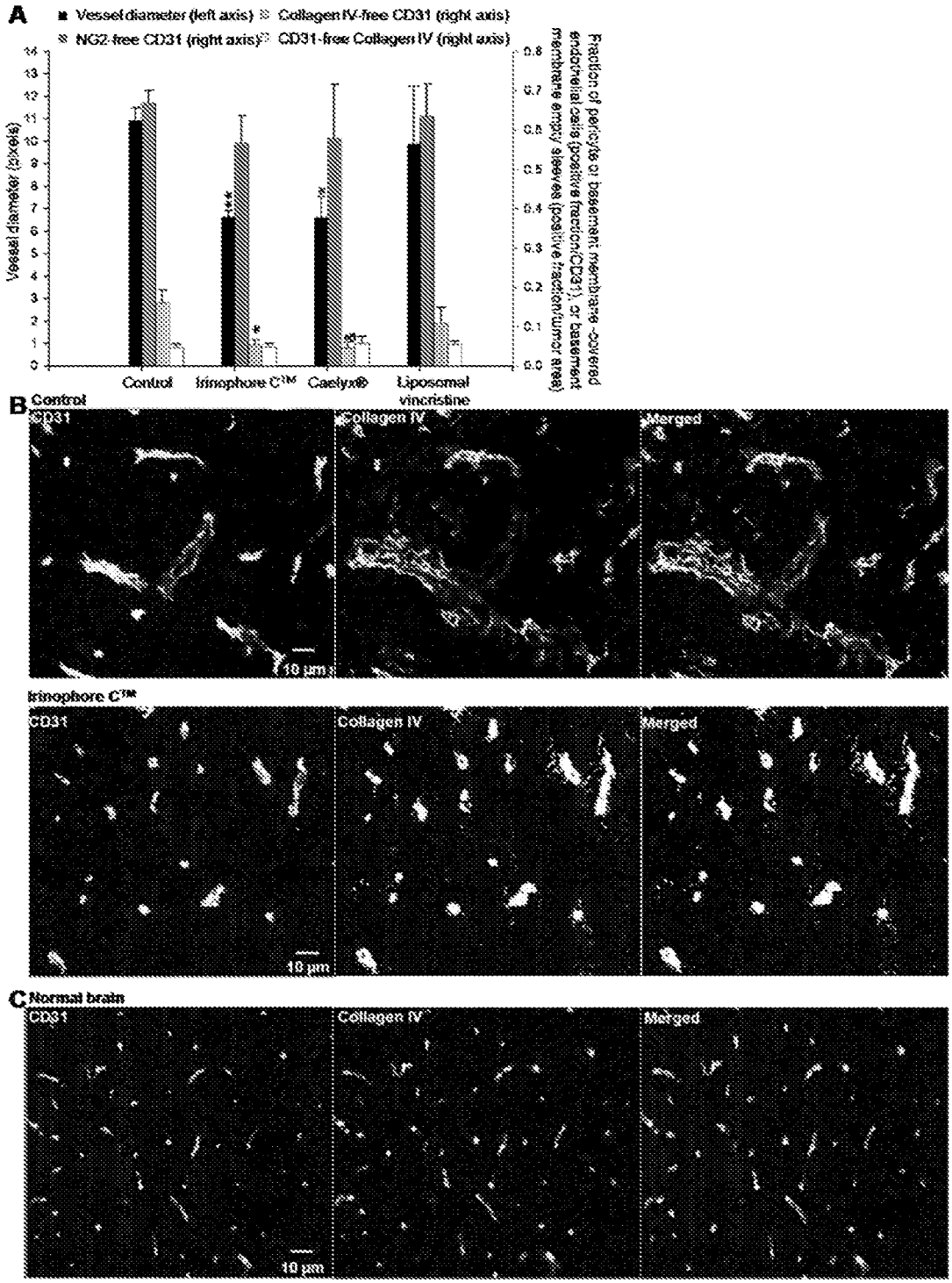

Figure 4 Irinophore C™, liposomal vincristine and Caelyx® treatments are associated with vascular normalization of the tumor vasculature in orthotopic GBM tumors. a) Vessel diameters (■, left axis) and the fraction of collagen IV-free CD31 pixels (■, right axis) in orthotopic GBM tumors were reduced by Irinophore C™ and Caelyx®. However, no changes were seen in the fraction of NG2-free CD31 positive endothelial cells (■, right axis) or Collagen IV-free CD31 positive endothelial cells (□, right axis). b) Representative images from untreated and Irinophore C™ treated tumors; similar images for normal brain tissue are shown for comparison. (c) Blood vessel diameters (CD31; green) are reduced by Irinophore C™ treatment. The basement membrane (collagen IV; yellow) is partially restored by treatment with Irinophore C™. The entire image represents non-necrotic and viable tissue. Statistical significances are indicated (*p-value ≤ 0.05; **p-value ≤ 0.01). Non-significant trends are indicated (&p-value = 0.064).

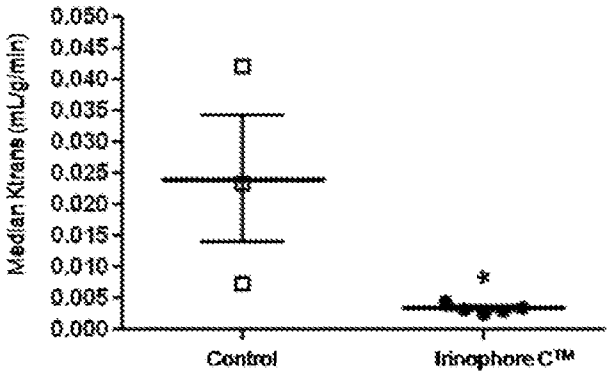

Figure 5 Irinophore C™ reduced K_{trans} measures compared to values obtained from control orthotopic tumors. Individual and median (thick line) K_{trans} values for untreated and Irinophore C™ treated orthotopic tumors with standard error of the mean (thin line). Statistical significance is indicated (*p-value ≤ 0.05).

anti-proliferative. Representative dose-response curves for d-HMVECs and HBMECs exposed to SN-38, the active metabolite of irinotecan, under proliferative and non-proliferative conditions are shown in Figure 6b. The data indicates that SN-38 is significantly more active against proliferating endothelial cells then non-proliferating cells. In an effort to highlight differences in drug activity under proliferating and non-proliferating conditions, drug concentrations decreasing d-HMVEC total nuclei count by 20% (fraction affected: Fa = 0.2), 50% (Fa = 0.5), 75% (Fa = 0.75) and 90% (Fa = 0.9) were calculated from the dose-response curves and compared for both proliferative and non-proliferative cells (Figure 7). For example, results obtained at Fa = 0.75 indicate that the greatest differential effects were observed when using SN-38 and vincristine, where the drug dose required to achieve a 75% decrease in nuclei count under proliferation conditions were at least 100- and 90.9-times lower, respectively, than the drug dose required to achieve the same effect level under non-proliferative conditions. These effects were much greater than those seen using the positive control compounds docetaxel and paclitaxel. In contrast, there was little or no difference in the concentrations of irinotecan or doxorubicin required to achieve a Fa of 0.75 under proliferating and non-proliferating conditions. Similar results were obtained when using HBMECs (Figure 8). It should be noted that the drug doses required to achieve a Fa value of 0.5 for SN-38 and vincristine was 45 to 5000 times greater for U251MG glioblastoma cells when compared to the proliferating endothelial cells (data not shown) and the increased specificity for proliferating endothelial cells has been noted previously for paclitaxel and SN-38 when compared against human colorectal and breast cancer cells [32,33].

Discussion

Studies over the last few decades have established that liposomal formulations of selected antineoplastic agents can be more effective than the same drug administered in free form. Liposomal formulations of anticancer drugs are known to have long circulation half-lives *in vivo*, and release the drug slowly over time [7,11]. Thus, the pharmacological properties of a drug given in its free form (e.g. via bolus injection or slow infusion) is changed dramatically by encapsulation in liposome. As a result, one might anticipate that the use of liposomal drugs will expose tumors to drugs for extended periods of time when compared to treatment with the free drug. This, of course, is well established in the literature and has been explained on the basis of the enhanced permeability and retention effect known to promote accumulation of intravenously administered liposomal drug formulations in tumors [34]. What is often not considered in studies with liposomal formulations is that these formulations constantly release the associated drug while in the circulation compartment, thereby extending the presence of the drug in the plasma compartment. This study tries to address whether part of the treatment benefits could be attributed to direct effects of the free drug (available in the blood compartment) on tumor vascular endothelial cells. The fact that these drug formulations are active against proliferating vasculature was anticipated, but not demonstrated to date. Liposomal drug formulations are known to accumulate and release drugs in close proximity to tumor blood vessels [14,15]. More intriguing, however, is the possibility that exposing the tumor vasculature to low concentrations of drug for extended periods may produce effects that are comparable to the vascular normalization effects described in the context of anti-angiogenic therapy [9,10] as discussed below.

In the present study, it is demonstrated that Irinophore C™, Caelyx® and liposomal vincristine are effective against GBM grown subcutaneously or orthotopically (in the brain). The tumor masses in treated animals were significantly smaller compared to control ($p < 0.001$; Figure 1a), indicating that the liposomal drugs used in this study are potent against GBM, regardless of the site of tumor growth. Analysis of the tumor tissue, and in particular the vascular morphology, also indicates that treatments affected the tumor vasculature to various degrees. Overall, Irinophore C™ impacted the vasculature to a greater extent than the other formulations, and generated tumors with blood vessels that were morphologically more mature. In the subcutaneous model, Irinophore C™ restored the basement membrane architecture, increased the pericyte coverage and reduced blood vessel diameters. The data

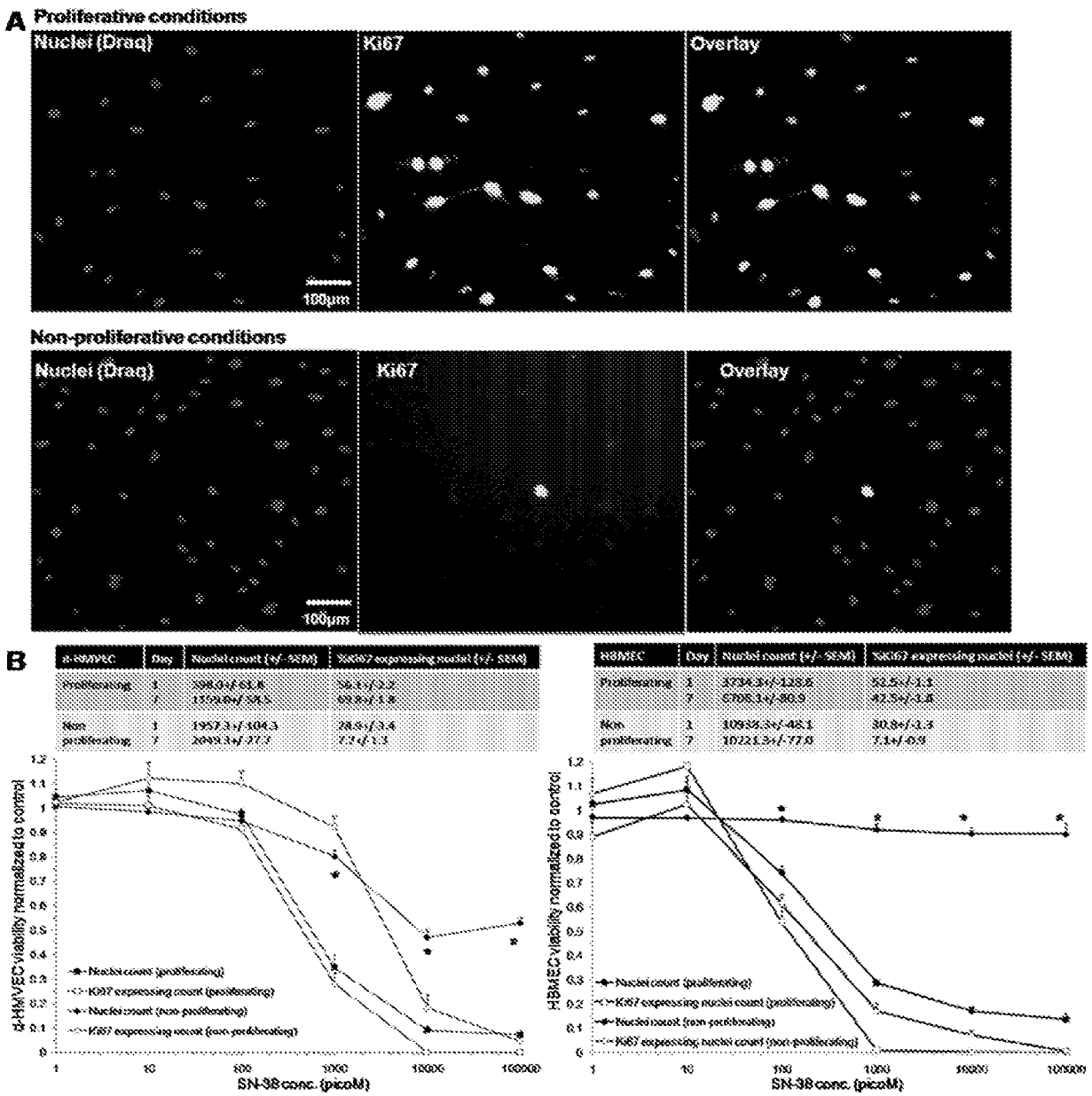

Figure 6 d-HMVEC and HBMEC were plated under proliferative conditions or non-proliferative conditions. a) Representative composite color images of d-HMVEC cells are shown; Draq (blue; nuclei), and Ki67 (green). Under proliferative conditions, the number of nuclei and Ki67 positive staining are similar; whereas under non-proliferative conditions, the number of nuclei with positive Ki67 staining is much lower. b) Total nuclei count and Ki67 expressing nuclei fraction of untreated cells for both cell lines under proliferative and non-proliferative conditions on day 1 and day 7 after plating (3 independent experiments; 3-21 replicates per experiment). Cells were exposed for 144hrs to increasing drug concentrations (1-100,000 picoM). Total nuclei count as well as nuclei expressing Ki67 expressing counts were normalized to counts obtained from control untreated cells. Representative data for d-HMVEC and HBMEC exposed to SN-38 is shown (3 independent experiments; 3 replicates per experiment +/- SEM). Statistical significance is indicated (*p-value < 0.0001) between total nuclei count of proliferative and non-proliferative cells.

suggest a restoration of the vessel architecture to a more normal state. In the more clinically relevant orthotopic model, Irinophore C™ treatment restored the basement membrane architecture and reduced blood vessel diameters of the tumor vasculature, again suggesting a restoration of the vessel architecture to a more normal state. Irinophore C™ treatment also increased the quantity of vessel staining in the center of tumors, suggesting a more homogenous distribution of blood across the entire tumor. Further, Irinophore C™ reduced K_{trans}

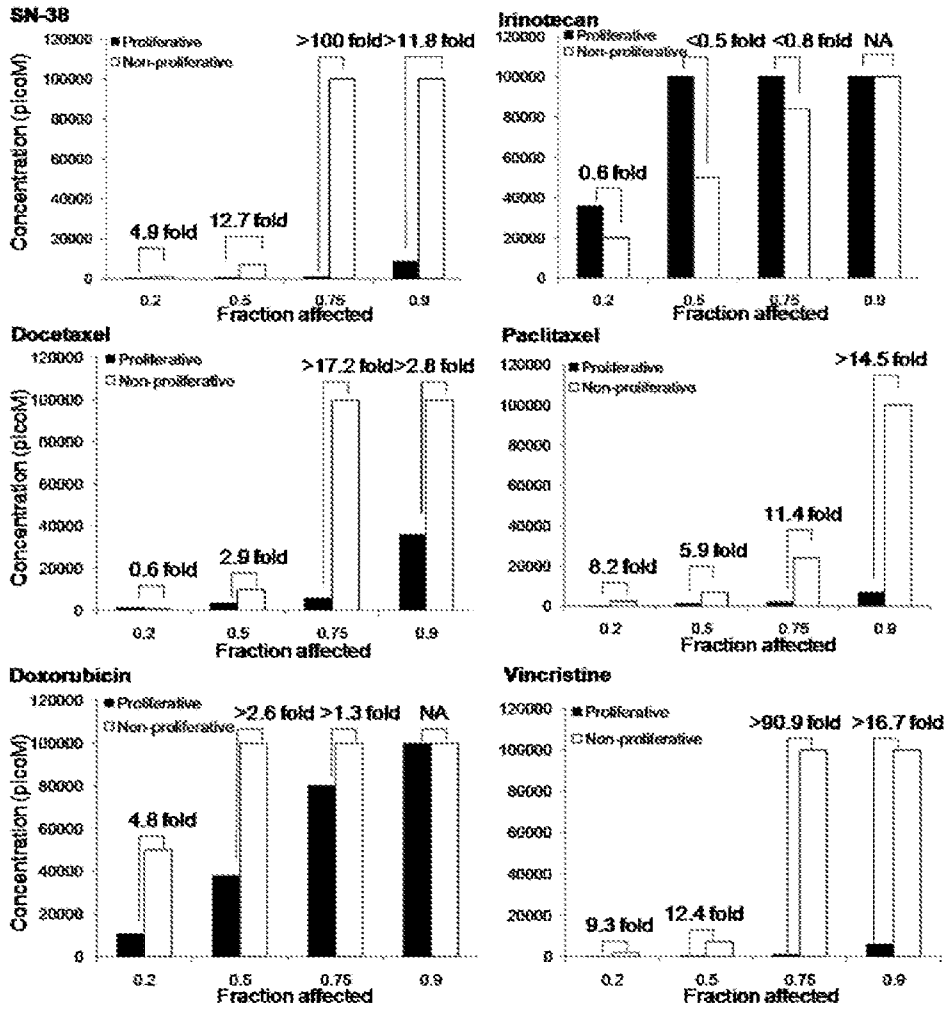

Figure 7 Proliferating HMVEC cells are more sensitive to SN-38, docetaxel, paclitaxel, doxorubicin and vincristine than non-proliferating cells. Concentrations at which a Fa of 0.2-0.9 was observed in d-HMVEC total nuclei count for both proliferative and non-proliferative conditions. The fold difference in drug concentration required to achieve the specified Fa is indicated above each pair of columns.

values calculated from Dynamic Contrast Enhanced (DCE)-MRI studies significantly. Based on changes in vessel morphological appearance, the drop in K_{trans} values was interpreted as a decrease in vessel permeability [35], and is consistent with the suggestion that Irinophore C™ treatment improved vascular function in the tumor. The larger variability in K_{trans} values determined in tumors from control animals reflects the random nature of chaotic and leaky blood vessels in individual tumors [36]. It had already been established in s.c tumors that Hoechst 33342 could be used as a marker for tumor vessel function by validation with K_{trans} measurements [8], but this had not been done for the orthotopic GBM tumor described here. It is shown here that the observed reduction in Hoechst 33342 staining after treatment while total CD31 staining remained constant correlates with a reduction in K_{trans} measures. Taken together, these observations strongly suggest an improvement in vascular function. The tumor blood vessels in tumors from animals treated with Irinophore C™ behave more like vessels in the normal brain where the blood-brain barrier is intact.

The concept of 'blood vessel normalization' was first postulated in the 70s [37] and more recently, the clinical potential of vascular normalization has been described [9,10]. As with most solid tumors, the microvasculature of gliomas is characterized by tortuous and fenestrated vessels with diameters that are larger than normal [38] and discontinuous basement membrane which rarely encloses pericytes [39]. In glioma [28,29,40], antiangiogenic therapies can stop the growth of tumor vessels, prune immature and inefficient tumor vessels and normalize surviving vasculature by increasing the fraction of pericyte-covered vessels, restoring the abnormally thick and irregular basement membrane and reducing the high vascular permeability of these vessels [9,10]. In

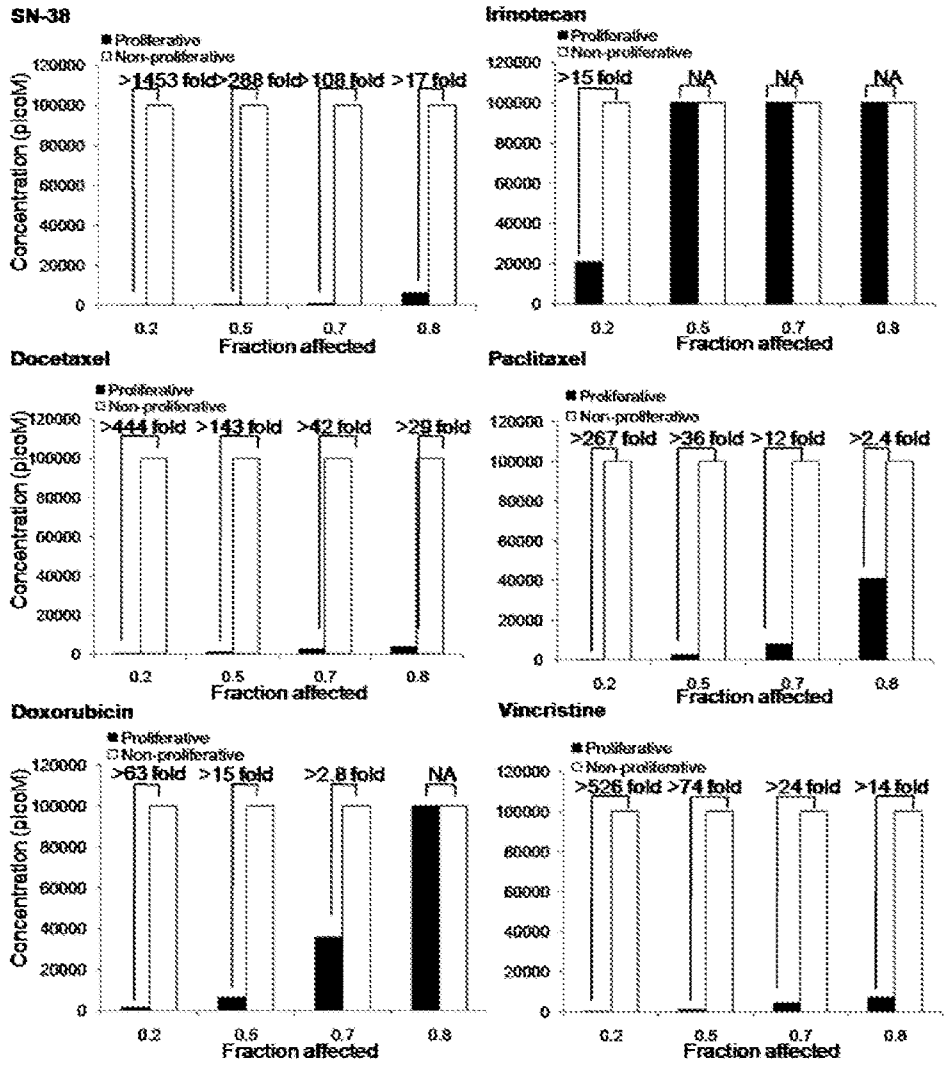

Figure 8 Proliferating HBMEC cells are more sensitive to SN-38, docetaxel, paclitaxel, doxorubicin and vincristine than non-proliferating cells. Concentrations at which a Fa of 0.2-0.9 was observed in HBMEC total nuclei count for both proliferative and non-proliferative conditions. The fold difference in drug concentration required to achieve the specified Fa is indicated above each pair of columns.

glioblastoma patients, a "vascular normalization index" was defined by changes in vascular permeability (K_{trans} values), microvessel volume and circulating collagen IV. It was found that this index was closely associated with overall survival and progression-free survival in response to Cediranib, a pan-VEGFR inhibitor [40]. Pre-clinically, the delivery of temozolomide in an intracerebral model of glioma increased after treatment with the angiogenesis inhibitor SU5416. This drug restored capillary architecture and decrease interstitial fluid pressure [41]. Such studies offer strong evidence that the tumor vasculature in GBM is a valid target, and that therapies which 'normalize' tumor vasculature may improve the delivery of a second drug at some point in the treatment regimen.

The studies described here, together with an earlier publication [8], offer strong evidence that liposomal formulations of selected drugs, and especially Irinophore C™, induce a normalization of the tumor vasculature. In this study, collagen IV and NG2 were used as markers for basement membrane and pericytes, respectively. However, there is no consensus in the field for a definitive marker of these parameters. Other markers used to evaluate basement membranes include nidogen or laminin, and desmin or α-smooth muscle actin for pericytes [9,30]. These caveats notwithstanding, the morphological changes observed were associated with changes in Hoechst 33342 uptake in the tumor and when using this parameter, remarkably different results were obtained

depending on the site of tumor growth (subcutaneous vs orthotopic). In the subcutaneous model, the liposomal treatments increased the amount of Hoechst 33342 staining in the tumor tissue (Figure 1c), while in the orthotopic tumors Hoechst 33342 staining was reduced (Figure 2c). As noted above, treatment effects were similar if blood vessel morphology parameters were used as a measured endpoint. While initially surprising, the Hoechst 33342 uptake data may actually be consistent with restoration of the blood-brain barrier, which is more impermeable to Hoechst 33342. It is well established that Hoechst 33342 is a p-glycoprotein substrate [42]. It does not accumulate in normal brain tissue because it cannot cross the blood brain barrier, but it is present in untreated orthotopic brain tumors which exhibit leakier blood vessel. This idea was further confirmed by K_{trans} measurements, which strongly suggested a vasculature normalization induced by Irinophore C™. This interpretation suggests that Hoechst 33342 is not an appropriate marker for tumor *perfusion* in orthotopic glioma models, as it was previously used in a s.c. tumor model [8]. It does, however, function as a *permeability* marker for perfused tumor associated blood vessels, which is reduced upon normalization. The impact of vascular normalization on tumor perfusion in orthotopic GBM tumors could not be assessed in the present study because MRI K_{trans} data and Hoechst 33342 staining data are not direct measures of perfusion in the brain tumor. However, data obtained in the subcutaneous model suggest that treatment with liposomal drugs does not reduce tumor perfusion, as measured by CD31/Hoechst 33342 double staining, and may even increase it, as suggested by data obtained from Caelyx®-treated s.c. tumors. Studies to measure the delivery of a second drug that can cross the BBB in liposomal drug-treated tumors are underway and will provide an indication of the impact of vascular normalization on vessel perfusion in the orthotopic model.

The idea that liposomal formulations of anti-cancer drugs, in addition to having a direct cytotoxic effect on the tumor cells, may also act as through anti-angiogenic mechanisms is intriguing. It seems reasonable to suggest that the extended drug release characteristics associated with the liposomal drug formulations used in this study [7,11] may have effects on blood vessels in a manner similar to metronomic dosing schedules - i.e. frequent, low dose administration of drugs with no prolonged drug-free breaks [43]. Metronomic dosing is now acknowledged to act specifically on the proliferating endothelial cells of tumor blood vessels [44] and was more recently shown to improve tumor perfusion and to decrease hypoxia in a pancreatic tumor model [45]. To examine this hypothesis, an *in vitro* assay was used to evaluate the activity of irinotecan, doxorubicin and vincristine (the drugs encapsulated by liposome examined in this study) against proliferating endothelial cells. The assay was adapted from one developed by Bocci et al. to examine the effects of metronomic drug exposure against endothelial cells [33]. Previous reports suggest that docetaxel and paclitaxel have potent activity against endothelial cells in an *in vitro* metronomic dosing regime [32,33,46], so these drugs were included in the assay as positive controls. The effects of SN-38 were also evaluated in the assay because SN-38 is a more active metabolite of irinotecan generated by tissue and plasma carboxylesterases *in vivo* [47,48]. Further it has already been established that following treatment with Irinophore C™, high levels of SN-38 are maintained in the plasma compartment for extended time periods [7]. SN-38 levels may play an important role in the anti-cancer activity of Irinophore C™.

The *in vitro* metronomic dosing assay presented in Figures 7 and 8 suggest that vincristine and SN-38, like the taxanes (docetaxel or paclitaxel), are highly active against proliferating endothelial cells (Figure 6a-b). In contrast, free irinotecan has little specificity for proliferating endothelial cells over non-proliferating cells *in vitro*. The data for free vincristine corroborate the effects on tumor vasculature seen with the liposomal form of the drug used here, while the results obtained with free irinotecan, which is not specific for proliferating endothelial cells, is actually contradictory. Irinophore C™ was the most active of the three liposomal formulations used. The results in Figure 7 and 8 would strongly suggest that the activity of Irinophore C™may be explained by the high plasma levels of SN-38 generated following administration of the formulation [7,16]. Thus it can be concluded from the studies presented here that the active metabolite of irinotecan, SN-38, may be the agent promoting vascular normalization in the models used here.

Interestingly, the *in vitro* assay suggests that doxorubicin should have little specificity on proliferating endothelial cells, yet i.v. administration of Caelyx® resulted in effects on the tumor vasculature that were comparable to those seen following administration of Irinophore C™. The reasons for this are unclear at present but may be related to disruptions in the production of hypoxia-induced VEGF caused by doxorubicin [49]. Previous studies completed using the rat intracranial 9L tumor model treated with a formulation of doxorubicin comparable to that used here [15] showed the presence of vascular breakdown and hemorrhage 48 hours after treatment. In contrast, the results summarized here were obtained using tumors harvested one week after the final treatment; thus the data here may reflect late effects on tumor vasculature. Further, 9L is a gliosarcoma cell line which exhibits a slower doubling time

(34.9 hrs [50]) than the U251MG glioblastoma cell line (20.9 hrs; data not shown) used in this study. The resulting 9L tumors are also histological distinct [50] when compared to the U251MG model. These differences will likely impact how tumors respond to agents capable of promoting vascular normalization. Studies assessing how vascular functions change in relationship to tumor growth rate are currently being completed.

Conclusion

In aggregate, data from this study indicates that liposomal formulations of irinotecan, doxorubicin and vincristine exert anti-angiogenic effects, as measured by endpoints assessing increases in mature blood vessels and improved vascular function. The normalization of tumor vessels appears to be transient in nature [36] but may create a window where blood flow is improved, leading to an opportunity to improve drug delivery for other drugs. The fact that all three formulations were therapeutically active in the orthotopic model suggests that vascular normalization did not prevent the drugs from accessing tumor cells, despite the fact that our interpretation of data obtained from Hoechst 33342 suggests a reduction in vessel permeability. Data from our laboratory showed that once irinotecan is released from the lipid carrier, the drug and its active metabolite SN-38 are capable of crossing a normal blood-brain barrier (Verreault M, Strutt D, Masin D, Anantha M, Waterhouse D, Yapp DT and Bally MB: Irinophore C™, a lipid-based nanoparticulate formulation of irinotecan, is more effective than free irinotecan when used to treat an orthotopic glioblastoma model, submitted for publication in March 2011). Vincristine was also shown to be able to cross a normal blood-brain barrier [51]. Thus, it can be speculated that vascular normalization would increase the delivery of drug that have dissociated from the liposome across the tumor vasculature, allowing higher levels of drug to diffuse into a greater volume of tumor tissue. Studies assessing the consequences of liposomal drug-induced vascular normalization on the delivery of a second drug capable of crossing the blood-brain barrier will provide important information regarding the impact of tumor vessel permeability on drug delivery. In the case of GBM, an obvious choice of such a drug is temozolomide. Pre-clinical studies to assess the impact of Irinophore C™ treatments on the delivery of temozolomide are currently on-going.

Abbreviations

d-HMVEC: adult dermal human microvascular endothelial cells; Fa: fraction affected; FBS: fetal bovine serum; GBM: glioblastoma; H&E: Hematoxylin and Eosin; HBMEC: Human brain microvascular endothelial cells; i.v.: intravenous; s.c.: subcutaneous;

Acknowledgements

The research described in this original paper was supported by grant funding from the Canadian Institutes of Health Research, the Cancer Research Society, Inc. and the National Cancer Institute of Canada (now the Canadian Cancer Society Research Institute). DTY was supported by Rethink Breast Cancer. The authors would like to acknowledge the staff and management personnel of the Animal Research Center at the BC Cancer Agency.

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Authors' contributions

MV carried out all parts of the experimental manipulations, data analysis and draft of manuscript. DS and DM were involved in the implantation of s.c. and orthotopic tumors and monitoring of the animals. MA and DW were involved in the development of Irinophore C™ formulation. AY and PK were part of MRI-DCE data acquisition and analysis. MBB and DTY were involved in the conception of the study, participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 5 October 2010 Accepted: 8 April 2011 Published: 8 April 2011

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2407/11/124/prepub

doi:10.1186/1471-2407-11-124
Cite this article as: Verreault *et al.*: Vascular normalization in orthotopic glioblastoma following intravenous treatment with lipid-based nanoparticulate formulations of irinotecan (Irinophore C™), doxorubicin (Caelyx®) or vincristine. *BMC Cancer* 2011 11:124.

Lipid-based nanoformulation of irinotecan: dual mechanism of action allows for combination chemo/angiogenic therapy

A number of studies have outlined the antiangiogenic effects of cytotoxic agents when administered frequently at low doses. These studies suggest that the effect of the cytotoxic agent is on the vasculature within the tumor and it is assumed that there is little or negligible cytotoxicity. Liposomal drug delivery systems have the ability to provide a dual mechanism of activity where tumor accumulation can deliver high local concentrations of the drug at the site of action with concomitant slow release of the drug from carriers in the blood compartment that results in antivascular effects, similar to that achieved when dosing frequently at low levels. Although this dual mechanism of activity may be linked to other lipid nanoparticle formulations of anticancer drugs, this article summarizes the evidence supporting direct (cytotoxic) and indirect (antivascular) actions of a liposomal formulation of irinotecan.

KEYWORDS: antiangiogenic therapy ▪ cytotoxic therapy ▪ dual mechanism ▪ irinotecan ▪ liposome ▪ metronomic dosing ▪ nanoformulation ▪ vascular normalization

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Cancer, as with other life-threatening diseases, is influenced by multiple molecular mechanisms as well as host microenvironmental factors. Tumors typically have a high degree of heterogeneity and their growth is based on enhanced survival capacity, the ability to resist apoptosis and the ability to proliferate endlessly in the absence of growth signals or in the presence of antigrowth signals [1]. Heterogeneity increases the number and the diversity of cellular targets, while also being reflected in the multiple and diverse signaling pathways within each given cancer cell, hence there is a need for combining cytotoxics, cytostatics and biological agents to provide optimal treatment responses. Multimodality therapeutic approaches must also exploit the balance between efficacy and toxicity. There are several strategies that can address tumor heterogeneity [2]: the classic approach of combining agents that have proven to be active as single agents [3]; the development of drug combination products [4]; and the identification of drugs and/or therapeutic targets that exhibit pleiotropic mechanisms of action [5,6]. Our group has an interest in identifying strategies that target both the proliferating tumor cells as well as the tumor-associated blood vessels [7,8].

Angiogenesis is the growth of new blood vessels from pre-existing blood vessels in response to VEGF and angiopoietin family members [9]. Tumor blood vessels are structurally and functionally abnormal, a result of excessive endothelial cell proliferation and a lack of supporting structural elements brought about by an imbalance in pro- and anti-angiogenic factors. This leads to tortuous, dilated and saccular blood vessels with increased resistance to blood flow as well as an irregular blood supply [10]. Angiogenesis is a key factor necessary for tumor growth beyond microscopic size and also contributes to tumor cell stress, such as transient hypoxia. In addition, the erratic nature of blood flow contributes to poor drug distribution in tumors. Therefore, one type of strategy for treating cancer, involving the use of drugs targeting angiogenesis, is a strategy designed to limit tumor growth. An unexpected outcome of this treatment strategy, however, was tumor vascular normalization.

Normalization of blood vessels refers to the elimination of excess endothelial cells and immature and inefficient blood vessels; in essence, correcting the disorganized vasculature brought on by rapid angiogenesis within the tumor. Tumor vessel normalization may occur following anti-VEGF therapy, such as bevacizumab, and has also been reported for other therapeutics, including trastuzumab [11] and low dose continuous chemotherapy, and we have also recently demonstrated this using various liposomal chemotherapeutics [12]. Jain has postulated that this normalized vasculature would result in enhanced chemotherapeutic delivery, a consequence that would primarily impact the delivery/distribution of small molecular weight drugs [10]. What is interesting is how this effect would influence delivery of nanoscaled drug delivery systems, which localize in sites of tumor

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growth due to the enhanced permeability and retention (EPR) effect. In fact, a recent study has shown that vascular normalization actually contributes to decreases in the EPR effect and associated decreases in lipid nanoparticle delivery to tumors [13].

A number of drug delivery strategies targeting tumors or specific cell targets within tumors have been evaluated. These strategies have largely focused on delivering more drug to sites where they can exert their effect on tumor cell populations and decreased delivery to sites of toxicity. In recognition of the role of the tumor microenvironment, the normal cells and associated stromal elements that surround the tumor, efforts have shifted to targeting nontumor cells such as epithelial cells, fibroblasts, immune cells, and endothelial cells. An ideal therapeutic strategy would include targeting of both the tumor cells and some element of stroma. This article will focus on liposomal delivery systems that are designed to achieve therapeutic effects by virtue of both antivascular as well as antitumor mechanism of activity.

Targeting tumor vasculature

Antiangiogenic therapy, or targeting of the vasculature within tumors, was first proposed as a treatment in 1971 by Dr Judah Folkman [14] and has now grown to a major focal area in cancer therapy. Bevacizumab is a humanized monoclonal antibody that binds and inactivates all VEGF isoforms in circulation [15] thereby inhibiting binding to the VEGF receptors. In 2004, Bevacizumab became the first approved antiantiogenic therapy in combination with intravenous 5-fluorouracil/oxaliplatin in the first-line treatment of colorectal cancer. Subsequently, bevacizumab has also been approved in combination with carboplatin and paclitaxel in first-line treatment of non-small-cell lung cancer, and with IFN-α in metastatic kidney cancer. There are numerous studies examining combinations with other agents, including irinotecan in which the efficacy of irinotecan has been shown to be enhanced by bevacizumab addition [16,17]. There are a number of experimental antiangiogenic agents in various stages of clinical development, as listed in Table 1, including both antibodies and small molecules.

Metronomic dosing

Antiangiogenic therapy is a critically important area of research, yet it is important to note that these agents are not being developed as standalone therapeutics, but are currently being used or tested in combination with traditional chemotherapeutic agents, which may in turn have their own intrinsic antiangiogenic properties when administered continuously at low doses or as a low dose on a repeat basis. This metronomic dosing was pioneered by Robert Kerbel and refers to chemotherapy given at frequent intervals and at low doses with no prolonged drug-free breaks [18]. The doses are low enough to reduce toxic side effects [19], and are thought to damage the newly forming endothelial cells within tumors. It is believed that this effect on tumor-associated endothelial cells occurs at doses below those required to exert cytotoxic/cytostatic effects on proliferating tumor cells. Regardless, the pruning of new blood vessels within the tumor exerts antitumor effects and eventually the remaining blood vessels within the tumor have a structure that is more closely associated with normal blood vessels. The advantages to this type of therapy lie in the continued exposure of the endothelial cells to the chemotherapeutic agent rather than short bursts of high drug concentration, known to cause toxic effects.

Studies of metronomic dosing have been conducted on a number of different drugs, including temozolomide [20], gemcitabine [21] and topotecan [22]; however, early efforts were focused on cyclophosphamide, which has been postulated to induce apoptosis in tumor endothelial cells leading to the collapse of angiogenic vessels and ultimate suppression of tumor growth. Clinical studies of daily oral cyclophosphamide in combination with low-dose methotrexate for treatment of metastatic breast cancer have shown promising results [23,24]. Of note, each of these drugs are administered orally and therefore lend themselves well to metronomic scheduling. This type of scheduling, however, is quite challenging when using chemotherapeutics not amenable to oral dosing or extended continuous infusions.

More recently, studies of metronomic dosing have included irinotecan in a number of indications including glioma [25] and colorectal cancer [26,27]. In the clinical setting, these studies have required the use of continuous irinotecan infusion [27] by implantation of a central venous catheter with a programmable pump that was refilled weekly. Allegrini *et al.* [27] demonstrated in their clinical study that the angiogenesis associated thrombospondin-1 (TSP-1) was markedly increased in response to metronomic irinotecan (in this group, irinotecan was dosed at 1.4 mg/m^2/day, representing a 75% dose reduction over earlier identified infusion dose levels as published by Herben *et al.* [28]). At the tested doses, the regimens were not toxic and efficacy

Table 1. Experimental antiangiogenic agents.

Category	Inhibitor	Mechanism of action	Ref.
Antibody	Volociximab	Chimeric anti-$\alpha_5\beta_1$ that blocks $\alpha_5\beta_1$ binding to fibronectin and causes endothelial cell apoptosis	[49]
Antibody	IM-1C11	Chimeric anti-KDR that binds the VEGF receptor KDR, therefore preventing VEGF binding and subsequent endothelial cell proliferation	[50]
Antibody	Etaracizumab	Humanized monoclonal antibody targeting the $\alpha_v\beta_3$ integrin that inhibits the adhesive interations of endothelial cells	[51]
Small molecule	Cilengitide	Targets the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$	[52]
Small molecule	Sorafenib	Multikinase inhibitor (VEGF receptor-2, VEGF receptor-3, PDGF receptor); currently indicated for treatment of hepatocellular and renal cell carcinoma	[53–56]
Small molecule	Sunitinib	Multikinase inhibitor that targets VEGFR1, VEGFR2, VEGFR3, PDGF receptor, fms-like tyrosine kinase 3, c-kit and rearranged during transfection (RET)	[57–59]
Small molecule	Cediranib	Selective inhibitor of the VEGF pathway	[60–62]
Small molecule	Pazopanib	Multikinase inhibitor that blocks VEGF receptor-1, -2, -3, PDGF receptor and cytokine receptor	[63,64]
Small molecule	Vandetanib	Blocks both the VEGF receptor and EGF receptor pathways as well as inhibiting the RET receptor tyrosine kinase activity	[65,66]

results were similar to those of other schedules of third/fourth-line treatment in patients with metastatic colorectal cancer (typically <10%). While results were promising in this study in terms of effects on vasculature as measured by TSP-1 and VEGF levels, efficacy was not improved over existing options.

Allegrini's group has since continued research into metronomic dosing of irinotecan and is now looking at combination with semaxinib, an experimental inhibitor of the Flk-1/KDR VEGF receptor tyrosine kinase. A study by Bocci *et al.* demonstrated that metronomic dosing of irinotecan with semaxinib not only decreased colorectal cancer xenograft tumor vessel density and modulated both VEGF and TSP-1 expression, but also significantly inhibited tumor growth [29]. This has also been demonstrated with irinotecan alone in the U87 xenograft model of glioblastoma by Takano *et al.*, in which tumors were grown subcutaneously and irinotecan was administered intraperitoneally daily at a dose of 1 or 4 mg/kg for 21 days from the day of tumor cell inoculation [25]. Tumor growth was significantly inhibited in a dose-dependent manner without evidence of toxicity using this schedule, and both VEGF and hypoxia inducible factor (HIF)-1α were significantly reduced.

Lipid-based delivery systems

Liposomes are spherical structures made up of phospholipids and cholesterol. The lipids spontaneously adopt bilayer structures that are separated by aqueous channels. Following hydration of dried lipids with a selected buffer, large (>1 micron) structures are formed with multiple lipid bilayers and these structures need to be processed further to generate unilamellar structures that are small (50–200 nm) and encompass a central aqueous core. Therapeutic agents can be encapsulated in the hydrophobic (partitioning in the lipid bilayer) as well as hydrophilic (encapsulation in the aqueous core) region of the liposome and this makes them very versatile as drug carriers. Passively targeted liposomal drugs (i.e., relying on the pharmacokinetic properties of the carrier only) often achieve vast increases in delivery of the associated drug to sites of disease, such as cancer, when compared with administration of free drug due to a phenomenon known as the EPR effect. The EPR effect is due to the leaky vasculature present within tumors, which allows for the accumulation of macromolecules with a diameter of less than 600 nm [30,31]. Since tumors often lack draining lymphatics, the regionally localized liposomes are retained at the site for extended periods of time. The ability of lipid-based formulations to localize in sites of tumor growth via the EPR effect is enhanced when that lipid-based formulation is designed to exhibit extended circulation lifetimes.

Liposomes and other delivery systems, such as micelles or block copolymers, lend themselves well to functionalization, or modification of the surface with targeting ligands [32,33], antibodies (enhanced targeting) [34] or polyethylene glycol (prevention of aggregation) [35]. As indicated above, however, liposomes are able to passively accumulate in tumor tissue by virtue of the leaky vasculature found in tumors, even without such modifications. Those liposomal systems with surface modifications may be taken into cells, for example by receptor-mediated endocytosis. Alternatively, encapsulated drug may be released

in close proximity to target cells with subsequent cellular uptake, or there may be fusion of liposomes with cellular membranes, with release of liposomal contents into the cytosol. The mechanism of liposome-assisted drug delivery is very dependent on composition. Most formulations advanced toward clinical testing are simple ones (no associated targeting ligands), where drug release from the liposome is required for the drug to access target cells. There are many lipid-based drug delivery systems currently in clinical testing, and a number of clinically approved products as indicated in Table 2. This highlights the utility of this technology as a clinically advanced approach to achieve improved therapeutic effects.

Combined modality therapy: targeting tumor cells & vasculature

The combination of antiangiogenic agents and traditional chemotherapy offers the ability to target both the tumor cells and vascular components of tumors, and in many instances, use of the antiangiogenic drug prior to chemotherapy has been shown to enhance the efficacy of chemotherapy due to vascular normalization achieved by the antiangiogenic drug [36]. However, given the demonstrated successes of metronomic dosing, one could envision development of drug carrier formulations of drugs known to exhibit antiangiogenic effects when given metronomically. This was postulated by Ng *et al.* [37] and tested in studies recently published from our laboratory [9]. More specifically, the use of metronomic dosing of irinotecan in the aforementioned glioma research [25] offered a tantalizing glimpse at a single therapy that could be both antiangiogenic as well as cytotoxic. In this paper, Takano *et al.* utilized nonformulated irinotecan at either 1 or 4 mg/kg daily intraperitoneal injection over 21 days and compared this regimen to a more conventional dosing schedule using 10 or 40 mg/kg. The results indicated that while conventional dosing did result in inhibition of glioma growth, it was associated with systemic toxicity. Treatment with the metronomic regimen resulted in both inhibition of tumor growth without toxicity and additionally inhibited angiogenesis.

The principle of metronomic dosing lies in the maintenance of drug levels in plasma over time with no prolonged drug-free periods, as mentioned above. This is also achieved when using appropriately designed liposomal drugs; formulations that slowly release drug from the carrier, which is retained in the vascular compartment over extended time periods. The extended circulation lifetime is needed to achieve enhanced tumor drug delivery by the EPR effect. Importantly, it is well established that drug within the carrier is slowly released over time, a release process that occurs in the tumor compartment as well as the blood compartment. A major benefit to the use of liposomal formulations, when compared with metronomic dosing, is the removal of the need for dosing on a frequent basis. The extended circulation life of drugs administered in liposomal form has been shown to result in marked improvements in efficacy in xenograft tumor models for vincristine [38,39], doxorubicin [40–42], irinotecan [43,44] and other cytotoxic compounds [45,46]. It is thus curious as to whether some of the therapeutic benefits achieved through use of these formulations are due to antiangiogenic mechanisms, in addition to direct cytotoxic effects on the tumor cells.

This has indeed been shown to be the case in a newly developed liposomal formulation of irinotecan (Figure 1), Irinophore C™, currently in late preclinical development. Irinophore C is exemplary of the benefit in circulation longevity

Table 2. Approved and investigational liposomal anticancer drugs.

Product name	Drug	Indication	Ref.
DaunoXome®	Daunorubicin	HIV-related Kaposi's sarcoma	[67]
Myocet®	Doxorubicin	Combination therapy with cyclophosphamide in metastatic breast cancer	[68]
Doxil®/Caelyx®	Doxorubicin	HIV-related Kaposi's sarcoma, metastatic breast cancer, metastatic ovarian cancer	[68]
CPX-351	Cytarabine:daunorubicin	Acute myeloid leukemia and first relapse acute myeloid leukemia (currently in Phase II trial)	[69]
CPX-1	Irinotecan HCl:floxuridine	Colorectal cancer (completed Phase II testing)	[70]
Marqibo®	Vincristine sulfate	Acute lymphoblastic leukemia and melanoma (ongoing pivotal clinical trials)	[71]
NanoVNB®	Vinorelbine	Vinorelbine responsive malignancies (completed Phase I testing)	[72]

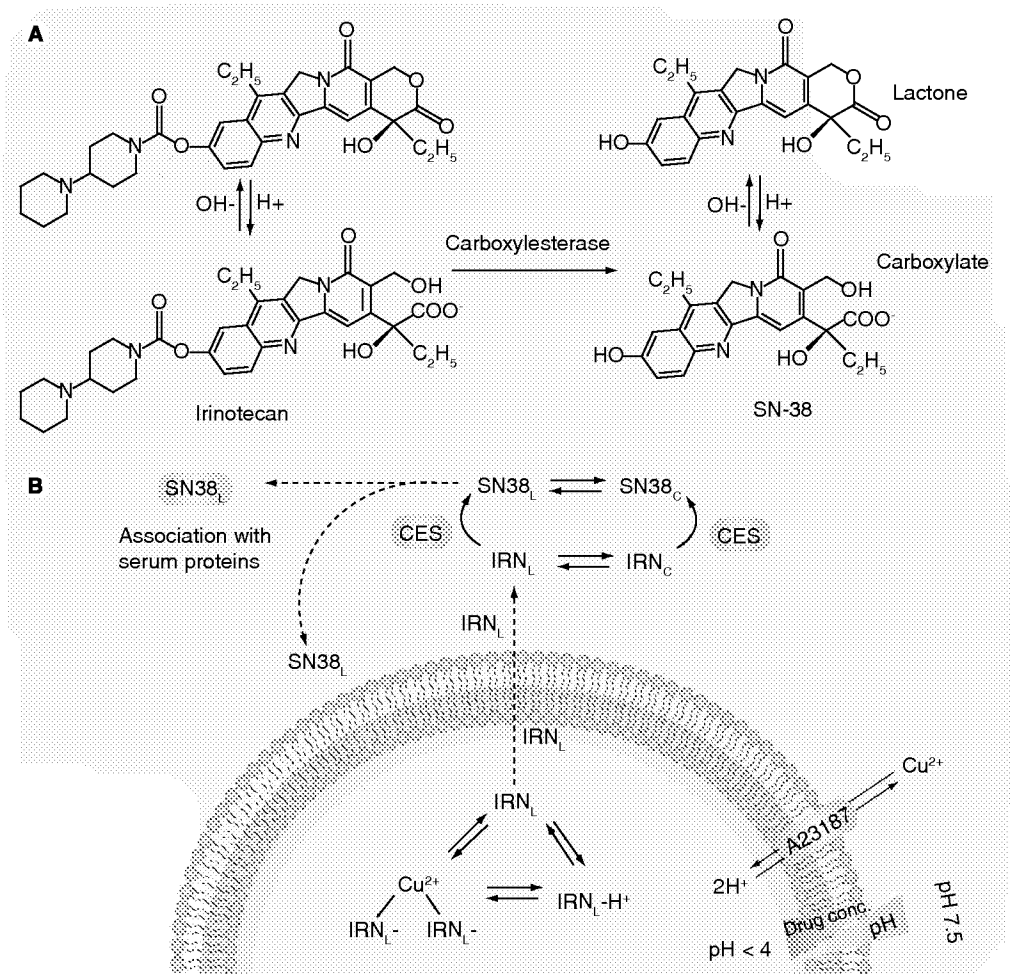

Figure 1. Liposomal encapsulation of irinotecan. (A) IRN and SN38 lactone and carboxylate conformations: conversion of the lactone to carboxylate form is favored at physiological pH; irinotecan conversion to SN38 is mediated by CES. **(B)** Representation of Irinophore C™ drug release and conversion: due to the low pH inside the liposomes, IRN is maintained in its lactone form (IRN_L). In addition to interacting with copper present in the formulation, it is anticipated that IRN_L may also interact with the inner and outer leaflet of the liposomal membrane, an interaction that is required for release of IRN_L. Once released, IRN_L is hydrolyzed through a pH-dependent reversible process to its carboxylate form (IRN_C). IRN is metabolized to SN38 by CES present in the liver (hCE1), the GI tract (hiCE) and tumor macrophages. The lactone form of SN38 ($SN38_L$) can also be hydrolyzed to its carboxylate form ($SN38_C$). It is possible that a fraction of $SN38_L$ may interact with the lipid carrier membrane or serum proteins; interactions that may decrease the rate of $SN38_L$ hydrolysis to $SN38_C$. CES: Carboxylesterase; IRN: Irinotecan.

afforded a drug by virtue of liposomal encapsulation in that the plasma area-under-the curve is improved 1000-fold following intravenous administration in mice [44]. Importantly, the carrier maintains irinotecan in the active lactone conformation [47] in contrast to administration of free irinotecan where the majority of drug is converted to the inactive carboxylate form at physiological pH. In addition, levels of the more active metabolite SN-38 (lactone) are increased up to 30-fold when irinotecan is administered in the Irinophore C formulation [44]. This is shown diagrammatically in Figure 2, in which the active form of the drug may be seen crossing from the blood vessel into the tumor interstitium.

In order to gain better understanding of the mechanism of action of antitumor activity of Irinophore C, the treatment-induced effects on the tumor microenvironment in a xenograft model of colorectal cancer were examined [6]. In this study, mice were treated once per week for 3 or 6 weeks with Irinophore C. Multimodality imaging techniques were used to assess hypoxia, cell density by Hoechst 33342 staining, K_{trans} (the volume transfer constant of a solute between the blood vessels and extracellular tissue compartment of the tumor), labeling of endothelial

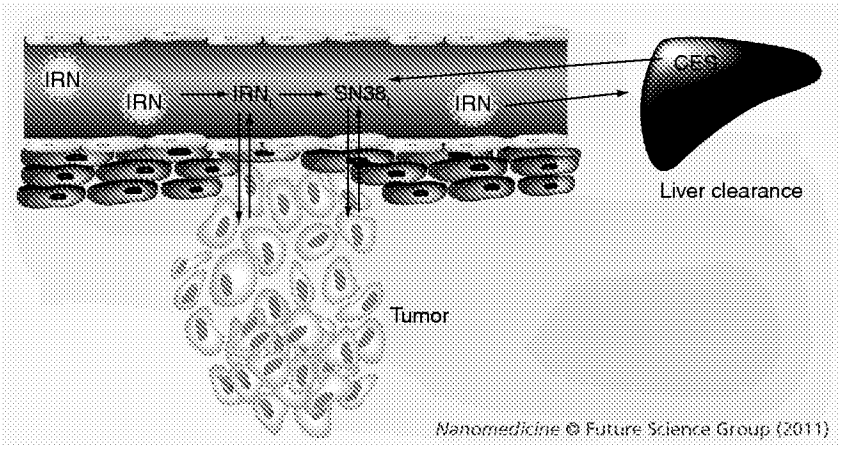

Figure 2. Representation of Irinophore C™ drug release and interaction with biological and tumor compartments. Following intravenous injections, it is known that appropriately designed lipid carriers are retained in the plasma compartment for extended periods compared with the free drug. Inevitably, a significant portion of drug-loaded carriers will accumulate in the liver and the spleen. This process will result in elimination of the carrier and associated drug from the plasma, while also contributing to irinotecan metabolism to SN38 via carboxylesterases of the liver and cells of the mononuclear-phagocytic system.
CES: Carboxylesterase; IRN: Irinotecan.

cells by CD31 staining and accumulation of second drug. It was shown that treatment, even at doses lower than that previously defined as an efficacious dose, resulted in inhibition of tumor growth. Noninvasive MRI revealed a decrease in K_{trans}, while cryosection staining showed higher perfusion of Irinophore C-treated tumors. Paradoxically, this study also demonstrated that tumor sections from Irinophore C-treated mice had a lower percentage of CD31-positive cells. This result, when combined with the finding that tumor hypoxia was decreased overall, led to the conclusion that Irinophore C given on a weekly schedule was acting comparably to metronomic drug dosing. Normalization of tumor vasculature occurred at the same time that the active drug component of the formulation was inhibiting the growth of tumor cells. To confirm the antiangiogenic mechanism of action, promoters and inhibitors of angiogenesis were assessed and it was shown that Irinophore C treatment caused downregulation of proangiogenic factors VEGF, IL-8 and HIF-1α while the antiangiogenic TIMP-1 and TSP-1 were upregulated (Figure 3) [6].

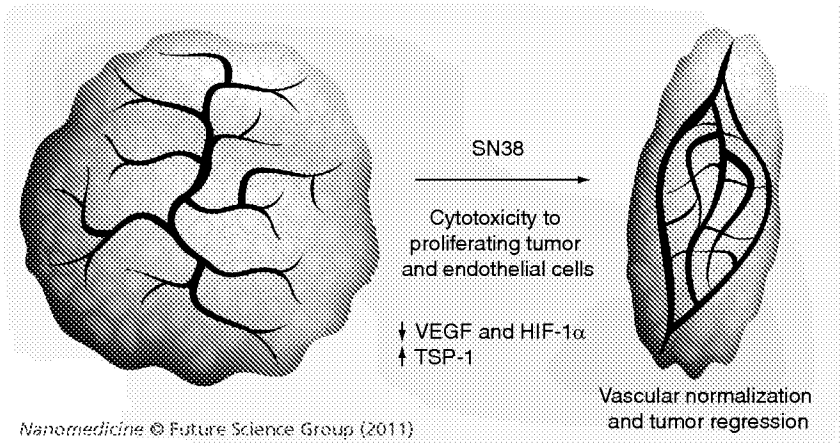

Figure 3. Tumor vasculature normalization. Extended exposure to SN38 provided by Irinophore C™ induces vascular normalization by a direct cytotoxicity on proliferating endothelial cells [12], and indirectly by triggering a reduction in VEGF and HIF-1α proangiogenic factors, and an increase in TSP-1 antiangiogenic factors [6]. Tumor vascular normalization may alter tumor metabolism and eventually increase tumor cell proliferation.
TSP-1: Thrombospondin.

A critical requirement of chemotherapy is the ability of a drug to reach the target tissue. In many instances, the tortuous nature of tumor vasculature prevents the optimal tissue distribution of systemically administered drug. Following vessel normalization, however, it is reasonable to assume that drug uptake in tumor tissue would be increased. This was indeed shown to be the case for mice pretreated with Irinophore C, in that approximately 1.5-fold higher levels of 5-fluorouracil and 2.7-fold higher levels of doxorubicin were found in tumor tissue as compared with saline pretreated controls [6].

Further validation of the concept of dual mechanism of action for liposomal formulations of cytotoxic drugs, specifically for Irinophore C, was provided by Verreault *et al.* [12]. This study demonstrated vascular normalization effects in an orthotopic glioblastoma model following intravenous administration of liposomal irinotecan (Irinophore C), doxorubicin (Caelyx®) or liposomal vincristine. This study assessed both efficacy and vascular function in stereotactically implanted glioblastoma cells in immunodeficient mice. Irinophore C treatment resulted in tumors with blood vessels that were morphologically more mature. In the subcutaneous model, Irinophore C restored the basement membrane architecture, increased the pericyte coverage and reduced blood vessel diameters, suggestive of a restoration of vessel architecture to a more normal state. In the more clinically relevant orthotopic model, Irinophore C treatment restored the basement membrane architecture and reduced the blood vessel diameters of the tumor vasculature, again suggesting a restoration of the vessel architecture to a more normal state. Irinophore C also increased the quantity of vessel staining in the center of tumors, suggesting a more homogenous distribution of blood across the entire tumor. Furthermore, the drop in K_{trans} values in the glioma model was interpreted as a decrease in vessel permeability consistent with the suggestion that Irinophore C treatment improved vascular

function in the tumor [12]. While a drop in K_{trans} is associated with decreased vessel permeability, we note that the tumor vasculature was more functional following Irinophore C treatment, which is of importance in glioma as only 25% (tumor center) – 75% (tumor periphery) of vasculature is typically functional in this tumor type [48]. The more functional blood vessels should improve the ability of injected compounds to extravasate at the tumor site, provided these compounds can normally cross the blood–brain barrier.

Future perspective

The results of these recent papers suggest that it is possible to recognize the benefits of combination therapy with a single, carefully designed therapeutic; one that targets the tumor cells directly with cytotoxic action and tumor-associated vascular endothelial cells for an antiangiogenic mechanism of action. Cancer therapy research has traditionally been conducted in 'silos' where one team researches cytotoxic effects and others research antiangiogenic effects or other microenvironment effects (for example). It is critically important to think outside of these silos and consider the multiple or combination therapeutic effects that may in fact be present when using a single therapeutic, and that perhaps this should be best considered in the context of rationally designed nanocarriers, such as liposomal or polymer-based carriers.

Financial & competing interests disclosure

The Irinophore C™ technology has been licensed from the BC Cancer Agency to Champions Oncology Inc. and as contributors to this work, some of the authors stand to benefit from future royalty payments that flow back to the BC Cancer Agency (DN Waterhouse, M Anantha and MB Bally). Work on Irinophore C has been supported by the Canadian Institutes of Health Research, the Terry Fox Research Institute, the Centre for Drug Research and Development, the National Cancer Institute of Canada, and the Cancer Research Society. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Targeting tumor vasculature

- Antiangiogenic therapy is of growing importance in treatment strategies for a number of cancer types targeting blood vessels within tumors. This can result in several outcomes:
  - Selective killing of tumor endothelial cells and subsequent starvation of the tumor.
  - Inhibition of proangiogenic factors, such as VEGF and HIF-1α.
  - Normalization of tumor vasculature and enhanced delivery of small molecular weight cytotoxic agents.
- Antiangiogenic therapies are typically paired with conventional chemotherapeutics in combination regimens due to lack of single agent activity.

Metronomic dosing

- Many traditional cytotoxic drugs can be administered in a metronomic fashion (multiple low doses) and act mechanistically as antiangiogenic agents.

Lipid-based delivery systems

- It is possible to mimic metronomic dosing by utilization of nanoformulations, such as liposomes, which have extended drug payout times, therefore reducing/eliminating the need for repeated dose administration.

Combined modality therapy: targeting tumor cells & vasculature

- Rationally designed nanoformulations of conventional chemotherapeutics are able to exert dual mechanisms of action: antiangiogenic effects exerted on the endothelial cells as well as direct cytotoxic effects exerted on the tumor cells.
- A liposomal irinotecan formulation has been shown to have multimechanistic activity:
  - Cytotoxic activity against tumor cells with demonstrated efficacy in a wide range of animal xenograft models.
  - Normalization of tumor vasculature.
  - Enhanced uptake of subsequently administered small molecular weight chemotherapeutic agent.

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Targeting hypoxia in cancer therapy

William R. Wilson and Michael P. Hay

Abstract | Hypoxia is a feature of most tumours, albeit with variable incidence and severity within a given patient population. It is a negative prognostic and predictive factor owing to its multiple contributions to chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, metastasis, resistance to cell death, altered metabolism and genomic instability. Given its central role in tumour progression and resistance to therapy, tumour hypoxia might well be considered the best validated target that has yet to be exploited in oncology. However, despite an explosion of information on hypoxia, there are still major questions to be addressed if the long-standing goal of exploiting tumour hypoxia is to be realized. Here, we review the two main approaches, namely bioreductive prodrugs and inhibitors of molecular targets upon which hypoxic cell survival depends. We address the particular challenges and opportunities these overlapping strategies present, and discuss the central importance of emerging diagnostic tools for patient stratification in targeting hypoxia.

Hypoxia influences many aspects of the biology of tumours and their responses to therapy. Initially, hypoxia arises because of oxygen diffusion limitations in avascular primary tumours or their metastases, but the tumour microvasculature (induced in part as a response to this hypoxia) is highly abnormal[1,2] and often fails to rectify the oxygen deficit. This persistent hypoxia reflects the spatial disorganization of tumour vascular networks, leading to intercapillary distances that are often beyond the diffusion range of oxygen (which is up to ~200 μm, depending on the local oxygen concentration in blood plasma). In addition to this diffusion-limited hypoxia, temporally unstable blood flow in tumour microvascular networks also leads to fluctuating perfusion-limited hypoxia[3].

The many effects of hypoxia on tumour biology include: selection of genotypes favouring survival under hypoxia–re-oxygenation injury (such as *TP53* mutations[4]); pro-survival changes in gene expression that suppress apoptosis[5] and support autophagy[6]; and the anabolic switch in central metabolism[7]. Hypoxia also enhances receptor tyrosine kinase-mediated signalling[8], tumour angiogenesis[9], vasculogenesis[10], the epithelial-to-mesenchymal transition[11], invasiveness[12] and metastasis[13], as well as suppressing immune reactivity[14]. In addition, hypoxia contributes to loss of genomic stability through the increased generation of reactive oxygen species (ROS)[15] and the downregulation of DNA repair pathways[16].

In part because of these effects on tumour development, hypoxia is implicated in resistance to therapy through multiple mechanisms (shown for cytotoxic agents in TABLE 1; see also Supplementary information S1 (tables)). Reflecting these major roles in cancer biology and therapy, there is compelling evidence that hypoxia can compromise clinical outcomes in human cancer (TABLE 2). However, as noted in TABLE 1, some changes in hypoxic cells can result in increased drug sensitivity; these exceptions caution against the frequent generalization in the literature that hypoxic cells are invariably chemoresistant.

The apparent extent of hypoxia in human tumours depends on the methods used to detect it; the most widely used methods are indicated in TABLE 2. Invasive oxygen electrodes provide the most direct measure and demonstrate extreme heterogeneity of oxygenation within and between tumours in every tumour type evaluated in patients[17]. Increasingly, evaluation of hypoxia in the clinic is shifting to the monitoring of endogenous markers, especially the transcriptional targets of the hypoxia-inducible factors (HIFs), and exogenous 2-nitroimidazole probes, such as pimonidazole, that bind covalently to SH-containing molecules (thiols) in hypoxic tissue[18,19]. The use of these markers to image hypoxia in a human tumour is illustrated in FIG. 1a, which shows the typically more restricted distribution of bound pimonidazole than the HIF1 target carbonic anhydrase 9 (CA9). This and other evidence indicates that metabolic activation of 2-nitroimidazole probes requires more severe hypoxia than does the HIF1 response. Quantitative understanding of hypoxia in tumours (and physiological hypoxia in some normal tissues) is far from complete, but the oxygen concentration dependencies

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doi:10.1038/nrc3064

At a glance

- Hypoxia represents a compelling therapeutic target, given that it has a major role in tumour development and resistance to therapy, and that the levels of hypoxia are more severe in most tumours than normal tissues.
- One approach to targeting hypoxia seeks to develop bioreductive prodrugs that are activated by enzymatic reduction in hypoxic tissue. These prodrugs are chemically diverse and represent two distinct strategies: activation under moderate hypoxia (as exemplified by tirapazamine) or only under severe hypoxia (as exemplified by PR-104). In the latter case, diffusion of the active drug to less hypoxic cells is essential.
- A second approach seeks small molecule inhibitors against molecular targets involved in the survival of hypoxic cells. Current interest focuses on the inhibition of the hypoxia-inducible factor 1 (HIF1), the unfolded protein response (UPR) and mTOR pathways, but the most important vulnerabilities in hypoxic cells are not well defined. Most molecularly targeted agents have been 'repurposed' from other applications, and have low selectivity as hypoxic cytotoxins.
- Both approaches face substantial challenges in relation to off-target effects, which, ironically, also present opportunities. For bioreductive prodrugs, activation by aerobic reductases can contribute to normal tissue toxicity, but this is exploitable in tumours that highly express these enzymes. For molecularly targeted agents, hypoxia-independent signalling through the same pathways may provide opportunities for additional antitumour activity.
- Both bioreductive prodrugs and molecularly targeted agents also need to overcome the problem of drug penetration through poorly perfused hypoxic tissue; strategies for addressing this requirement are being developed.
- The current generation of bioreductive prodrugs generate DNA-reactive cytotoxins, making them difficult to combine with conventional chemotherapy because of overlapping toxicity. This challenge is stimulating the development of bioreductive prodrugs that release molecularly targeted agents as their effectors, potentially combining the best features of both approaches.
- Given the marked heterogeneity in hypoxia between tumours of the same type, the clinical exploitation of hypoxia using all of these approaches will require their co-development with companion diagnostics for hypoxia (and for other determinants of sensitivity).

for some of the critical biological processes considered in this Review are illustrated schematically in FIG. 1b. These differences in oxygen concentration thresholds have important implications for targeting hypoxic cells, as have differences in the spatial distribution and duration of hypoxia and the genetic and environmental context in which hypoxia occurs. In particular, these factors will dictate the choice of hypoxia-targeted therapy that best complements existing agents used to treat the oxic cell population in tumours.

The compelling evidence for hypoxia in tumour tissue and its therapeutic importance makes hypoxia a high priority target for cancer therapy. In this Review we describe recent progress in developing small molecule drugs to kill hypoxic cells, including bioreductive prodrugs that are activated selectively under hypoxia, and drugs that inhibit molecular targets in hypoxic cells. We focus here on agents that kill hypoxic cells directly, rather than inhibitors of hypoxia-dependent processes such as angiogenesis.

Bioreductive prodrugs

Chemical classes and mechanisms of action. The concept of activating prodrugs selectively in tumours, to achieve targeted delivery of cytotoxins, has a long history. The first clear demonstration was the reactivation of β-glucuronide metabolites of an aniline nitrogen mustard in tumours with high β-glucuronidase activity[20], but such approaches have struggled with the challenge of finding tumours with high enough expression of the activating enzymes to achieve useful selectivity. Hypoxia is potentially a more generic feature, with a clear basis for tumour selectivity, although expression of the activating enzymes is also critically important in this context.

Five different chemical moieties (nitro groups, quinones, aromatic *N*-oxides, aliphatic *N*-oxides and transition metals) have the potential to be metabolized by enzymatic reduction under hypoxic conditions, and thus provide the basis for the design of bioreductive prodrugs for exploiting tumour hypoxia. The mechanisms by which bioreductive prodrugs are selective for hypoxic cells are summarized in FIG. 2A; most often these mechanisms involve the re-oxidation by oxygen of the initial free radical intermediate formed by a one-electron reduction of the prodrug, thus generating superoxide. This futile redox cycling ensures that steady-state concentrations of the prodrug radical are kept low in oxic cells, resulting in hypoxia-selective cell killing provided that the prodrug radical (or its downstream products) is more cytotoxic than superoxide or the unreduced prodrug.

Inhibition of drug reduction by oxygen through this redox cycling mechanism was first demonstrated for nitro compounds[21] and was subsequently shown to be responsible for the hypoxia-selective cytotoxicity of nitroimidazoles[22]. This bioreductive mechanism is distinct from hypoxic cell radiosensitization by the same compounds[23], which is due to the ability of these compounds to replace oxygen in oxidizing ionizing radiation-induced DNA free radicals to generate cytotoxic DNA strand breaks[24]. This first proof-of-principle demonstration of the hypoxia-selective cytotoxicity of bioreductive prodrug activity stimulated the search for ways of linking nitroreduction to the formation of more potent cytotoxins, illustrated by PR-104 and TH-302 (FIG. 2B), and for other redox moieties capable of hypoxia-selective metabolic activation.

The potential for using quinones in this context can be traced to the discovery in the 1960s that the DNA-crosslinking anticancer antibiotic mitomycin C is activated by reduction of its indoloquinone moiety[25,26]. Sartorelli's group subsequently designed simpler quinone bioreductive alkylating agents[27], which were proposed to exploit the more reducing environment in tumours relative to normal tissues[28]. It was later shown that the bioreductive activation of quinones occurs selectively under hypoxia[29] through a redox cycling mechanism[30] analogous to that for nitro compounds, but with two sequential one-electron reductions (first to the semiquinone and then to the hydroquinone).

Subsequently, three other chemical moieties capable of hypoxia-selective metabolic reduction by tumour cells have been discovered. Martin Brown[31] showed that the aromatic *N*-oxide tirapazamine (TPZ; FIG. 2B) is 50–200-fold more toxic to hypoxic than oxic cells in culture[31] owing to one-electron reduction to a DNA-damaging free radical (originally thought to be the TPZ radical itself, but now considered to be an

Bioreductive prodrugs
Biologically inactive molecules that are converted to an active drug by enzymatic reduction.

Superoxide
A free radical formed by a one-electron reduction of oxygen, including by electron transfer from a prodrug free radical. Despite its name, superoxide itself is not highly reactive and is generally less toxic than the reduced prodrug, so its generation represents a detoxification mechanism in aerobic cells.

Table 1 | **Mechanisms of resistance (and sensitivity) of hypoxic cells to cytotoxic therapy***

Effect of hypoxia	Resistance or sensitivity?	Mechanism	Agents affected	Example
Lack of oxidation of DNA free radicals by O_2	Resistance	Failure to induce DNA breaks	Ionizing radiation	2–3-fold increase in ionizing radiation dose required for equivalent cell kill
			Antibiotics that induce DNA breaks	Bleomycin
Cell cycle arrest in G1 or G2 phase	Resistance	Repair before progression to S or M phase	Cycle-selective chemotherapy drugs	5-Fluorouracil
Cell cycle arrest in S phase	Sensitivity	Collapse of stalled replication forks	PARP inhibitors‡	Veliparib (ABT-888)
Distance from vasculature (indirect)	Resistance	Compromised drug exposure	Drugs extensively bound in tumour cells	Taxanes
Extracellular acidification (indirect)	Resistance	Decreased uptake	Basic drugs	Doxorubicin
	Sensitivity	Increased uptake	Acidic drugs	Chlorambucil
Resistance to apoptosis	Resistance	Genetic selection of *TP53* mutants	Multiple	
		Downregulation of BID and BAX	Multiple	Etoposide
Genomic instability	Resistance	Mutagenesis	Multiple	*DHFR* amplification and methotrexate
Suppression of DNA repair	Resistance	Downregulation of MMR	DNA methylating agents	
	Sensitivity	Downregulation of NER	Bulky DNA monoalkylating and crosslinking agents	
		Downregulation of HR	DNA crosslinking agents	Cisplatin
HIF1 stabilization	Resistance	Expression of ABC transporters	ABC transporter substrates	MDR1 and doxorubicin
		Downregulation of NHEJ	Agents that induce DSBs	Etoposide

BAX, BCL2-associated X protein; BID, BH3 interacting domain death agonist; *DHFR*, dihydrofolate reductase; DSB, double strand break; HIF1, hypoxia-inducible factor 1; HR, homologous recombination; MDR1, multidrug resistance protein 1; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PARP, poly(ADP-ribose) polymerase. *See also Supplementary information S1 (tables) for tables with references. ‡Also sensitized by downregulation of HR under hypoxia.

oxidizing hydroxyl[32] or benzotriazinyl[33] radical arising spontaneously from the TPZ radical) (FIG. 2B). Later, Laurence Patterson[34] and ourselves[35] independently demonstrated that inhibition by oxygen of the bioreduction of aliphatic *N*-oxides to the corresponding tertiary amines can also be used as a basis for hypoxia-activated prodrugs, in these examples through increasing DNA binding affinity of intercalators (illustrated for banoxantrone (also known as AQ4N) in FIG. 2B). For the aliphatic *N*-oxides, hypoxic selectivity stems from inhibition of two-electron reductases by oxygen (FIG. 2A), rather than redox cycling. Examples of the fifth class (transition metals) include cobalt(III)[36,37] and copper(II)[38] complexes capable of hypoxia-selective bioreductive activation through one-electron reductions of the metal centres to unstable cobalt(II) or copper(I) complexes that then dissociate to release cytotoxic ligands.

Bioreductive prodrugs under recent or ongoing clinical development (FIG. 3; TABLE 3) include examples of each of these chemotypes (except transition metal complexes, for which hypoxic cell killing has only been reported in cell culture). Other than TPZ and apaziquone (also known as E09), for which Phase III clinical trial results are pending, the compound currently most advanced in clinical testing is TH-302 (FIG. 2B). This 2-nitroimidazole-based nitrogen mustard prodrug has shown promising activity in a Phase I study[39] and is being evaluated in multiple Phase I and II trials, including a randomized Phase II trial with gemcitabine in pancreatic cancer (www.ClinicalTrials.gov identifier NCT01144455). The clinical status of the other compounds is discussed below in relation to unique features of their mechanisms of action. These prodrugs illustrate diverse strategies for exploiting oxygen-sensitive biotransformations to achieve cytotoxic activation (FIG. 2B), and are representative of other prodrugs reviewed previously[40–43]. The prodrugs also differ in their quantitative oxygen dependence (K_{O2}, the K_i for inhibition by oxygen), the activating reductases and the nature of the resulting DNA lesions (TABLE 3). A recent addition is a chloromethylbenzindoline prodrug, SN29730, which generates a potent DNA minor groove alkylator on nitroreduction and has high hypoxic potency and selectivity *in vitro* and *in vivo*[44]. A common feature of all these prodrugs is that interference with the DNA replication fork appears to be the main mechanism of cytotoxicity, as illustrated by the dependence of the hypoxic cytotoxicity of TPZ[45] — and the alcohol metabolite of PR-104, PR-104A[46] — on homologous recombination (HR) repair, which is required for the resolution of damage at the replication fork[47].

Replication fork
The branch-point structure that forms between two DNA template strands during DNA replication at which nascent DNA synthesis is ongoing.

Homologous recombination (HR)
High-fidelity repair of DNA lesions, including double-strand breaks, in S and G2 phases of the cell cycle, using a sister chromatid as a template.

Identifying and exploiting the activating reductases. Targeting hypoxia with bioreductive prodrugs depends on tumour expression of the appropriate activating reductases. Most of the one-electron reductases responsible for the redox cycling (and hence the hypoxic selectivity) of prodrugs appear to be NAD(P)H-dependent flavoproteins with low substrate affinities and specificities as xenobiotic metabolizing enzymes; their identification represents an important ongoing challenge (BOX 1).

Reductases that catalyse concerted two-electron reductions provide an alternative pathway for bioreductive prodrug activation (FIG. 2A) and represent both an opportunity and challenge for tumour targeting. These enzymes fall into two broad groups. Haemoproteins, such as cytochrome P450s (CYPs), especially CYP3A4, can catalyse the two-electron reduction of AQ4N[48]. A recently identified extrahepatic CYP, CYP2S1, also reduces AQ4N[49], which is notable given that this enzyme is upregulated by HIF1 (REF. 50). The one-electron reductase inducible nitric oxide synthase (iNOS; also known as NOS2) is also upregulated under hypoxia (BOX 1), and can similarly catalyse the two-electron reduction of AQ4N through its CYP-like haem domain[51]. Importantly, although these haem-dependent reductions of *N*-oxides do not generate an oxygen-sensitive radical intermediate, they are nonetheless inhibited by oxygen[49,51], presumably through competitive binding of O_2 and the *N*-oxide to the haem prosthetic group. This process is therefore potentially exploitable for targeting hypoxia, although the K_{O2} is not well defined, and whether this pathway is fully suppressed under oxic conditions is unclear.

A second group of two-electron reductases catalyse hydride (H^-) transfer from NAD(P)H and are not inhibited by oxygen. These can bypass the oxygen-sensitive free radical intermediate during reduction of quinones, nitro compounds and aromatic *N*-oxides. The best studied enzyme of this class is NAD(P)H dehydrogenase [quinone] 1 (NQO1; also known as DT-diaphorase), which catalyses the facile two-electron reduction of quinones including apaziquone and the aziridinylbenzoquinone RH1 to their hydroquinones[52]. NQO1 also reduces the dinitrobenzamide CB 1954 (tretazicar) to its active 4-hydroxylamine metabolite[53]. Although CB 1954 is a poor substrate for human NQO1, it is efficiently reduced by its paralogue NQO2 using dihydronicotinamide riboside (NRH) as a cofactor[54]. NQO2 also catalyses aerobic reduction of RH1 (REF. 55). In addition, the NADH-dependent two-electron reductase aldo–keto reductase 1C3 (AKR1C3) has recently been shown to reduce PR-104A (but not other bioreductive prodrugs) in some human tumour cell lines under aerobic conditions[56].

Aerobic two electron reductions by these enzymes represent 'off-target' activation in the context of hypoxia and are likely to contribute to the normal tissue toxicity of some quinones and nitro compounds, as illustrated by the resistance of *Nqo1* knockout mice to mitomycin C-induced myelotoxicity[57] and the expression of NQO1 in many normal human tissues[58]. However, this activation may also be therapeutically exploitable in tumours that highly express these enzymes. *NQO1*, *NQO2* (REF. 59) and *AKR1C3* (REFS 56,60) are each transcriptionally regulated, through their antioxidant response elements (AREs), by the transcription factor nuclear

Table 2 | **Representative examples of the prognostic and predictive significance of hypoxia in human cancer***

Measure of hypoxia	Probe	Clinical setting	Outcome for hypoxic tumours
Oxygen concentration	Eppendorf oxygen electrode	Chemoradiation of advanced HNSCC	Worse OS
		Radiotherapy of soft tissue sarcomas before surgery	Worse DFS owing to a higher rate of distant metastasis
		Brachytherapy of localized prostate cancer	Decreased biochemical control (shown by PSA levels)
		Cervical carcinoma	Worse DFS in node-negative patients owing to a higher rate of distant metastases
Endogenous markers	HIF1α	Node-negative breast cancer	Worse OS
	HIF1α	BRCA1 mutant breast cancer	Worse DFS
	HIF2α, CA9	CHART trial in HNSCC	Worse local control and OS
	CA9	Adjuvant chemotherapy of breast cancer	Worse OS
	Osteopontin	Radiotherapy for HNSCC	Nimorazole (hypoxic radiosensitizer) improved local control and OS
	Lysyl oxidase	Breast cancer	Worse metastasis-free survival
	Hypoxic gene signature	HNSCC and breast cancer	Worse outcome, multiple end points
	Hypoxic gene signature	Hepatocellular carcinoma	Worse OS
Exogenous probes	Pimonidazole	Radiotherapy for advanced HNSCC	Worse local control
	EF5	Post-surgical radiotherapy of HNSCC	Worse DFS

CA9, carbonic anhydrase 9; CHART, continuous hyperfractionated accelerated radiotherapy; DFS, disease-free survival; EF5, etanidazole pentafluoride; HIF, hypoxia-inducible factor; HNSCC, head and neck squamous cell carcinoma; OS, overall survival; PSA, prostate specific antigen. *See also Supplementary information S1 (tables) for tables with references.

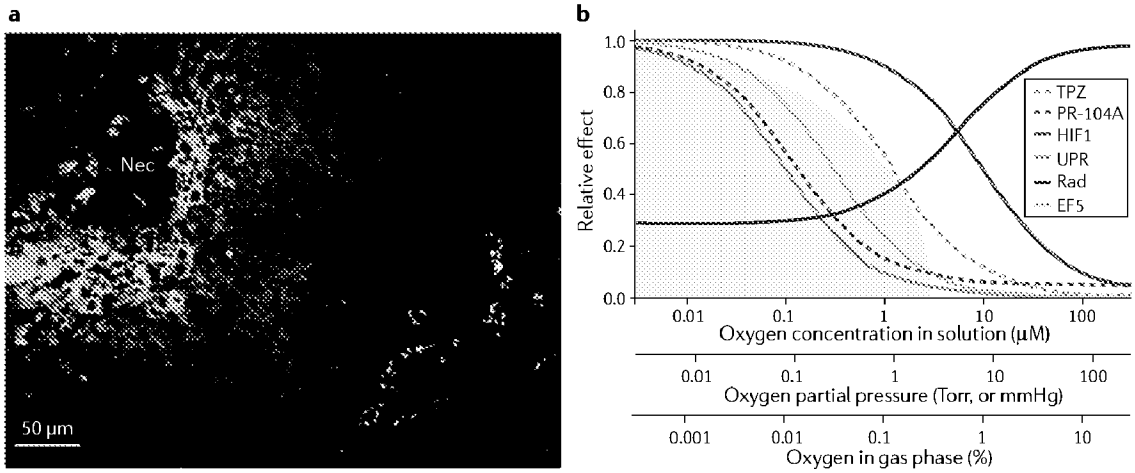


Figure 1 | **Oxygen dependence of hypoxia-responsive processes in tumours. a** | Pseudocolour immunofluorescence showing the difference in distribution of covalently bound pimonidazole (green), an exogenous 2-nitroimidazole hypoxia marker, and hypoxia-inducible factor 1 (HIF1)-regulated carbonic anhydrase 9 (CA9; red), an endogenous marker of hypoxia. This distribution is shown relative to blood vessels (white) and necrosis (Nec) in a representative region of a human squamous cell carcinoma of the larynx. **b** | Schematic representation of quantitative oxygen dependencies for ionizing radiation, bioreductive activation of prodrugs and imaging agents, and biological responses to hypoxia. Three commonly used units for oxygen concentration are shown on the *x* axis, assuming that the culture medium is in equilibrium with humidified gas mixtures at atmospheric pressure[77]. The curves are based on representative oxygen sensitivity parameters for clonogenic cell killing by: ionizing radiation (Rad)[185], tirapazamine (TPZ)[78] and PR-104A[83]. Also shown is binding of the 2-nitroimidazole etanidazole pentafluoride (EF5) to intracellular proteins[186]. Biological responses to hypoxia are time- and cell-type-dependent; the indicative relationships shown here are based on acute stabilization of HIF1 in HT1080 cells[186] and evidence that the unfolded protein response (UPR) is rapidly induced only under severe hypoxia[110,187]. Part **a** is reproduced, with permission, from REF. 150 © (2009) Elsevier Science.

factor erythroid 2-related factor 2 (NRF2; also known as NFE2L2). NRF2, in turn, is controlled by a redox-sensitive cytoplasmic repressor Kelch-like ECH-associated protein 1 (KEAP1), and independently by PRKR-like endoplasmic reticulum kinase (PERK; also known as eIF2AK3)[61]. Both of these signalling pathways provide the potential for indirect upregulation of NRF2-regulated reductases under hypoxia through increased ROS (especially under conditions of fluctuating hypoxia), leading to KEAP1 inactivation or activation of unfolded protein response (UPR) signalling through PERK (see below). High expression of NQO1 is the major driver for clinical development of apaziquone as an intravesicular (topical) therapy for non-invasive bladder cancer[62], and RH1 is also being explored for treatment of tumours with high NQO1 expression[63]. The combination of CB 1954 with the synthetic reducing cofactor caricotamide (also known as EP-0152R), an NRH analogue, has recently been explored for the treatment of NQO2-expressing hepatocellular carcinomas (HCCs). Similarly, high expression of AKR1C3 in some non-small-cell lung cancers and HCCs[56] has led to pilot clinical studies of PR-104 in these cancers, and evaluation is ongoing for acute myeloid leukaemia (AML), based on the high expression of *AKR1C3* mRNA in leukaemic cells from some patients with AML[64]. In each case, the additional hypoxia-selective activation by one-electron reductases is potentially beneficial, including in leukaemias and multiple myeloma, given recent evidence for hypoxia secondary to their expansion in the bone marrow[65,66].

TPZ is also a substrate for NQO1, but uniquely sidesteps the complications of two-electron reduction in that its mono-oxide and non-oxide reduction products (X and Y in FIG. 2A) are relatively non-toxic[67]. This attractive feature of the aromatic *N*-oxides is retained in second-generation TPZ analogues such as SN30000 (REF. 68).

Bioreductive prodrug micropharmacokinetics: the extravascular transport problem. Limited extravascular penetration of drugs, an important contributor to the chemoresistance of solid tumours[69], becomes more crucial when the target cells are confined to hypoxic zones distant from functional blood vessels. The problem is particularly severe for bioreductive prodrugs, given that they are designed to be metabolized as they diffuse into hypoxic zones; if this metabolism is too facile, exposure of the most hypoxic cells will inevitably be compromised. This probably underlies the much lower hypoxic selectivity of TPZ in tumours than in low-density cell cultures[70]. The first suggestion that metabolic consumption of TPZ compromises its tissue penetration came from studies showing loss of activity in hypoxic multicellular spheroids[71]. This was confirmed in more quantitative studies[72,73] using another three-dimensional cell culture model, multicellular layers (MCLs), a model that is more amenable to the direct measurement of drug diffusion.

The importance of prodrug penetration in determining hypoxic cell killing in tumours is illustrated by a comparison of 15 TPZ analogues with widely different extravascular transport properties[74]. In this study the

Multicellular spheroids
Spherical clusters of cells that grow large enough to become diffusion-limited, and thus model some features of the tumour microenvironment.

Multicellular layers
(MCLs). Three-dimensional cell cultures that model the extravascular compartment of tumours. Grown on collagen-coated micro-porous membranes, they allow measurement of drug diffusion and metabolism in tumour-like tissue.

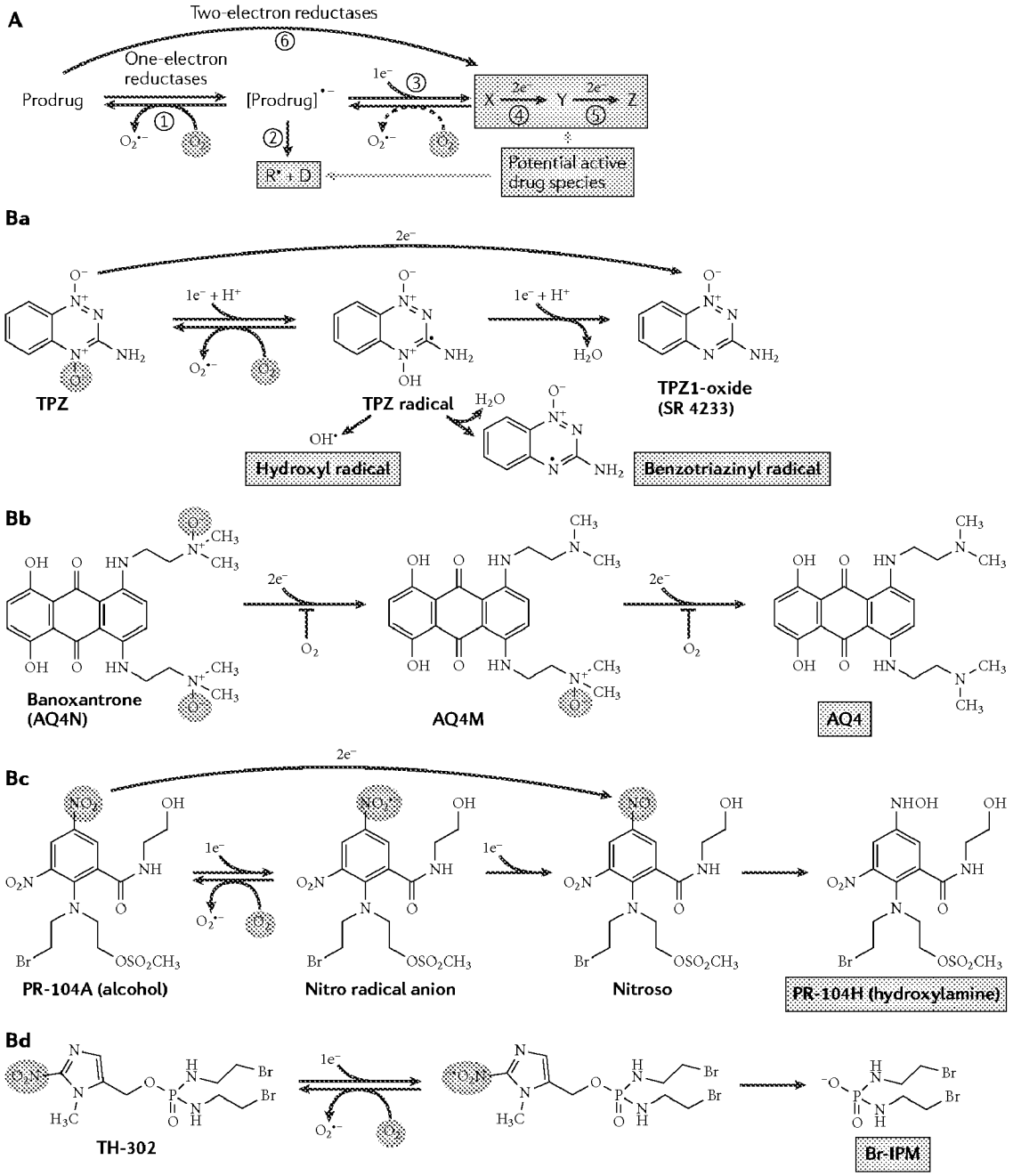


Figure 2 | **Mechanisms of metabolic activation of bioreductive prodrugs.** The cytotoxic metabolites are shown in blue. **A** | Generalized scheme showing competing one-electron and two-electron reductions of prodrugs. One-electron reduction generates a prodrug radical that can be re-oxidized by oxygen (reaction 1) in oxic cells, but generates active drug (blue boxes) in hypoxic cells, either by fragmentation of the prodrug radical (reaction 2) or by its further reduction, usually by disproportionation (reaction 3) and subsequent reduction of the two electron reduction product, X (reactions 4 and 5). Some prodrugs are also reduced by a concerted two-electron reduction (reaction 6), thus bypassing the oxygen-sensitive prodrug radical. Two-electron reduction is typically insensitive to oxygen, with important exceptions (see main text). **B** | Examples of well-studied prodrugs that exploit bioreduction in different ways to elicit selective killing of hypoxic cells. **Ba** | Reduction of an aromatic *N*-oxide to generate a DNA-reactive free radical; **Bb** | reduction of an aliphatic *N*-oxide to unmask a DNA intercalator; **Bc** | nitroreduction as an electronic switch to activate a reactive centre, thus generating an activated nitrogen mustard; and **Bd** | nitroreduction to initiate fragmentation to a non-radical cytotoxin, such as a nitrogen mustard.

tissue diffusion coefficient and bioreductive metabolism kinetics of each prodrug was measured using MCLs grown from HT29 human colon adenocarcinoma cells. These measurements were used to develop a spatially resolved pharmacokinetic and pharmacodynamic model describing pharmacokinetics (concentration–time profiles) and pharmacodynamics (cell killing probability) as a function of position in a tumour microvascular network. Hypoxic

Bystander effect
In the context of bioreductive prodrugs, the killing of adjacent cells that lack prodrug-activating ability through local diffusion of the active drug.

cell killing in HT29 tumour xenografts was well predicted by the model, but only when extravascular transport was included explicitly. This study demonstrated that prodrug reduction kinetics need to be optimized to balance the competing requirements of metabolic stability (for maximal tissue penetration) and metabolism to the cytotoxic metabolite (for maximal cytotoxicity in hypoxic cells).

Until recently the penetration problem has largely been ignored during the development of bioreductive prodrugs, many of which have been found to lack activity as hypoxic cytotoxins in xenograft models despite marked hypoxic selectivity in low-density cell cultures. Some progress has been made in defining the physicochemical properties (such as lipophilicity, molecular weight and hydrogen bond donors and acceptors) that determine diffusion coefficients using MCLs, at least for TPZ analogues[75]. This has assisted the design of new analogues with higher tissue diffusion coefficients, making it possible to accommodate higher rates of bioreductive metabolism without compromising penetration[76]. These features are illustrated by SN30000 (TABLE 3), which has higher activity than TPZ against hypoxic cells in multiple xenograft models[68].

Finessing bioreductive prodrug activation:* K *values and bystander effects. Bioreductive prodrugs can act as direct oxygen sensors through redox cycling or other mechanisms of reductase inhibition by oxygen, as outlined above. However, their quantitative oxygen dependence is crucially important for their ability to complement other anticancer agents such as ionizing radiation (FIG. 1b), and differs among prodrugs.

The elimination of hypoxic tumour cells at 'intermediate' oxygen concentrations (~1–10 μM oxygen) is arguably more important than the most severely hypoxic or anoxic cells, which are less frequent and probably less likely to contribute to tumour regrowth after therapy. Two different bioreductive prodrug strategies are being explored for targetimg these moderately hypoxic cells, each with different strengths and weaknesses. One strategy is to use prodrugs with relatively high K_{O_2} to provide activation under moderate hypoxia. The only bioreductive prodrugs demonstrated to be activated under such conditions are TPZ[77,78] and its analogues, such as SN30000 (REF. 68), which have K_{O_2} values of ~1 μM in cell culture (TABLE 3).

The other strategy is to confine prodrug activation to more severely hypoxic cells (K_{O_2} ~0.1 μM), which has the advantage of restricting activation to pathologically hypoxic regions in tumours and thus avoiding activation under physiological hypoxia in normal tissues. This also limits the metabolic loss of prodrugs during diffusion into hypoxic zones. These very low K_{O_2} values — although difficult to measure experimentally because of technical limitations in controlling and quantifying low oxygen concentrations in respiring cell cultures — seem to be typical of quinones[79], nitro compounds[80] and cobalt complexes[81]. These bioreductive prodrugs can be expected to spare many radioresistant and chemoresistant hypoxic cells at oxygen concentrations above the drugs' K_{O_2}. In this case it may be crucially important that the active bioreductive metabolites can diffuse to cells at higher pO_2 (known as the bystander effect). Such local diffusion has been demonstrated for CB 1954 and dinitrobenzamide mustards using anoxic MCL co-cultures in which 'activator' cells overexpressing NADPH–cytochrome P450 reductase (CYPOR; also known as POR) facilitate the killing of 'target' cells that are less able to activate the prodrugs[82]. PR-104A provides an example of a bioreductive prodrug with this profile (a low K_{O_2} and efficient bystander killing)[83]. Which of these strategies (high K_{O_2} versus low K_{O_2} plus bystander effect) is preferable may depend on tumour-specific features such as the depth and spatial distribution of hypoxia (for example, whether most moderately hypoxic cells are contiguous with more severely hypoxic cells) and on treatment-specific features such as the oxygen dependence and extravascular penetration of any other agents used in combination.

Beyond DNA-reactive cytotoxins as effectors for bioreductive prodrugs. A common feature of all bioreductive prodrugs currently in development (TABLE 3) is that their active metabolites are DNA-reactive cytotoxins that damage the replication fork. Although the DNA replication fork can be considered the most successful chemotherapy target to date[84], toxicity to proliferating normal tissues is an inescapable consequence. Existing chemotherapy and chemoradiation protocols are already titrated to maximal myelotoxicity, which limits the opportunities to add the current generation of bioreductive prodrugs to standard therapies. This makes it attractive to consider adapting bioreductive prodrug design to release a broader range of active metabolites, including non-genotoxic inhibitors of molecular targets. Early examples were 2-nitroimidazole prodrugs that, on chemical reduction, release the poly(ADP-ribose) polymerase 1 (PARP1) inhibitor 5-bromoisoquinolone[85] and the prototypical cyclooxygenase inhibitor aspirin[86]. More recently a similar approach has been used to release the tubulin-stabilizing drug combretastatin A4 (REF. 87) and the lysyl oxidase inhibitor β-aminoproprionitrile by bioreduction of prodrugs under hypoxia[88]. In addition, quaternary ammonium nitroheterocyclic bioreductive triggers[89] have been used to release non-myelotoxic, irreversible pan-ERBB inhibitors under hypoxia[90]. The prototype of this new class, SN29966, provides marked activity as a monotherapy against human tumour xenografts, a result that is suggested to reflect the ability of this prodrug to exploit fluctuating hypoxia because of its long residence time in tumours[90].

Molecular targets in hypoxic cells

The identification of molecular mechanisms that mediate cellular responses to hypoxia has stimulated interest in targets that might compromise the survival of hypoxic cells if inhibited. The two main oxygen-responsive signalling pathways that mediate adaptation to hypoxia are centred on the HIF family of transcription factors[3,91,92] and the UPR[93], whereas mTOR presents a less well-defined opportunity to target hypoxic cell survival (FIG. 4).

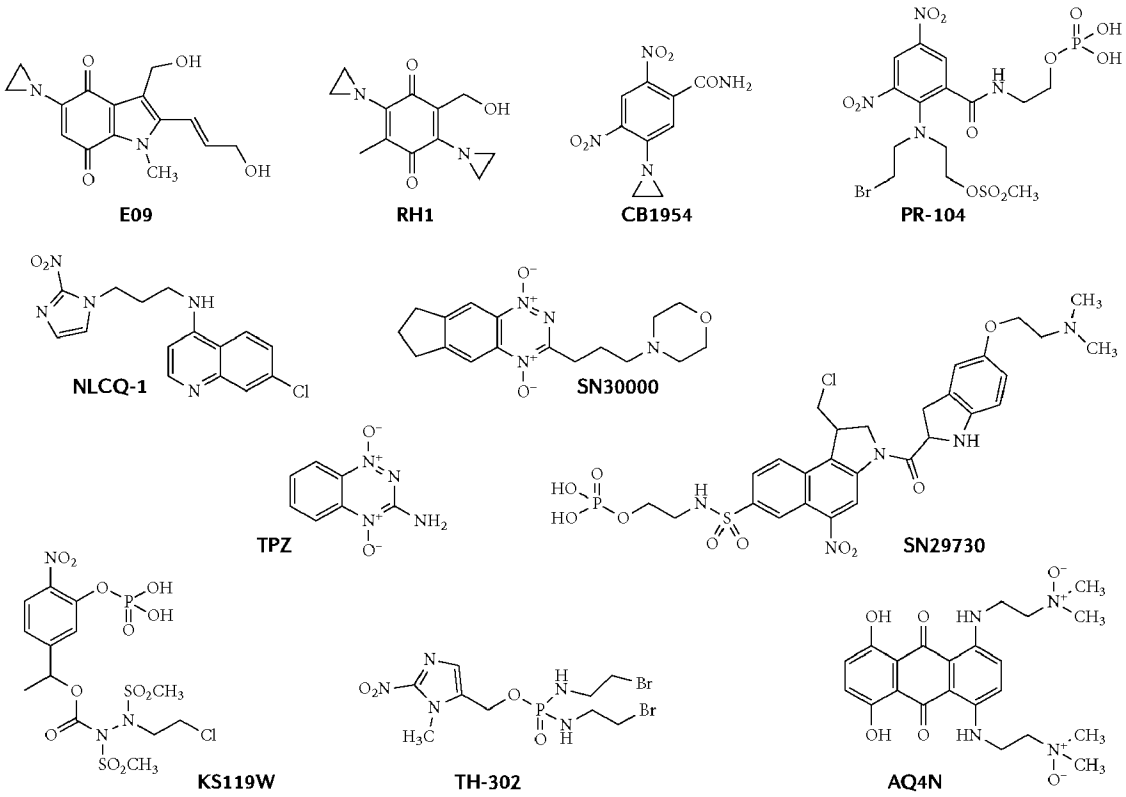


Figure 3 | **Structures of bioreductive prodrugs.** Structures of the prodrugs presented in TABLE 3 and in the main text are shown.

HIFs. Regulation of HIF1α and HIF2α (also known as EPAS1) by oxygen-dependent dioxygenases such as prolyl hydroxylase domain (PHD) enzymes, the primary oxygen sensors, leads to a broad, adaptive response to hypoxia. This response includes the transcription of genes involved in angiogenesis (such as vascular endothelial growth factor A (*VEGFA*)), metabolic adaption (such as *SLC2A1*, which encodes the glucose transporter GLUT1), tolerance of acidosis (*CA9*), cell survival (for example, insulin-like growth factor 1 (*IGF1*)) and metastasis (such as lysyl oxidase (*LOX*))[92]. HIF1α activity may also be influenced by many factors in addition to hypoxia[92], hence targeting HIF1α or its downstream products may additionally kill pseudo-hypoxic tumour cells. Nonetheless, even if not strictly specific to hypoxia, HIF1 inhibitors clearly have considerable potential to suppress resistance to therapy through multiple mechanisms, including the prevention of HIF1-dependent enhancement of endothelial cell radioresistance through cycling hypoxia[94] and blocking of the vasculogenic response to ionizing radiation-induced hypoxia[10].

HIF1α overexpression and its association with poor treatment response and outcome has been demonstrated in an extensive range of human tumours[19,95] (TABLE 2). Multiple components of the HIF1 signalling pathway have been identified as candidate drug targets[96,97] and a wide range of pharmacological approaches have been proposed; surveys of these have been published recently[92,95] (TABLE 4). Several novel agents have undergone Phase I evaluation (such as EZN-2968 (www.ClinicalTrials.gov identifier NCT00466583) and PX-478 (www.ClinicalTrials.gov identifier NCT00522652)), but currently there is no clear clinical evidence of antitumour activity due to HIF1 inhibition. Other agents have been 'repurposed' from their original applications (such as the antibiotic geldanamycin[98]), and have limited specificity for HIF1α. In addition, many new agents have been discovered through phenotypic screens (inhibition of HIF1α signalling) but their direct molecular targets and ability to selectively kill hypoxic cells are not yet well defined. A further interesting strategy for the selective killing of HIF1-expressing cells is the incorporation of a PHD-sensitive oxygen degradation domain (ODD) from HIF1α into cytotoxic proteins, such as a procaspase 3 fusion protein containing both an ODD and a protein transduction domain[99].

The UPR. The elucidation of the role of the UPR in oxygen sensing and hypoxic cell survival has extended the potential molecular targets for drugging hypoxic cells[100]. Oxygen is the preferred terminal electron acceptor in the redox relay required for disulphide bond formation in protein folding[101]. Severe hypoxia leads to increased levels of unfolded proteins in the endoplasmic reticulum (ER), leading to the induction of the UPR (FIG. 4). The UPR is mediated by three signalling pathways: the PERK–eukaryotic translation initiation factor 2A (eIF2A)–activating transcription factor 4 (ATF4) pathway, the inositol-requiring

Pseudo-hypoxia
The induction of molecular responses analogous to those caused by hypoxia but triggered by other conditions.

Table 3 | **Bioreductive prodrugs of DNA-reactive cytotoxins recently or currently in clinical development**

Prodrug	Current clinical status	Company or institution	Chemical class	Mechanism of activation*	Mechanism of cytotoxicity	One-electron reductases	Two-electron reductases	K_{O2} (μM)
Tirapazamine (SR 4233)	Phase III, cervix (closed)	SRI International/ NCI	Aromatic *N*-oxide	1, 3 [R•]	Complex DNA damage	CYPOR, iNOS	NQO1‡	~1
Apaziquone (E09)	Phase III, bladder (closed)	Spectrum	Quinone	1, 4 [X,Y]	ICL	CYPOR	NQO1	
TH-302	Phase I/II, multiple (active)	Threshold	Nitro	1, 3 [D]	ICL	CYPOR		~10§
PR-104	Phase I/II, leukaemia (active)	Proacta and University of Auckland	Nitro	1/2, 4, 5, 6 [Y,Z]	ICL	CYPOR, iNOS, MTRR, NDOR1	AKR1C3	~0.1
Banoxantrone (AQ4N)	Recent Phase I/II	Novacea	Aliphatic *N*-oxide	2, 5 [Y]	TOPOII	iNOS	CYP3A4, CYP2S1	
Caricotamide (EP-0152R) plus tretazicar (CB1954)	Phase II, HCC (discontinued)	BTG	Nitro	1/2, 4, 5, 6 [Y,Z]	ICL	CYPOR, iNOS	NQO1, NQO2	
RH1	Recent Phase I	CRUK	Quinone	1, 4 [X,Y]	ICL		NQO1, NQO2	
NLCQ-1	Preclinical	Evanston Hospital	Nitro	1, 4, 5	TOPOII or multiple?	CYPOR		~1§
SN30000 (CEN-209)	Preclinical	Centella and University of Auckland	Aromatic *N*-oxide	1, 3 [R•]	Complex DNA damage	CYPOR		~1
SN29730	Preclinical	University of Auckland	Nitro	1, 4, 5, 6 [Z]	Adenine *N*3 alkylation	CYPOR		
KS119W	Preclinical	Yale University	Nitro	1, 4, 5, 6 [D]	Guanine *O6* ICL	B5R, CYPOR		

See FIG. 3 for chemical structures. AKR1C3, aldo-keto reductase 1C3; B5R, NADH-cytochrome *b5* reductase, CRUK, Cancer Research UK; CYP, cytochrome P450; CYPOR, NADPH–cytochrome P450 reductase; HCC, hepatocellular carcinoma; ICL, DNA interstrand crosslink; iNOS, inducible nitric oxide synthase; MTRR, methionine synthase reductase; NCI, US National Cancer Institute; NDOR1, NADPH-dependent diflavin oxidoreductase 1; NQO, NAD(P)H dehydrogenase [quinone]; TOPOII, topoisomerase II. *Reaction numbers refer to FIG. 2A. Active cytotoxins (X,Y etc in FIG. 2A) are shown in square brackets. ‡Detoxifying. §Gas phase O_2 concentration[66] (K_{O2} values of 2-nitroimidazoles are typically much lower based on solution oxygen concentrations). See also Supplementary information S1 (tables) for tables with references.

enzyme 1 (IRE1; also known as ERN1)–X-box binding protein 1 (XBP1) pathway and the ATF6 pathway. These pathways activate responses to suppress protein synthesis, stimulate protein degradation in the ER, and activate apoptosis and autophagy to resolve ER stress[93]. An additional mechanism of activation of UPR by hypoxia is the stabilization of ATF4 through loss of its oxygen-dependent PHD3-mediated degradation[102]. Gene knockout and RNA interference studies have demonstrated that the PERK–eIF2A–ATF4 and IRE1–XBP1 pathways contribute to hypoxic cell survival[102–104].

Two drug strategies are being pursued to kill hypoxic cells selectively through UPR targets (TABLE 4). One approach seeks to inhibit the UPR by targeting PERK, ATF4 and IRE1. High-throughput screens and *in vivo* luminescence-based assays for UPR inhibitors have been reported[105], as have first-generation inhibitors of the endonuclease domain of IRE1 (REFS 106,107). Further drug discovery will be facilitated by the availability of crystal structures of the endonuclease domain of yeast IRE1 (REF. 108). A second approach seeks to exacerbate ER stress in order to overwhelm the UPR on the assumption that the UPR is near its capacity in hypoxic cells. Evidence that the ER stressors thapsigargin and bortezomib elicit hypoxia-selective cytotoxicity *in vitro* supports this approach[109].

Cap-dependent translation
Translation initiated by binding of the eIF4F complex to the methyl-7-G(5′)pppN structure (cap) at the 5′ end of the mRNA.

mTOR. As a key node for the integration of the signals regulating cellular energy and nutrient status, mTOR presents a potential target for hypoxic cell killing. Under hypoxia, mTOR complex 1 (mTORC1) kinase activity is restricted through multiple mechanisms (FIG. 4), resulting in the suppression of protein synthesis to an extent that depends on the severity and duration of hypoxia[110]. The mechanisms include activation of the tuberous sclerosis 1 (TSC1)–TSC2 complex through the HIF1 target gene DNA-damage-inducible transcript 4 (*DDIT4*; also known as *REDD1*)[111] and through increased AMP-activated protein kinase (AMPK) activity under hypoxia[110,112]. In addition, hypoxia induces the HIF1 target gene *BNIP3*, which inhibits mTORC1 through RAS homologue enriched in brain (RHEB)[113]. The resulting suppression of mTORC1 has multiple effects on transcription and translation, the latter in part owing to hypophosphorylation of eIF4EBP1, which leads to sequestration of eIF4E and thus inhibition of cap-dependent translation. This results in preferential cap-independent translation of a subset of mRNAs including *HIF1A* and *VEGFA*. Hypoxia has been proposed to have a dual role in tumour cell survival through modulation of mTORC1 (REF. 93). In small, early stage tumours, moderate hypoxia inhibits tumour growth through mTORC1 suppression, providing a selective pressure for abrogation of the pathway. In larger, late stage tumours, mTORC1 suppression by hypoxia may be an

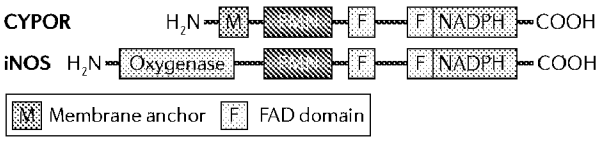
adaptive response in the face of energy limitations, thus favouring hypoxic cell survival. If so, the consequences of further inhibiting mTORC1 in hypoxic cells are difficult to predict. Several studies have explored the activity of mTOR inhibitors in hypoxic cells (TABLE 4). Rapamycin provided hypoxia-selective antiproliferative effects on HT29 cells and, when combined with low dose irinotecan, gave increased hypoxic cell killing *in vitro* and increased tumour control *in vivo*[114]. Treatment with WYE 125132, a potent and specific mTOR kinase inhibitor, gave substantial tumour control in a range of models and blocked HIF1α and HIF2α accumulation under hypoxic conditions, leading to reduced hypoxic adaptation[115].

Targets downstream of the primary hypoxia-sensing pathways. The hypoxia-induced HIF, UPR and mTOR signalling pathways are highly interactive networks that influence many downstream gene products and processes that have potential as therapeutic targets. Here we outline some of the downstream targets under consideration for selective killing of hypoxic cells.

Recent studies have shown that the UPR activates autophagy to ameliorate hypoxic stress[6,116], and that inhibition of autophagy with chloroquine or 3-methyladenine causes selective hypoxic cell killing[6].

Metabolic reprogramming in tumour cells, most famously demonstrated by the shift to aerobic glycolysis (known as the Warburg effect), is in part mediated by HIF1 (REF. 117) and mTOR[7], and is therefore linked to hypoxia. This metabolic switch is also regulated by many other signalling nodes (especially by MYC, p53 and the PI3K–AKT pathway) and reflects the re-gearing of metabolism to support biosynthetic programmes and antioxidant defences to drive tumour cell growth[7,118]. Although the shift from oxidative phosphorylation is not confined to hypoxic cells, the dependence on glycolytic ATP generation creates a vulnerability for these cells because they can no longer call on the residual mitochondrial oxidative phosphorylation, which still contributes significant ATP generation in aerobic tumour cells[119]. This reliance on glycolysis makes hypoxic tumour cells highly sensitive to suppression of glycolytic flux, hence glucose analogues that inhibit glycolysis (TABLE 4) produce striking hypoxia-selective cytotoxicity *in vitro*[120]. The most widely studied compound of this class, 2-deoxy-D-glucose (2DG), is phosphorylated by hexokinases to the corresponding 6-phosphate. This phosphorylated analogue inhibits both hexokinases and phosphoglucose isomerase (GPI), which catalyses the next step in glycolysis[119]. The 2-fluoro analogue of 2DG is a more potent glycolytic inhibitor and hypoxic cytotoxin[121]. 2DG has been evaluated in clinical trials, but the results have not been reported; toxicity to other highly glucose-dependent tissues (such as the brain, retina and testes) represents a potential challenge in the further clinical development of this approach.

Box 1 | **Identity of prodrug-activating one-electron reductases**



Enzymes that catalyse one-electron transfer to prodrugs are central players in hypoxia-selective bioreduction (FIG. 2A). Their identification is an urgent priority to enable profiling of individual tumours, but has proven challenging. The best characterized enzyme is the diflavin reductase NADPH–cytochrome P450 reductase (CYPOR; also known as POR), which catalyses an intramolecular redox shuttle in which a hydride ion (H^-) is transferred from the NADPH domain to the FAD domain, which then transfers electrons to the terminal one-electron donor flavin mononucleotide (FMN) domain (see the figure). CYPOR reduces non-mitochondrial cytochrome P450s (CYPs) and has broad substrate specificity for xenobiotics with one-electron reduction potentials that are similar to or higher than its FMN and FAD redox centres, including many bioreductive prodrugs (TABLE 3).

The nitric oxide synthases (NOSs) have diflavin (FMN and FAD) reductase domains that are homologous to CYPOR, but NOSs reduce an intramolecular haem prosthetic group in the oxygenase domain, which is responsible for nitric oxide synthesis. As for CYPOR, the transferred electron can be intercepted by small molecule electron acceptors such as tirapazamine (TPZ) and quinones[163,164]. Interest has focused on the inducible NOS (iNOS; also known as NOS2) isoform because it is highly expressed in some tumours[165,166] including by macrophages that accumulate in hypoxic zones[167]. Notably, iNOS is upregulated under hypoxia through the binding of hypoxia inducible factor 1 (HIF1) to the transcription factor interferon regulatory factor 1 (IRF1)[168,169]. This leads to localized iNOS expression in hypoxic regions of tumours[170], which provides an additional mechanism of hypoxic selectivity for its substrates. However, given that iNOS expression in tumours is often predominantly stromal[166], this enzyme will be best exploited by bioreductive prodrugs that generate cytotoxic metabolites with an efficient bystander effect. In this regard it is notable that the prodrugs AQ4N[171], CB 1954 (REF. 172) and PR-104A[173] are activated by iNOS under hypoxia; each provides efficient bystander effects and thus has potential for exploiting hypoxic expression of iNOS in the tumour stroma. The tropism of macrophages for hypoxic regions of tumours is also being exploited for the delivery of prodrug-activating enzymes, using adenoviral transduction of *CYPOR* and hypoxia response element (HRE)-regulated *CYP2B6* to activate cyclophosphamide[174]. Increased hypoxic activation of TPZ has previously been demonstrated by transduction of tumour cells with HRE-driven *CYPOR*[175], suggesting the potential for further enhancing hypoxic targeting by bioreductive prodrugs by combining these approaches.

PR-104A can also be activated under hypoxia by the other members of the diflavin reductase family, NADPH-dependent diflavin oxidoreductase 1 (NDOR1) and methionine synthase reductase (MTRR)[173]. Other flavoproteins capable of one-electron prodrug activation include NADH-cytochrome *b*5 reductases[176], ferredoxin reductase (FDXR)[177], xanthine oxidase[55] and xanthine dehydrogenase, which is also capable of two-electron reduction[178]. However, much needs to be learned about the relative activity of these and other reductases in hypoxic regions of human tumours.

There is much interest in inhibiting other targets that can be rate-limiting for glycolysis, and which might offer greater tumour selectivity, including the HIF1-regulated facultative glucose transporter GLUT1, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFBs) and the tumour-specific pyruvate kinase M2 (PKM2) isoform. Elevated GLUT1 levels has been described in a wide range of tumour types and has been demonstrated to be a negative prognostic indicator[122]. Many experimental GLUT1 inhibitors, such as phloretin, have multiple molecular targets or act indirectly, but recent examples (fasentin[123] and

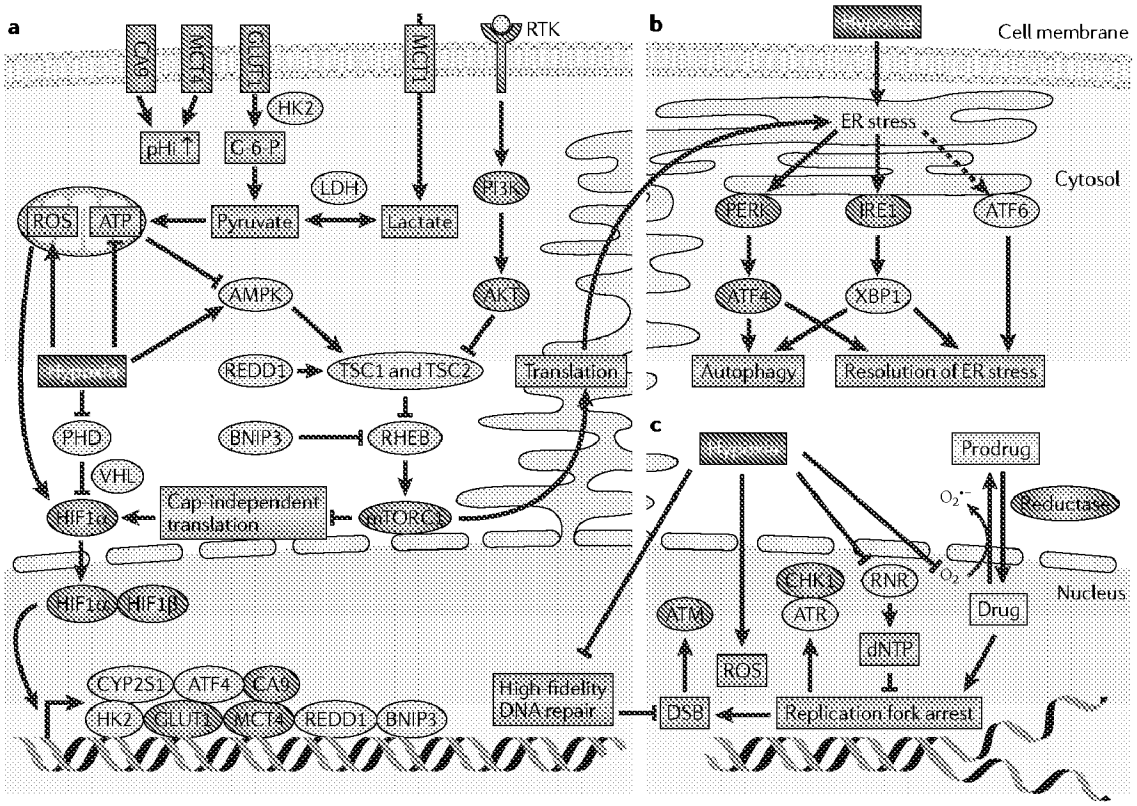


Figure 4 | **Potential molecular targets for killing hypoxic cells in the oxygen-responsive signalling pathways that mediate adaptation to hypoxia. a** | The hypoxia-inducible factor (HIF)–mTOR central metabolism module. Hypoxia inhibits prolyl hydroxylase domain (PHD)-mediated degradation of HIF1α, which allows its dimerization with HIF1β (also known as ARNT) and transcription of a range of genes associated with metabolic reprogramming (including hexokinase 2 (*HK2*) and the glucose transporter GLUT1 (encoded by *SLC2A1*)) and control of intracellular pH (pHi), such as monocarboxylate transporter 4 (*MCT4*) and carbonic anhydrase 9 (*CA9*). Also, the ability of aerobic tumour cells to use lactate in place of glucose for oxidative phosphorylation has been suggested to allow glucose to diffuse to hypoxic cells, which are highly glucose-dependent, defining the lactate transporter MCT1 as a potential target (potential target proteins are shown in green). Hypoxia induces the formation of reactive oxygen species (ROS), which stabilize HIF1α. Hypoxia also inhibits mTOR complex 1 (mTORC1) through the HIF1-dependent transcription of DNA damage-inducible transcript 4 (*DDIT4*, which encodes REDD1) and *BNIP3* and through AMP-activated protein kinase (AMPK) signalling. This inhibition results in the hypophosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (eIF4EBP1), which favours cap-independent translation of a subset of transcripts, including *HIF1A* and provides an mTOR–HIF1 regulatory loop. Receptor tyrosine kinases (RTKs) also modulate HIF1α translation through mTOR and other pathways in some cell lines and can also influence hypoxic survival responses. **b** | The unfolded protein response (UPR) module. Hypoxia, through the lack of oxygen to act as the ultimate electron acceptor in disulphide bond formation, impairs protein folding in the endoplasmic reticulum (ER). This leads to activation of the UPR, through PRKR-like endoplasmic reticulum kinase (PERK; also known as eIF2AK3), inositol-requiring enzyme 1 (IRE1; also known as ERN1) and potentially activating transcription factor 6 (ATF6), which supports hypoxic cell survival. **c** | DNA damage response module. Severe hypoxia inhibits ribonucleotide reductase (RNR), leading to replication fork arrest and protective ataxia telangiectasia and Rad3-related (ATR) signalling. Production of ROS in hypoxic cells, and especially on re-oxygenation, leads to DNA double-strand breaks (DSBs), which activate ataxia telangiectasia mutated (ATM) signalling. Thus, DNA damage signalling pathways provide potential targets for hypoxia-selective cell killing. Hypoxia also reduces high fidelity DNA repair (by, for example, homologous recombination (HR), which leads to sensitivity to poly(ADP-ribose) polymerase (PARP) inhibitors). In addition, hypoxia permits activation of bioreductive prodrugs, mainly by preventing redox cycling of the prodrug radical anions generated by one-electron reductases. The resulting cytotoxic drugs typically induce DNA replication fork damage, exacerbated by suppression of HR in hypoxic cells, leading to cell death. CYP2S1, cytochrome P450 2S1; G-6-P, glucose-6-phosphate; LDH, lactate dehydrogenase; RHEB, RAS homologue enriched in brain; TSC, tuberous sclerosis; VHL, von Hippel-Lindau tumour suppressor; XBP1, X-box binding protein 1.

Table 4 | **Representative examples of pharmacological approaches to molecular targets in hypoxic cells***

Pathway	Target	Agent	Class
HIF1α expression	HIF antisense mRNA	EZN-2968	RNA oligonucleotide
	Topoisomerase I	Topotecan	Camptothecin analogues
	Multiple	PX-478	Melphalan *N*-oxide
	Translation	Digoxin	Cardiac glycoside
	HSP90	Geldanamycin and tanespimycin (17-AAG)	Benzoquinone ansamycin antibiotics
HIF1 transcription	HIF–p300 binding	Chetomin and analogues	Dithiodiketopiperazine
	Thioredoxin 1	PX12	Imidazole disulphide
		PMX290	Indoloquinol
	DNA binding	Echinomycin	DNA intercalator
HIF1 target gene products	CA9 and CA12	Aryl sulphonamides	Sulphonamide zinc binders
	GLUT1	Glufosfamide	Glucose isophosphoramide mustard
		2-GLU-SNAP	Glucose SNAP conjugate
		Fasentin	Oxobutanilide
		STF-31154	Unknown
	HK2	5TDG, 2DG, 2FDG	Glycolysis inhibitors
	MCT1	α-cyano-4-hydroxycinnamate	Lactate transport inhibitor
Receptor tyrosine kinases	VEGFR	Bevacizumab	Monoclonal antibody
	EGFR	Gefitinib and erlotinib	ATP competitive kinase inhibitors
		Cetuximab	Monoclonal antibody
RAS–MAPK signalling	BRAF	Sorafenib	ATP competitive kinase inhibitor
mTOR	mTORC1	Rapamycin and everolimus	Allosteric binders of FKBP12-rapamycin binding domain
		WYE-125132	ATP-competitive mTOR kinase inhibitor
	Autophagy	Chloroquine	Lysosomal pH
UPR	HSP90	Geldanamycin and 17-AAG	Benzoquinone ansamycin antibiotic
	IRE1	Salicaldehydes	IRE1 inhibitor
	26S proteasome	Bortezomib	Boronic acid tripeptide
		Nelfinavir and ritonavir	HIV protease inhibitors
	SERCA	2,5-Dimethyl celecoxib	Celecoxib analogue

CA, carbonic anhydrase; DG, deoxy-D-glucose; EGFR, epidermal growth factor receptor; FDG, fluorodeoxyglucose; FKBP12, FK506 binding protein 12; GLUT1, glucose transporter 1; HIF, hypoxia-inducible factor; HK2, hexokinase 2; HSP90, heat shock protein 90; IRE1, inositol-requiring enzyme 1 (also known as ERN1); MCT1, monocarboxylate transporter 1; mTORC1, mTOR complex 1; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SNAP, S-nitroso-acetyl-penicillamine; UPR; unfolded protein response; VEGFR, vascular endothelial growth factor receptor. *See also Supplementary information S1 (tables) for tables with references.

STF-31154 (REF. 124)) target GLUT1 directly. The shift to glycolysis is accompanied by increased generation of pyruvate and its conversion to lactate by lactate dehydrogenase A (LDHA). The lactate transporter monocarboxylate transporter 1 (MCT1) has been suggested as a target for killing hypoxic cells by glucose starvation, through a novel mechanism of metabolic symbiosis[125]. This study showed that aerobic tumour cells expressing MCT1 can use lactate as a preferred substrate for respiration, and further demonstrated that inhibition of MCT1 by α-cyano-4-hydroxycinnamate increases glucose consumption *in vitro* and tumour radiosensitivity[125]. The proposed model is that the stimulation of glucose consumption in aerobic tumour cells compromises glucose penetration into hypoxic regions, leading to the selective death of hypoxic cells in tumours. However, laboratory tools such as α-cyano-4-hydroxycinnamate are not particularly selective for the MCTs[126] and one class of selective MCT1 inhibitors has been identified as an immunomodulator[127], raising concerns about the selectivity of such an approach for targeting hypoxic cells.

One of the consequences of the glycolytic shift, driven in part by hypoxia, is that increased generation of metabolic acids further compromises hypoxic cell survival. Disruption of pH homeostasis by targeting MCTs (such as MCT1 and MCT4) and carbonic anhydrases in hypoxic tumour cells has been proposed as a tumour-selective approach[128]. MCT4 is upregulated in a HIF1α-dependent manner[129] and increased expression of MCT4 in tumour cells has been demonstrated[130]. MCT4 export of lactate and H^+ prevents intracellular acidification and assists in the remodelling of the extracellular milieu, but specific inhibitors of MCT4 have yet to be reported.

Carbonic anhydrases are metalloenzymes that catalyse the reversible hydration of carbon dioxide to carbonic acid. The expression of CA9 and CA12 is

controlled by HIF1 (REF. 131) and CA9 is also regulated through the UPR by ATF4 (REF. 132). Despite generating H^+ and HCO_3^- with equivalent stoichiometry at the extracellular catalytic domain of these transmembrane proteins, linked bicarbonate transporters raise the intracellular pH to protect hypoxic cells[128]. Silencing both CA9 and CA12 resulted in marked inhibition of the growth of LS174 human colon carcinoma cell xenograft tumours[131]. Extensive drug development efforts have identified a range of compounds with varying selectivity for CA9 and CA12; several compounds inhibited tumour growth and metastasis selectively in CA9-positive tumour models[133].

Molecular targets in DNA damage response and repair pathways. Inhibitors of DNA damage signalling and DNA repair have the potential to exploit changes in these pathways in hypoxic cells[134–136]. Three approaches have recently been considered. The first is to exploit activation of the DNA damage response in hypoxic cells. Severe hypoxia rapidly induces replication arrest through a HIF1- and p53-independent mechanism[137]. Recent evidence indicates this is due to depletion of dCTP, dGTP and dATP pools[138], reflecting the requirement of class 1a (eukaryotic) ribonuncleotide reductases for molecular oxygen[139]. Single-stranded DNA at stalled replication forks then induces ataxia telangiectasia and Rad3-related (ATR)–CHK1 signalling, which is required to maintain replication fork integrity. Consistent with this, knockdown of CHK1 is selectively toxic to hypoxic cells[140]. This ATR-mediated replication arrest is reversible if cells are re-oxygenated within a few hours, but re-oxygenation then induces ROS-mediated DNA damage, including double-strand breaks that activate the kinase ataxia-telangiectasia mutated (ATM)[141], potentially providing sensitivity to inhibitors of ATM signalling.

A second strategy is to exploit defects in DNA repair in hypoxic cells. ATR- and ATM-mediated signalling in hypoxic cells can help to facilitate DNA repair. For example, hypoxia stimulates CHK2-mediated Ser988 phosphorylation of BRCA1[142], which stimulates its activity in HR. However, hypoxia also downregulates expression of key HR proteins such as RAD51 and BRCA1 through HIF1-independent repression of transcription and translation[136]. In addition, hypoxia suppresses RAD51 expression in breast cancer initiating cells through HIF1-dependent upregulation of the Polycomb protein enhancer of zeste homologue 2 (EZH2)[143]; *RAD51* mRNA has also recently been shown to be downregulated in hypoxic regions of 9L gliomas by laser-capture microdissection of etanidazole pentafluoride (EF5)-stained tissue[144]. Hypoxia-mediated suppression of HR in chronically hypoxic cells[145,146] confers an increased sensitivity to DNA-damaging cytotoxins[146], which may make a significant contribution to the activity of bioreductive prodrugs that deliver such cytotoxins to hypoxic cells. Notably, hypoxia-induced downregulation of HR creates the same phenotype that sensitizes BRCA1 or BRCA2 homozygous mutant cells to PARP1 inhibition. Recently a synthetic lethal interaction has been demonstrated for hypoxia and genetic deletion or chemical inhibition of PARP1, analogous to that for BRCA1 or BRCA2 mutations, and the PARP1 inhibitor veliparib (also known as ABT-888) has been shown to selectively reduce the proportion of radioresistant (that is, hypoxic) cells in RKO colon carcinoma xenografts[147]. The authors point to the potential for synthetic lethal interactions between hypoxia and inhibitors of other repair pathways downregulated by hypoxia.

A third strategy is to pharmacologically reactivate p53 to restore hypoxia-mediated apoptosis[135]. Small molecules that are in development for p53 reactivation include APR-246 (also known as PRIMA-1), which restores transcriptional activity of mutant p53, and Nutlin-3 and RITA, which interfere with MDM2-mediated p53 degradation[148]. RITA also induced a DNA damage response that appears to contribute to its stimulation of p53-dependent apoptosis, but cell killing was similar in hypoxic and aerobic cells[149].

Hypoxia and personalized cancer medicine

As in other aspects of cancer medicine, emerging technologies for profiling individual tumours have the potential to revolutionize the development of hypoxia-targeted agents. Indeed, the heterogeneity in tumour hypoxia at the broader human population level, even within a single disease subtype, means that successful development of hypoxia-targeted agents is probably a forlorn hope unless hypoxic tumours can be identified prospectively. Studies with advanced head and neck squamous cell carcinomas (HNSCCs), in which hypoxia has been demonstrated to be a negative prognostic factor using every type of diagnostic tool available (TABLE 2), are instructive in this regard. A large, relatively homogenous (stage T2–T4 laryngeal) series of HNSCC samples showed evidence of hypoxia by both pimonidazole and CA9 immunostaining in the majority of tumours, but with extreme variability[150]. The need to quantify (not just to detect) hypoxia is illustrated by a meta-analysis of oxygen-electrode studies, which suggested that hypoxia compromised overall survival in patients with advanced HNSCC undergoing chemoradiation treatment but only in the subset of patients with the most extensive hypoxia[151]. This situation is different from the subcutaneous xenograft models widely used in preclinical studies, in which essentially all tumours display extensive hypoxia; these models thus tend to over-represent the target (and will over-predict activity) relative to autochthonous tumours in humans.

Thus there is currently much interest in the further development of hypoxia diagnostics as predictive biomarkers[18,19,152,153]. Although studies using invasive methods (TABLE 2) have been important in establishing the significance of tumour hypoxia at the population level, broader clinical application for stratifying patients will require less-invasive tools such as positron emission tomography (PET) imaging (BOX 2). There is also great potential for minimally invasive serum-based diagnostics and global gene expression signatures for the identification of hypoxia (TABLE 2).

Synthetic lethal interaction
In genetics, an interaction between two non-lethal mutations that, in combination, confer lethality. In chemical genetics, this term can refer to interaction between a drug and mutation that confers greater drug-sensitivity than with the wild type.

Autochthonous tumours
Tumours that arise in the host being studied, as distinct from tumours introduced by transplantation.

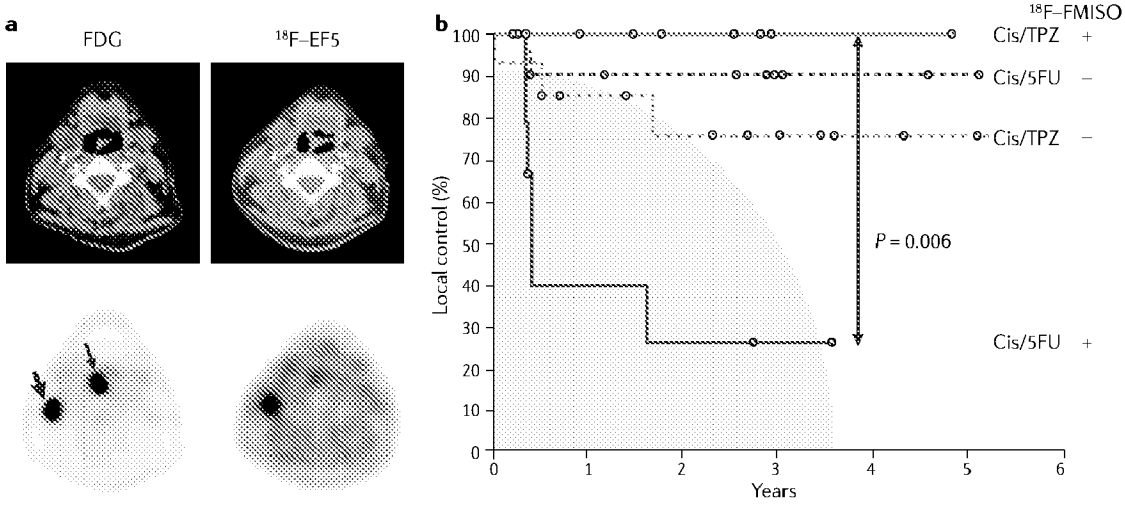
The presence of hypoxia is a necessary but not sufficient condition for hypoxia-targeting, given that there are other crucially important determinants of sensitivity to such agents. For bioreductive prodrugs, the molecular targets are in effect the specific reductases in hypoxic cells for which these compounds are substrates. Although identification of these enzymes is incomplete (BOX 1), their activity clearly varies widely between tumours. The need for reductase profiling to identify tumours potentially responsive to bioreductive prodrugs has long been recognized[154], but only now are the tools becoming available to address this requirement. In addition, there is a further set of molecular targets, for the active drug metabolites, which brings into play many potential mechanisms of drug resistance. Given that most bioreductive prodrugs generate DNA damage that is repaired by HR, the validation of biomarkers for this repair pathway (currently driven by predicting the sensitivity to PARP inhibitors and cytotoxic chemotherapy[155–157]) has strong potential to affect their development.

Clearly, the diagnostic tools for selecting patients for treatment with hypoxia-targeted drugs need to be matched to the specific therapeutic agent. Thus, one would expect the preferred diagnostic for a bioreductive prodrug to be an exogenous probe that is activated through bioreductive metabolism (by similar enzymes and with similar oxygen-dependence to the therapeutic agent). As an example, binding of the 2-nitroimidazole probe EF5 reports activity of the one-electron reductases that activate SN30000, as well as reporting hypoxia, making it a potential dual probe for both of these stratification biomarkers[158]. By contrast, endogenous markers of hypoxia-responsive signalling pathways will be more appropriate for agents that target such pathways. It is noteworthy that there tends to be poor correlation between different hypoxia markers in both

Box 2 | **PET imaging for tumour hypoxia**

The variability in levels of hypoxia among individual tumours, even within a single disease subtype, calls for tools that can be used to quantify tumour hypoxia in a clinical setting. Positron emission tomography (PET) methods are undergoing active development in this context[152]. One strategy depends on radiolabelled antibodies against carbonic anhydrase 9 (CA9)[179,180], which would be of value for the selection of patients for treatment with CA9-targeted therapeutics[133]. To the extent that CA9 can be considered a specific hypoxia-inducible factor 1 (HIF1) reporter[132,181], and that HIF1 activity is regulated by hypoxia[92], this approach also has potential for monitoring hypoxia.

The most widely studied PET strategy depends on entrapment of 2-nitroimidazole probes — such as fluoromisonidazole (FMISO), fluoroazomycinarabinofuranoside (FAZA) and etanidazole pentafluoride (EF5) — in hypoxic cells as a result of their bioreductive metabolism[152]. The mechanism is analogous to that for one-electron (oxygen-inhibited) metabolic activation of bioreductive prodrugs, subsequently generating nitroso and hydroxylamine metabolites (X and Y in FIG. 2A), which react covalently with intracellular thiols. The resulting protein adducts can be detected by immunohistochemistry (FIG. 1a), which requires a tumour biopsy, but ^{18}F-labelled versions of the same compounds have been adapted for non-invasive PET imaging. The PET–computerized tomography (CT) scan shown in part **a** of the figure demonstrates a difference in ^{18}F–EF5 entrapment in two lesions in the same patient that both rapidly metabolize ^{18}F–fluorodeoxyglucose (FDG), suggesting that the lesion marked with the wide arrow is more hypoxic than that marked with the thin arrow. The related 2-nitroimidazole probe ^{18}F–FMISO has been used to evaluate hypoxia in a small subset of patients in clinical trials of the bioreductive prodrug tirapazamine (TPZ) combined with cisplatin (cis) and radiotherapy, versus 5-fluorouracil (5FU) combined with cisplatin and radiotherapy for advanced head and neck squamous cell carcinoma (HNSCC). As shown in part **b** of the figure, a retrospective analysis demonstrated a marked advantage of the TPZ-containing regimen compared to the 5FU-containing regimen in patients with hypoxic tumours (solid lines,^{18}F–FMISO-negative)[183]. This notable result points the way for future trials of hypoxia-targeted agents, but, regrettably, stratification for hypoxia was not used in subsequent unsuccessful Phase III trials of TPZ in this same setting[184]. Part **a** of the figure is reproduced, with permission, from REF. 182 © (2008) Society of Nuclear Medicine, Inc. Part **b** of the figure is modified, with permission, from REF. 183 © (2006) The American Society of Clinical Oncology.



Network medicine
Analysis of biological networks to derive understanding of disease and therapy.

preclinical and clinical studies[150,159]. Ultimately, paired diagnostics and therapeutics will need to be validated in prospective clinical trials, despite the logistical and regulatory challenges that this presents.

Conclusions and perspective

This Review has considered the two main approaches to the selective killing of hypoxic cells in tumours, with different strengths and weaknesses. Bioreductive prodrugs achieve striking selectivity between aerobic and severely hypoxic cells in culture, typically with potency differentials in the order 10–1,000-fold. By contrast, inhibition of molecular targets in hypoxic cells typically gives much more modest cytotoxicity differentials. However, these targeted inhibitors offer a more benign toxicity profile, which is distinctly different from that of cytotoxic therapy, and therefore have greater opportunity for combination with current standards of care. Compatibility with existing therapy is fundamentally important for the clinical translation of these targeted drugs, given that hypoxic cells represent only a minority subpopulation in most tumours (although a critically important one). Therefore, monotherapy activity is not a realistic expectation for hypoxia-selective agents that are strictly on-mechanism unless exceptional requirements can be met, such as a very long residence time in tumours (to exploit fluctuating hypoxia) or efficient, long-range bystander killing. Bioreductive prodrugs that generate molecularly targeted drugs as effectors, rather than DNA-damaging cytotoxins, arguably offer an opportunity to combine the best features of both classes of drug (high hypoxic selectivity and more benign toxicity), but are at an early stage of development.

Although much has already been learned about the molecular responses to hypoxia, the identification of the most useful molecular targets in hypoxic cells is far from complete. While new targets with roles in hypoxic cell survival continue to be identified, the highly interactive nature of the PHD–HIF, mTOR, UPR–autophagy and DNA damage response modules (FIG. 4) makes it difficult to identify the vulnerabilities of hypoxic cells that can best be exploited as drug targets. The results of unbiased whole-genome screens, analogous to the RNA interference screens used to identify synthetic lethal interactions with chemotherapy[160], are eagerly awaited. Ideally, these screens will compare multiple cancer cell lines with normal cells, under hypoxia, to reveal targets that provide selectivity for hypoxia in the context of cancer genomes, and will be interpreted in a network medicine framework[161]. A better definition of the preferred molecular targets will make it feasible to design small molecules of greater specificity, and to move beyond the repurposing of drugs that have been developed for other applications, an approach that currently characterizes this field (TABLE 4). In a similar fashion, improved understanding of the human reductases that activate prodrugs will provide opportunities for structure-based design to improve specificity for enzymes that confer tumour selectivity.

Many of the challenges in targeting hypoxic cells are similar for both bioreductive prodrugs and molecularly targeted inhibitors; both need to be designed to address the stringent micropharmacokinetic requirements for efficient penetration to cells distant from blood vessels. This critical issue is still rarely addressed explicitly. Both classes of drugs also need to address, and where possible exploit, off-target effects (such as the aerobic reduction of bioreductive drugs, and the inhibition of hypoxia-independent HIF1 responses to ionizing radiation[162]). An associated challenge is the potential toxicity resulting from physiological hypoxia in normal tissues; there is still little understanding of the contribution of such hypoxia to the dose-limited toxicities of bioreductive prodrugs. In addition, clinical development of all hypoxia-targeted agents suffers from a lack of information about the clinical settings in which hypoxic cells contribute to treatment failure. The notable exception is in chemoradiation treatment of HNSCC, for which there is overwhelming evidence from multiple hypoxic biomarkers that hypoxia compromises outcome (TABLE 3). An additional challenge is the lack of a drug-development culture in the field of radiation oncology, which is the setting in which the impact of hypoxia is most clearly understood.

Perhaps the most crucial requirement for hypoxia-targeting strategies is the development of improved predictive tools for patient stratification. These tools need to evaluate not only hypoxia, but also many other determinants of sensitivity, as discussed above. Ultimately, tumour and host genomic analyses will revolutionize the matching of hypoxia-targeted therapeutics to individual patients. However, extracting information on physiological features such as the severity of hypoxia from genomic data will be challenging, so functional assays such as PET imaging are likely to play a major part in the foreseeable future. Together, this individualized phenotyping has the potential to identify clinical niches for the diverse types of cytotoxins that are already identified as hypoxia-selective, and provide a rational basis for their clinical development.

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Acknowledgements

We thank our collaborators (R. Anderson, W. Denny, K. Hicks, C. Guise, A. Patterson, F. Pruijn, J. Smaill, M. Tercel and J. Wang) for many fruitful discussions that have helped to inform the views expressed here. The authors acknowledge financial support from the Health Research Council of New Zealand (W.R.W.) and the Maurice Wilkins Centre for Biodiscovery (M.P.H.).

Competing interests statement

The authors declare competing financial interests: see Web version for details.

DATABASES

ClinicalTrials.gov: http://www.clinicaltrials.gov
NCT00566683 | NCT00225652 | NCT01134445

US National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary
apaziquone | AP8 346 | banoxantrone | borrezomib | CB 1954 | carbetamide combination | chloroquine | combretastatin A4 | EZN 2968 | goldanamycin | genexitabine | irinotecan | mitomycin C | pimonidazole | PR 104 | PX-478 | rapamycin | RSU | TH 302 | thymitaq | tirapazamine | veliparib

FURTHER INFORMATION

William R. Wilson's homepage: http://www.fmhs.auckland.ac.nz/sms/acsrc/reig/default.aspx
Michael P. Hay's homepage: http://www.fmhs.auckland.ac.nz/sms/acsrc/medicinal_chemistry/hay/default.aspx

SUPPLEMENTARY INFORMATION

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MOLECULAR AND CELLULAR BIOLOGY, Oct. 2002, p. 7184–7192 Vol. 22, No. 20
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.20.7184–7192.2002

Structural Basis for Activation of Fibroblast Growth Factor Signaling by Sucrose Octasulfate

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Received 9 May 2002/Returned for modification 9 July 2002/Accepted 19 July 2002

Sucrose octasulfate (SOS) is believed to stimulate fibroblast growth factor (FGF) signaling by binding and stabilizing FGFs. In this report, we show that SOS induces FGF-dependent dimerization of FGF receptors (FGFRs). The crystal structure of the dimeric FGF2-FGFR1-SOS complex at 2.6-Å resolution reveals a symmetric assemblage of two 1:1:1 FGF2-FGFR1-SOS ternary complexes. Within each ternary complex SOS binds to FGF and FGFR and thereby increases FGF-FGFR affinity. SOS also interacts with the adjoining FGFR and thereby promotes protein-protein interactions that stabilize dimerization. This structural finding is supported by the inability of selectively desulfated SOS molecules to promote receptor dimerization. Thus, we propose that SOS potentiates FGF signaling by imitating the dual role of heparin in increasing FGF-FGFR affinity and promoting receptor dimerization. Hence, the dimeric FGF-FGFR-SOS structure substantiates the recently proposed "two-end" model, by which heparin induces FGF-FGFR dimerization. Moreover, the FGF-FGFR-SOS structure provides an attractive template for the development of easily synthesized SOS-related heparin agonists and antagonists that may hold therapeutic potential.

Fibroblast growth factors (FGFs; FGF1 to FGF22) regulate a wide array of physiological processes including embryogenesis, cell growth, differentiation, angiogenesis, tissue repair, and wound healing (30). The diverse activities of FGFs are mediated by four receptor tyrosine kinases (FGFR1 to FGFR4), each composed of an extracellular ligand-binding portion consisting of three immunoglobulin-like domains (D1 to D3), a single transmembrane helix, and a cytoplasmic portion with protein tyrosine kinase activity (18).

Receptor dimerization is an obligatory event in FGF signaling and requires heparin or heparan sulfate proteoglycans (28). Two contrasting mechanisms for FGF receptor (FGFR) dimerization have emerged from the recent crystal structures of FGF-FGFR-heparin complexes. In the "two-end" model, deduced from the FGF2-FGFR1-heparin crystal structure, two 1:1:1 FGF-FGFR-heparin ternary complexes form a symmetric dimer (40). Each FGF binds to both receptors, and there is a direct contact between the two FGFRs. Within each ternary complex, heparin interacts extensively with FGF and FGFR, thereby enhancing FGF-FGFR affinity. Heparin also binds to the FGFR across the twofold dimer and thereby fortifies the interactions of FGF and FGFR from one ternary complex with FGFR in the other ternary complex. Thus, heparin fulfils an adapter role in receptor dimerization.

In the model derived from the FGF1-FGFR2-heparin structure (33), a single heparin oligosaccharide bridges two FGF molecules into a dimer that in turn brings two receptor chains together. Heparin makes a different set of contacts with the two ligands and binds to one receptor only, resulting in the distinctive asymmetry of the dimer. Unlike the configuration in the two-end model, each FGF contacts a single FGFR and there is no direct FGFR-FGFR contact. The total lack of protein-protein interface between the two FGF-FGFR monomers in the dimer means that heparin is absolutely necessary for receptor dimerization in this model.

Besides heparin, a number of chemically diverse low-molecular-weight sulfated sugars such as sucrose octasulfate (SOS) have been reported to potentiate FGF action. SOS has been shown to mimic heparin action in supporting FGF-induced neoangiogenesis and cell proliferation in vitro (2, 11, 23, 29, 45, 49). Moreover, SOS facilitates wound healing by enhancing FGF-induced angiogenesis (39). The molecular mechanism by which SOS stimulates FGF signaling is not fully understood. Since SOS binds and protects FGFs against high temperature and low pH (1, 11, 45), it has been suggested that SOS enhances FGF signaling by prolonging the half-life of FGF.

However, because receptor dimerization is mandatory for activation of FGF signaling, we reasoned that the heparin-like activity of SOS must involve FGFR dimerization as well. In this report, we first confirm the heparin-like activity of SOS in an FGF-dependent-differentiation assay. Next, we demonstrate that SOS induces FGF-FGFR dimerization in vitro. Finally, we determine the crystal structure of the dimeric FGF-FGFR-SOS complex. Analysis of this dimeric structure reveals that SOS induces FGF-FGFR dimerization with a mode and stoichiometry reminiscent of the two-end model. Thus, we con-

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clude that SOS stimulates FGF signaling by imitating heparin in increasing FGF-FGFR affinity and promoting dimerization. Our structural finding gives strong credence to the two-end dimerization model.

MATERIALS AND METHODS

Organ culture and in situ hybridization. For embryo staging, the day of evidence of a vaginal plug was considered as 0.5 day postcoitum (dpc). Calvaria were dissected from 16.5-dpc mouse embryos (NMRI strain). The dura mater was left intact in all explants. Explants were cultured for 9 days in a Trowell-type organ culture system on 0.8-μm-pore-size Costar filters supported by metal grids. The serum-free culture medium consisted of BGJb medium (Gibco) containing 0.1% bovine serum albumin, 40 μg of transferrin/ml, 2 mM Glutamax, 150 μg of ascorbic acid/ml, and antibiotics (14). The explants (four or five specimens per group) were cultured in the absence or presence of FGF2 (50 ng/ml), heparin (10 μM; molecular weight, 3,000; Sigma), or SOS (50 or 200 μM), alone or in combination.

After 9 days of culture, the calvaria were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for paraffin embedding. Six-micrometer serial sections were prepared for hybridization with a ^{35}S-UTP-labeled riboprobe as previously described (46). An *Eco*RI-linearized 0.35-kb osteocalcin template was transcribed with T7 RNA polymerase and used as the probe.

Dimerization of FGF-FGFR complexes by SOS in vitro. The ligand binding portion of FGFR1 consisting of immunoglobulin-like domain 2 (D2) and D3 was produced in *Escherichia coli* and refolded in vitro as previously described (37). The refolded FGFR1 ectodomain was then mixed with FGF2, and the resulting 1:1 FGF2-FGFR1 complexes were purified to homogeneity by size exclusion chromatography as previously described (37). The purified 1:1 FGF2-FGFR1 complexes were mixed at various molar ratios with SOS or selectively desulfated SOS analogs, and the mixtures were analyzed on a Superdex 200 size exclusion column in a 25 mM HEPES-NaOH buffer (pH 7.5) containing 150 mM NaCl.

Crystallization and structure determination. Dimeric 2:2:2 FGF2-FGFR1-SOS complexes were generated by mixing purified 1:1 FGF2-FGFR1 complexes with SOS at a molar ratio of 1:1. Crystals were grown by vapor diffusion at 20°C by the hanging-drop method. Two microliters of protein solution (10 mg/ml in 25 mM HEPES-NaOH [pH 7.5], 150 mM NaCl) was mixed with an equal volume of crystallization buffer (12 to 16% polyethylene glycol 5000, 0.2 M ammonium sulfate, and 15% glycerol in 0.1 M HEPES-NaOH [pH 7.5]). The FGF2-FGFR1-SOS crystals belong to orthorhombic space group $P2_12_12_1$ with a solvent content of 56% and the following unit cell dimensions: a = 64.2 Å, b = 122.4 Å, and c = 219.5 Å. Diffraction data were collected from a flash-frozen crystal on a charge-coupled device detector at beamline X4A at the National Synchrotron Light Source, Brookhaven National Laboratory. The data were processed with DENZO and SCALEPACK (32). A molecular replacement solution was found for the four copies of the FGF2-FGFR1 complex in the asymmetric unit with the program AmoRe (26) and the FGF2-FGFR1 structure (PDB identification code, 1CVS) (37) as the search model. The initial model for SOS was taken from the FGF1-SOS crystal structure (PDB identification code, 1AFC) (50). The parameters for the SOS molecule were generated by using the HIC-Up server (22). Simulated annealing, positional, and temperature factor refinements were performed with the crystallography and NMR system (6). Bulk solvent and anisotropic B-factor corrections were applied. Tight noncrystallographic symmetry restraints were imposed throughout the refinement for the backbone atoms of FGF2, D2, and D3. Model building into the $2F_o$-F_c and F_o-F_c electron density maps was performed with program O (19). The refined model consists of 4 FGF2 molecules (residues 16 to 144), 4 FGFR1 molecules (residues 149 to 359), 4 SOS molecules, 3 sulfate ions, and 42 water molecules.

Chemical synthesis of desulfated SOS analogs. (i) Synthesis of 1′,3′,4′,6′-tetra-*O*-sulfo-β-D-fructofuranosyl-3,4,6-tri-*O*-sulfo-α-D-glucopyranoside, heptasodium salt (2-hydroxysucrose hepatasulfate). 2-*O*-Lauryl-β-D-fructofuranosyl α-D-glucopyranoside (320 mg, 0.6 mmol) (8) and the trimethylamine · SO_3 complex (880 mg, 6.3 mmol) were stirred under N_2 in 20 ml of dry dimethylformamide at 50°C for 12 h. The trimethylamine · SO_3 complex (880 mg) was again added, and the suspension was kept at 50°C for an additional 12 h. CH_3OH-H_2O (1:1 [vol/vol]) was added, and the suspension was layered on a column of Sephadex LH-20 and eluted with the same solvent system. The combined product was passed through Dowex 50 (Na^+). After evaporation, the colorless glass was dissolved in water and lyophilized to give 1′,3′,4′,6′-tetra-*O*-sulfo-β-D-fructofuranosyl-2-*O*-lauryl-3,4,6-tri-*O*-sulfo-α-D-glucopyranoside, heptasodium salt as a white powder in 96% yield. The product was characterized by ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR. 1′,3′,4′,6′-tetra-*O*-sulfo-β-D-fructofuranosyl-2-*O*-lauryl-3,4,6-tri-*O*-sulfo-α-D-glucopyranoside, heptasodium salt (120 mg) in 2.5 ml of 0.5 N NaOH was stirred overnight at 4°C. The reaction was quenched with 3 ml of 0.5 N HCl and extracted with $CHCl_3$ (three times with 5 ml). The aqueous layer was passed through Dowex 50 (Na^+), evaporated under high vacuum, redissolved in water, and lyophilized to give a white powder (2-hydroxysucrose hepatasulfate; 96% yield). The product was characterized by ^{1}H NMR and ^{13}C NMR.

(ii) Synthesis of 1′,3′,4′,6′-tetra-*O*-sulfo-β-D-fructofuranosyl-2,3-di-*O*-sulfo-α-D-glucopyranoside, hexasodium salt (4,6-dihydroxysucrose hexasulfate). Sulfonation of 4,6-*O*-isopropylidine (20) (as described above) afforded 1′,3′,4′,6′-tetra-*O*-sulfo-β-D-fructofuranosyl-4,6-*O*-isopropylidine-2,3-di-*O*-sulfo-α-D-glucopyranoside, hexasodium salt, which was characterized by ^{1}H NMR and ^{13}C NMR. 1′,3′,4′,6′-tetra-*O*-sulfo-β-D-fructofuranosyl-4,6-*O*-isopropylidine-2,3-di-*O*-sulfo-α-D-glucopyranoside (20 mg, 0.02 mmol) and 60% acetic acid (2 ml) were stirred under N_2 at 80°C for 20 min. The product was passed through Dowex 50 (Na^+). After evaporation, the colorless glass was dissolved in water and lyophilized to give 4,6-dihydroxysucrose hexasulfate as a white powder in 95% yield. The product was characterized by ^{1}H NMR and ^{13}C NMR.

Coordinates. The atomic coordinates of the FGF2-FGFR1-SOS structure will be deposited in the Protein Data Bank for immediate release upon publication.

RESULTS

SOS can replace heparin in promoting FGF-dependent cellular responses. A large body of literature has documented that SOS can mimic heparin in potentiating FGF-induced cell proliferation and neoangiogenesis (2, 11, 23, 29, 45, 49).

To extend these studies, we decided to assess the heparin-like activity of SOS in yet another FGF-dependent system. Recent advances in human genetics have identified FGF signaling as an essential regulator of skeletal development (24). Activation of FGFR has been shown to promote the fusion of calvarial sutures by stimulating the differentiation of sutural mesenchyme into osteoblasts (21). Thus, we evaluated the ability of SOS to induce FGF-dependent fusion of calvarial sutures. Cultures of developing calvarial bones were treated with FGF2 and/or SOS and then analyzed by in situ hybridization for osteocalcin mRNA, a molecular marker for differentiated osteoblasts (5). In untreated cultures, the overlapping parietal and frontal bones in the coronal suture (Fig. 1a) and the osteogenic fronts of the parietal bones in the sagittal suture (Fig. 1b) were widely separated by undifferentiated osteocalcin-negative mesenchyme. Osteoblasts in the bone plates expressed basal levels of osteocalcin mRNA. Treatment with SOS (50 μM) or heparin (10 μM) alone resulted in no morphological changes relative to the untreated cultures (Fig. 1c, d, i, and j). However, in the presence of high concentrations of SOS (200 μM), the frontal and parietal bones at the coronal and sagittal sutures exhibited an increase in osteocalcin expression and the bone plates were separated by less undifferentiated sutural mesenchyme (Fig. 1e and f). Prolonged exposure to FGF2 alone produced morphological changes similar to those with 200 μM SOS alone (Fig. 1g and h). We attribute these modest effects to the presence of endogenous FGF2 and heparan sulfate proteoglycans in the organ cultures. Combination of FGF2 with heparin (10 μM) or SOS (50 μM) augmented the effects of FGF2 on sutural differentiation and osteocalcin expression (Fig. 1k to n). In both cases, nearly all of the mesenchyme between the osteogenic fronts in the sagittal sutures had differentiated into osteoblasts (Fig. 1l and n). Treatment with FGF2 and 200 μM SOS led to a dramatic increase in osteocalcin expression and complete closure of the coronal and sagittal sutures (Fig. 1o and p). Thus, these organ

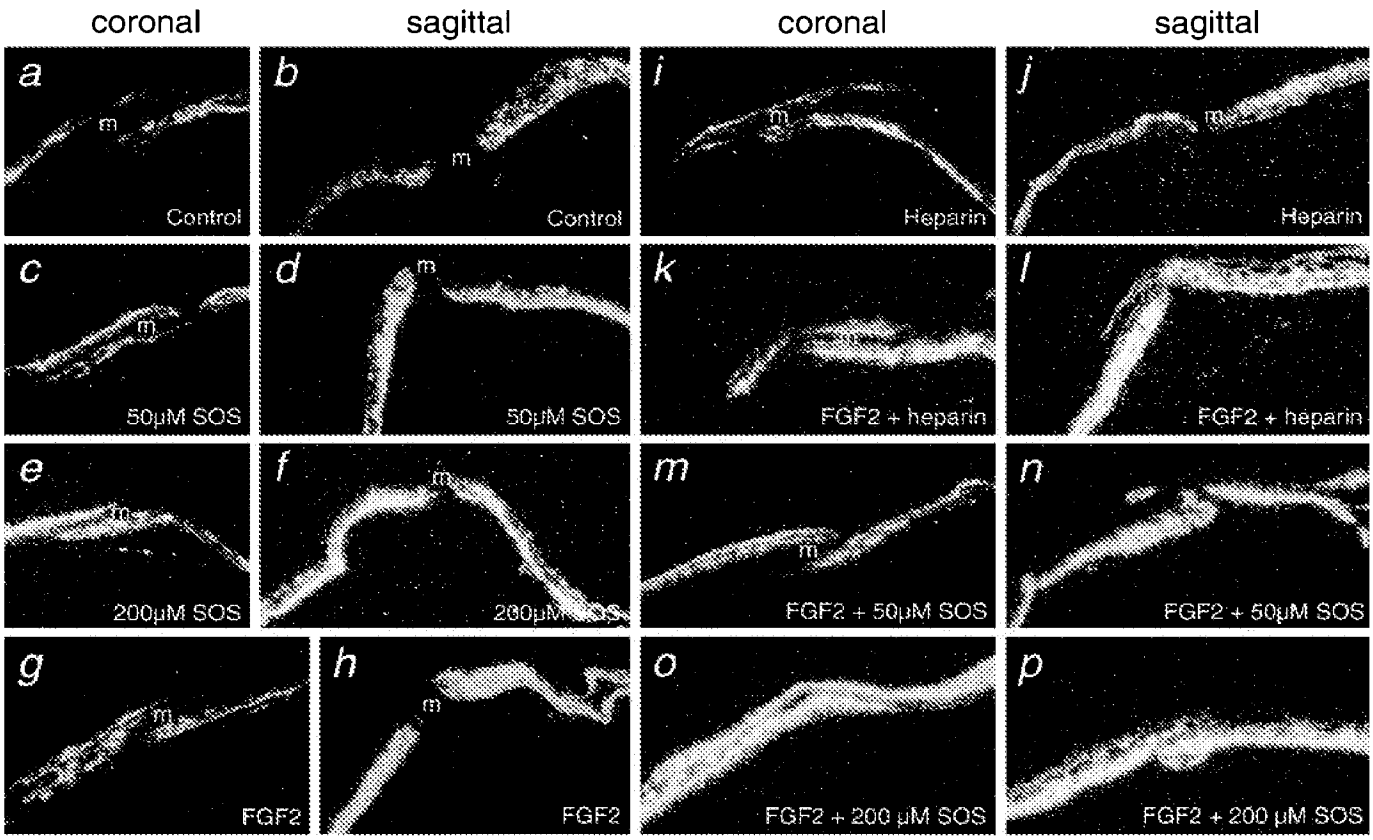

FIG. 1. Morphological changes and osteocalcin expression in 16.5-dpc mouse calvaria after culture in vitro in the presence or absence of FGF2, heparin, and SOS, alone or in combination. Frontal sections were taken at the coronal (a, c, e, g, i, k, m, and o) and sagittal (b, d, f, h, j, l, n, and p) sutures. Note the dramatic increase in osteocalcin expression in the presence of FGF2 and 200 μM SOS (o and p). m, mesenchyme.

culture data reaffirm the ability of SOS to imitate heparin action in stimulating FGF signaling.

SOS can dimerize the FGF-FGFR complex. Because activation of FGF signaling is strictly dependent on receptor dimerization, it was pertinent to check whether SOS is capable of dimerizing the FGF-FGFR complex. A 1:1 binary FGF2-FGFR1 complex was purified and mixed with SOS at various molar ratios. The resulting mixtures were analyzed by size exclusion chromatography. SOS induced dimerization of the FGF2-FGFR1 complex in a concentration-dependent manner (Fig. 2a to c). At a complex-SOS molar ratio of 1:1, we observed quantitative dimerization of the FGF2-FGFR1 complex (Fig. 2c).

To elucidate the structural mechanism by which SOS dimerizes FGF2-FGFR1 complexes, we crystallized the purified 2:2:2 FGF2-FGFR1-SOS dimer. Orthorhombic crystals containing two FGF2-FGFR1-SOS dimers in the asymmetric unit were obtained. Data collection and refinement statistics are given in Table 1.

SOS dimerizes the FGF-FGFR complex in a manner reminiscent of the two-end model. Each dimer exhibits a twofold symmetric assembly of two 1:1:1 FGF2-FGFR1-SOS ternary complexes reminiscent of the dimeric FGF2-FGFR1-heparin structure (Fig. 3a and c) (40). Within the dimer, each FGF binds to both FGFRs and the two FGFRs contact each other through their D2 portions. The C-terminal ends of the two receptors are predicted to insert into the plasma membrane at the membrane-proximal side of the dimer and are about 50 Å apart. This distance is similar the distance between the membrane insertion points of the ligand-induced erythropoietin receptor dimers (47). A deep canyon, the hallmark of the two-end model, is formed on the membrane-distal side of the dimer between the adjoining FGFR D2s and wanes as it reaches the ligands.

Two SOS molecules are observed to bind in this heparin-binding canyon (Fig. 3a). The F_o-F_c electron density is strong and well defined for one of the two SOS molecules (Fig. 3b) and weaker for the other molecule. Nevertheless, the observed electron densities for both molecules are sufficiently strong to reveal that the two SOS molecules bind in a symmetric head-to-head fashion as the two heparin molecules do in the FGF2-FGFR1-heparin structure (40). This difference in electron density suggests that SOS binds tighter to one site than the other in the dimer. As with the dimeric FGF2-FGFR1 structure, the FGF2-FGFR1-SOS dimer exhibits a slight asymmetry in the orientation of the D2s (37). This asymmetry results in SOS binding tighter to one half of the heparin-binding canyon than to the other half.

Compared to heparin, SOS occupies mainly the deep portion of the heparin-binding canyon consisting of D2s of both FGFR1s and the FGF2 heparin-binding site adjacent to the receptors (Fig. 3a and c). This is in contrast to the crystal

TABLE 1. X-ray data collection and refinement statistics

Parameter	Value
Data Collection	
Resolution (Å)	30.0–2.6
Observations	764,014
Unique reflections	53,698
Completeness[a] (%)	99.9 (100.0)
R_{sym}[a,b]	7.8 (33.2)
Refinement	
No. of atoms	
Protein	10,823
SOS	220
Water	42
SO_4^{2-}	15
Resolution (Å)	25.0–2.6
Reflections	52,014
R_{cryst}/R_{free}[c] (%)	24.1/27.8
Root mean square deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.4
B factors[d] (Å^2)	1.00
Average B factors (Å^2)	
All atoms	40.91
Protein	39.97
SOS	76.06

[a] The overall (30.0- to 2.6-Å) value is given first, with the value for the highest-resolution shell (2.69 to 2.6 Å) given in parentheses.
[b] $R_{sym} = 100 \times \Sigma|I - \langle I \rangle|/\Sigma I$.
[c] $R_{cryst} = 100 \times \Sigma||F_O| - |F_c||/\Sigma|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively ($F_o > 0_\sigma$). R_{free} was determined from 5% of the data.
[d] For bonded protein atoms.

structure of SOS bound to FGF only (50), where SOS binds to the FGF high-affinity heparin-binding site, which topologically corresponds to the distal shallow portion of the heparin-binding canyon. Moreover, opposite orientations of SOS, with respect to FGF, between the binary FGF-SOS and ternary FGF-FGFR-SOS structures are observed. Despite this disparity, the overall conformations of SOS for these two structures are similar.

Within each FGF2-FGFR1-SOS ternary complex, SOS makes four hydrogen bonds with FGFR1 and five hydrogen bonds with FGF2 (Fig. 4). As for heparin, concurrent binding of SOS to FGF and FGFR clearly promotes FGF-FGFR affinity. Interactions of SOS with FGFR1 involve Lys-163 and Lys-177, which protrude from the heparin-binding surface of D2, and the sulfate groups of both the five- and six-member rings of SOS (Fig. 4). These very same lysines bind heparin in the FGF2-FGFR1-heparin structure (40). At the SOS-FGF2 interface, a total of five hydrogen bonds between Lys-26 and Lys-135 of FGF2 and the sulfate groups of SOS are made (Fig. 4). Likewise, in the FGF2-FGFR1-heparin structure, these very same lysines also bind heparin (40). Compared to heparin, SOS makes five and nine fewer hydrogen bonds with FGFR1 and FGF2, respectively. Thus, the structure indicates that SOS enhances FGF2-FGFR1 affinity, albeit with lower efficacy than heparin.

Like heparin in the dimeric FGF2-FGFR1-heparin structure, SOS also interacts with D2 of the adjoining FGFR1 across the twofold axis. Five hydrogen bonds between SOS and D2 are made at this interface, just one less than at the corresponding interface in the FGF2-FGFR1-heparin structure.

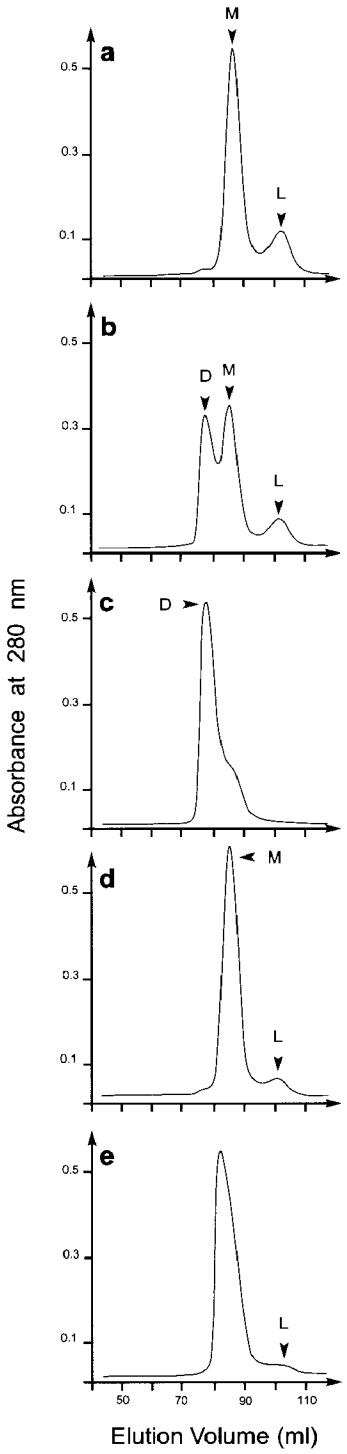

FIG. 2. SOS can dimerize an FGF2-FGFR1 complex. Aliquots of a purified 1:1 FGF2-FGFR1 complex (2 mg) were mixed at various molar ratios with SOS or selectively desulfated SOS analogs, and the reaction mixtures were analyzed by size exclusion chromatography. (a) Control (no SOS added); (b), 1:0.5 complex-SOS; (c) 1:1 complex-SOS; (d) 1:1 complex–4,6-dihydroxysucrose hexasulfate; (e) 1:1 complex–2-hydroxysucrose heptasulfate. M and D, elution positions of monomers and dimers, respectively; L, position of free FGF2 ligand, which results from the dissociation of FGF2-FGFR1 complexes due to protein dilution during size exclusion chromatography. Note that the addition of SOS reduces the free FGF2 peak as SOS increases FGF2-FGFR1 affinity.

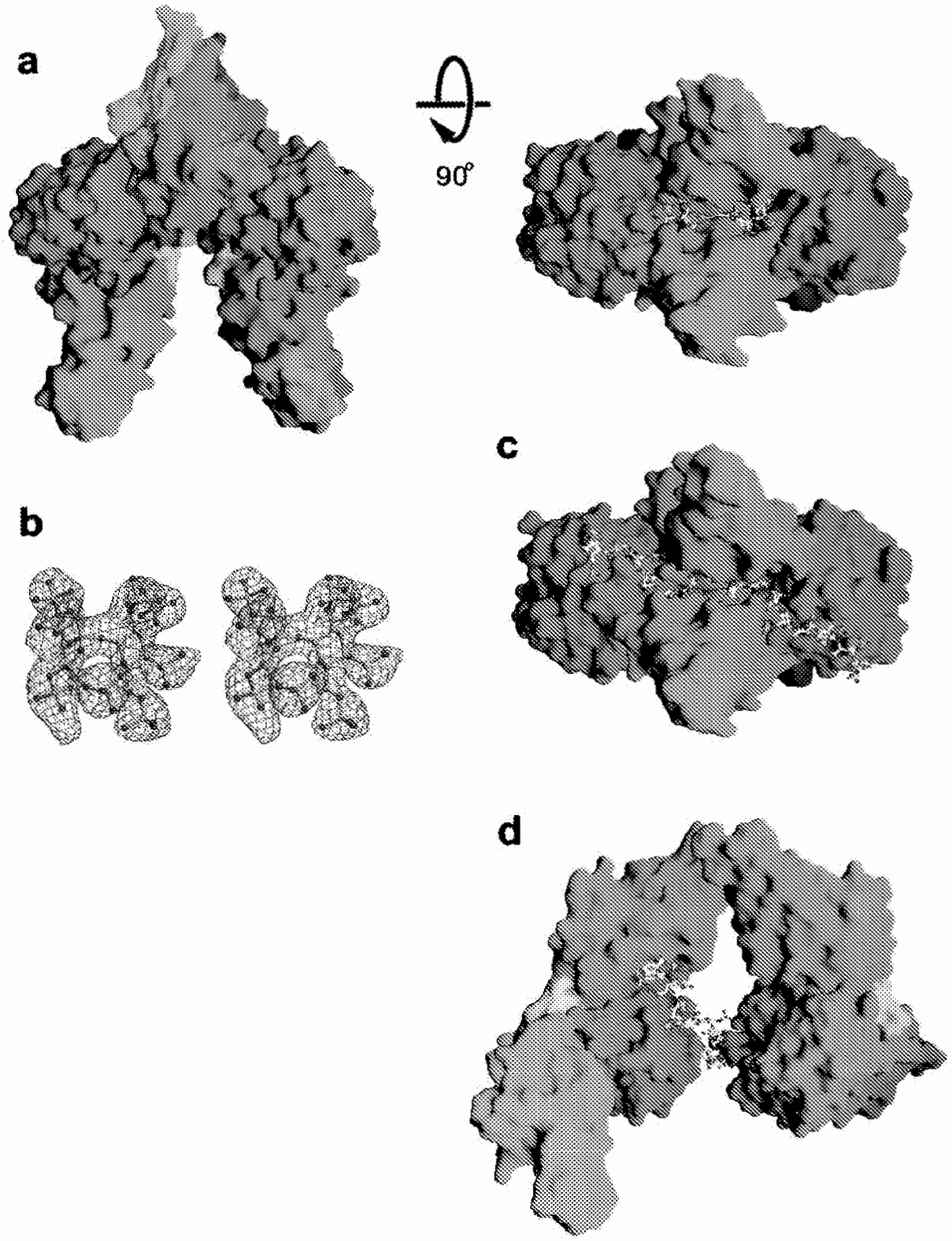

FIG. 3. Crystal structure of the FGF2-FGFR1-SOS complex. (a) Molecular surface representation of the 2:2:2 FGF2-FGFR1-SOS dimer in the asymmetric unit. Color coding is as follows: D2, green; D3, cyan; D2-D3 linker, gray; FGF2, orange. The D2 closer to the viewer is rendered partially transparent in the left view. SOS molecules are rendered in ball and stick. Atom coloring is as follows: red, oxygen; yellow, sulfur; blue, nitrogen; white-gray, carbon. The figure was made with GRASP (27). (b) Stereo view of the F_o-F_c electron density map computed after simulated annealing with SOS omitted from the atomic model. The map is computed at 2.6-Å resolution and contoured at 2.6 σ. Atom coloring is as above. This figure was made with Bobscript (10). (c) Molecular surface representation of the 2:2:2 FGF2-FGFR1-heparin dimer (the symmetric two-end model) (40). (d) Molecular surface representation of the 2:2:1 FGF1-FGFR2-heparin dimer (the asymmetric alternative model) (33).

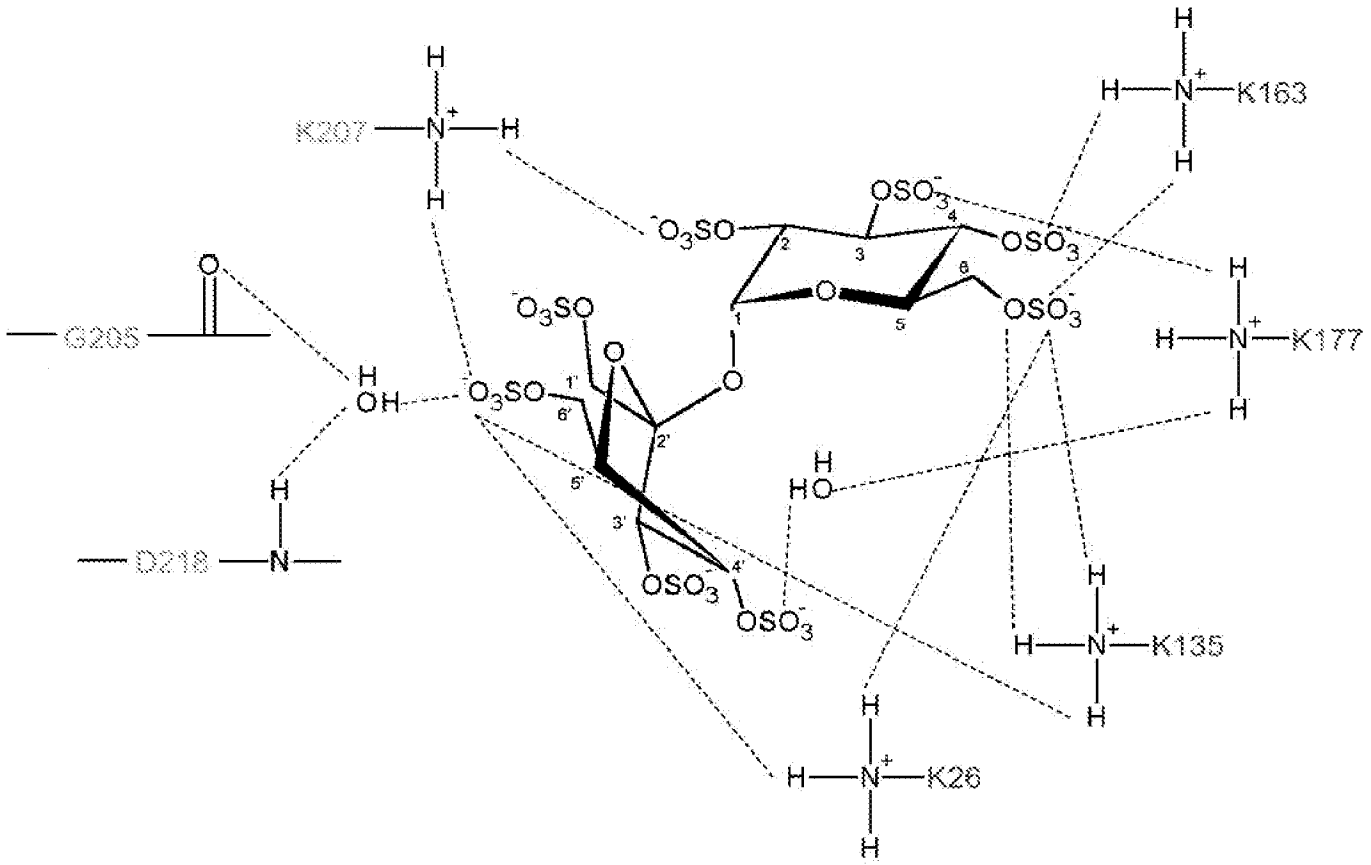

FIG. 4. Schematic diagram of the interactions between SOS, FGF2, and FGFR1. Dashed lines, hydrogen bonds. The backbone atoms of SOS are numbered according to International Union of Pure and Applied Chemistry nomenclature. The type and number of interacting residues are colored based on the molecule to which they belong. Coloring is as follows: FGF2, red, D2 of the primary FGFR1, blue; D2 of the adjoining FGFR1, green.

Lys-207 of FGFR1 D2 makes two hydrogen bonds with 2-sulfate (in the six-member ring) and 6′-sulfate (in the five-member ring) of SOS (Fig. 4). In addition, two water-mediated hydrogen bonds between the backbone atoms of FGFR1 D2 and the 6′-sulfate of SOS from the adjoining complex are made (Fig. 4). In the FGF2-FGFR1-heparin structure, Lys-207 is also implicated in heparin binding (40). These hydrogen bonds sustain the contacts between FGF and FGFR from one ternary complex with FGFR D2 in the adjoining ternary complex. Thus, the structure demonstrates that SOS also mimics heparin in promoting receptor dimerization.

To provide biochemical support for the observed mode of FGF-FGFR dimerization by SOS, we synthesized two SOS analogs lacking selected sulfate groups and tested these analogs for the ability to induce dimerization of FGF2-FGFR1 complexes in vitro (Fig. 2d and e). One analog, 4,6-dihydroxysucrose hexasulfate, was totally incapable of dimerizing the FGF2-FGFR1 complex (Fig. 2d). Since the 4 and 6 sulfates of SOS are involved in augmenting FGF2-FGFR1 affinity (Fig. 4), these data indicate that stabilization of the FGF-FGFR complex is a requisite for receptor dimerization. In the presence of the other analog, 2-hydroxysucrose heptasulfate, no peak corresponding to the FGF2-FGFR1-SOS dimer was observed as well (Fig. 2e). However, in this case the, FGF2-FGFR1 complex eluted slightly earlier than the control (Fig. 2a), indicating that the average Stokes radius of the FGF2-FGFR1 complex is slightly larger in the presence of the analog. This suggests that 2-hydroxysucrose heptasulfate may weakly dimerize the FGF2-FGFR1 complex. Indeed, according to the structure, the presence of the 6′-sulfate in this analog would allow this analog to minimally interact with adjoining FGFR and cause marginal receptor dimerization (Fig. 4). Taken together, the reduced abilities of the two SOS analogs to induce receptor dimerization confirm the structural mechanism by which SOS dimerizes the FGF-FGFR complex.

DISCUSSION

Prior studies have shown that SOS can promote FGF signaling (2, 11, 29, 39, 45). In this report, we extend these studies by demonstrating that SOS, like heparin, evokes FGF-dependent differentiation in organ cultures. To provide a molecular basis for the heparin-like activity of SOS, we first demonstrated that SOS can dimerize FGF-FGFR complexes in vitro. We then determined the crystal structure of the dimeric FGF2-FGFR1-SOS complex. This structure reveals the symmetric association of two 1:1:1 FGF2-FGFR1-SOS ternary complexes reminiscent of the FGF2-FGFR1-heparin structure. Analysis

of the dimer unequivocally illustrates that SOS, merely a sulfated disaccharide, functionally imitates heparin by enhancing FGF-FGFR affinity and dimerization. Thus, we conclude that SOS promotes FGF signaling by promoting FGF-dependent FGFR activation.

Beyond providing a molecular basis for the heparin-like activity of SOS, the dimeric FGF2-FGFR1-SOS structure helps resolve the present uncertainty concerning the exact mode of FGFR dimerization. As introduced above, two competing models for FGFR dimerization by heparin and FGF have been proposed (Fig. 3c and d). Despite fundamental differences in their proposed mechanisms of receptor dimerization, both models have a common feature. In both models, one can readily identify a common ternary complex consisting of one FGF, one heparin, and D2 of one FGFR chain. Within this ternary complex, heparin interacts with FGFR through the terminal disaccharide unit at its nonreducing end and with FGF via the segment preceding the terminal disaccharide unit. Thus, both models convincingly corroborate, at the molecular level, the experimentally documented role of heparin in stabilizing the binary FGF-FGFR complex (31, 48).

The two models diverge when the predicted minimal heparin chain length capable of inducing FGF-FGFR dimerization is considered. In the two-end model a hexasaccharide is sufficient for promoting FGF-FGFR affinity and dimerization (40). Moreover, this model predicts that sugars as small as a disaccharide can have activity, as a disaccharide is still predicted to provide the minimal number of contacts with FGF and FGFR that are absolutely essential for increased ligand-receptor affinity and dimerization. In contrast, the total lack of protein-protein contacts between the two FGF-FGFR protomers in the Pellegrini model demands a heparin span of at least eight sugars for minimal receptor activation. This octasaccharide is predicted to cross-link the two ligands and to marginally engage one of the FGFR D2s (Fig. 3d) (33).

The dimeric FGF2-FGFR1-SOS structure is entirely consistent with and further reinforces the two-end dimerization model. Accumulating structural data demonstrate that FGF in the absence of heparin can form a low-affinity 1:1 complex with FGFR (17, 36, 37, 42). This complex has the potential to dimerize with another 1:1 binary complex through the concerted binding of FGF and FGFR from one complex to FGFR in another complex, albeit at high protein concentrations (37). However, at physiological concentrations these binary complexes tend to break up and thus fail to dimerize. According to the two-end model, the binding of heparin or SOS to binary FGF-FGFR complexes generates tight ternary complexes (FGF-FGFR-heparin or FGF-FGFR-SOS), which are less likely to dissociate than the binary FGF-FGFR complexes. These stabilized ternary complexes now have sufficient opportunity to dimerize through the concerted binding of FGF and FGFR from one ternary complex to FGFR from another ternary complex. Thus, a major role of heparin or SOS in FGF-FGFR dimerization is to generate stable FGF-FGFR complexes, which then provide sufficient interface for the binding of a second FGFR molecule. The inability of 4,6-dihydroxysucrose hexasulfate to dimerize the FGF2-FGFR1 complex is consistent with this hypothesis (Fig. 2d). In addition to enhancing FGF-FGFR affinity within the ternary complex, heparin and SOS interact with the heparin-binding sites in FGFR D2 of the adjoining ternary complex. These interactions further promote dimerization by fortifying the interactions of FGF and FGFR in one ternary complex with FGFR from the adjoining ternary complex.

It is difficult to reconcile the heparin-like activity of SOS with the Pellegrini model, where a heparin-linked FGF dimer is the sole driving force for receptor dimerization (33). SOS does not seem to dimerize FGFs in solution (2, 9, 41). Moreover, the FGF1-SOS crystal structure shows that only a single SOS molecule binds to the high-affinity heparin-binding site of FGF1 (50). Even if one were to assume that SOS dimerizes FGFs, it is not clear how a SOS sandwiched between two FGFs would be able to simultaneously engage the receptor as well.

In addition to SOS, other small polysulfonated molecules, including *myo*-inositol hexasulfate (MIHS) and sulfated β-cyclodextrin, have been reported to potentiate FGF actions (12, 25, 35). Like SOS, MIHS and β-cyclodextrin also bind to and stabilize FGFs (7, 35). Notably, MIHS and β-cyclodextrin also do not induce dimerization of FGFs (15, 25). Thus, it is unlikely that these molecules promote FGF signaling by dimerizing FGFs. We suggest that these molecules, like SOS and heparin, directly promote FGF-induced FGFR dimerization and activation.

Since a small sugar such as SOS can mimic heparin action, the criteria for the sulfation and length of heparin sufficient for FGFR dimerization need to be reevaluated. The smallest heparin molecule suggested to promote FGFR dimerization is a hexasaccharide (40). Although SOS is only two sugars long, its high sulfate content enables it to interact with the heparin-binding sites on FGF and FGFR in a manner sufficient for FGFR dimerization. Thus, high levels of sulfation can reduce the length requirement for supporting receptor dimerization and activation. This hypothesis is perhaps best supported by the ability of sulfated monosaccharide MIHS to induce FGF-dependent FGFR dimerization and activation (25, 35).

The structural data presented in this paper also afford a potential molecular mechanism for the ulcer-healing activity of sucralfate, the aluminum salt of SOS. Sucralfate is used to treat gastric and duodenal ulcers (43). Folkman et al. have shown that, unlike that of the conventional antiulcer drugs, the ulcer-healing activity of sucralfate does not involve adjustment of the stomach pH or antimicrobial activity (11). Instead, they showed that SOS is the active component of sucralfate and postulated that SOS heals ulcers by binding to and prolonging the half-lives of FGFs in the acidic milieu of the stomach, thereby promoting FGF-induced neoangiogenesis. In addition, our structural data imply that SOS exerts its ulcer-healing activity by promoting FGF-dependent FGFR dimerization and activation, which can occur in the vascular endothelium.

FGFs and FGFRs are also implicated in a variety of human skeletal disorders, including dwarfism and the craniosynostosis syndromes (24). In adult organisms, FGFs are thought to be involved in physiological angiogenesis and wound healing as well as in pathological angiogenesis, such as in tumor neovascularization and diabetic retinopathy (3, 13, 16). Consequently, the FGF2-FGFR1-SOS structure provides a template for the development of new therapeutic agents that modulate FGF signaling, particularly in light of limitations in the synthesis of homogeneously sulfated heparin oligosaccharides (34). Be-

cause the synthesis of homogeneously sulfated sucrose derivatives is straightforward, SOS derivatives are attractive candidates for novel therapeutics (4, 38, 44).

ACKNOWLEDGMENTS

We thank Craig Ogata for synchrotron beamline assistance. Beamline X4A at the National Synchrotron Light Source, a Department of Energy facility, is supported by the Howard Hughes Medical Institute. We are grateful to Irma Thesleff for advice and inspiration in calvaria culture, to Tilmann Wurtz for the osteocalcin probe, and to Daniel Bar-Shalom and BM Research (Denmark) for providing SOS. We thank Omar Ibrahimi for critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (DE-13686 to M.M.; HL-62244 and HL-52622 to R.J.L.), the Swedish Medical Research Council (2789 and 14100 to A.G.L.), and the Jubileum Kliniken (to A.G.L.).

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Electronic Acknowledgement Receipt

EFS ID:	38228085
Application Number:	15809815
International Application Number:	
Confirmation Number:	5137
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Customer Number:	153749
Filer:	Mary Rucker Henninger/Richard King
Filer Authorized By:	Mary Rucker Henninger
Attorney Docket Number:	263266-421428
Receipt Date:	07-JAN-2020
Filing Date:	10-NOV-2017
Time Stamp:	18:52:19
Application Type:	Utility under 35 USC 111(a)

Payment information:

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File Listing:

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(Not for submission under 37 CFR 1.99)

Application Number	15809815
Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

	1	ADIWIJAYA B, et al., "Population Pharmacokinetics of Liposomal Irinotecan in Patients With Cancer," Clin Pharmacol Ther. 102(6):997-1005 (2017).
	2	CHEN L, et al., "Effect of Baseline Carbohydrate Antigen 19-9 (CA19-9) Level on Overall Survival (OS) in NAPOLI-1 Trial: a Phase 3 Study of MM-398 (nal-IRI), with or without 5-Fluorouracil and Leucovorin (5-FU/LV), versus 5-FU/LV in Metastatic Pancreatic Cancer (mPAC) Previously Treated with Gemcitabine-based Therapy." Poster handout at the Gastrointestinal Cancers Symposium of the ASCO meeting of January 21-23, 2016, San Francisco, California, 2 pages.

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	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

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Population Pharmacokinetics of Liposomal Irinotecan in Patients With Cancer

BS Adiwijaya[1], J Kim[1], I Lang[2], T Csőszi[3], A Cubillo[4], J-S Chen[5], M Wong[6], JO Park[7], JS Kim[8], KM Rau[9], B Melichar[10], JB Gallego[11], J Fitzgerald[1], B Belanger[1], I Molnar[1] and WW Ma[12]

Nanoliposomal irinotecan (nal-IRI) is a liposomal formulation of irinotecan with a longer half-life ($t_{1/2}$), higher plasma total irinotecan (tIRI), and lower SN-38 maximum concentration (C_{max}) compared with nonliposomal irinotecan. Population pharmacokinetic (PK) analysis of nal-IRI was performed for tIRI and total SN-38 (tSN38) using patient samples from six studies. PK-safety association was evaluated for neutropenia and diarrhea in 353 patients. PK-efficacy association was evaluated from a phase III study in pancreatic cancer NAPOLI1. Efficacy was associated with longer duration of unencapsulated SN-38 (uSN38) above a threshold and higher C_{avg} of tIRI, tSN38, and uSN38. Neutropenia was associated with uSN38 C_{max} and diarrhea with tIRI C_{max}. Baseline predictive factors were race, body surface area, and bilirubin. Analysis identified PK factors associated with efficacy, safety, and predictive baseline factors. The results support the benefit of nal-IRI dose of 70 mg/m^2 (free-base; equivalent to 80 mg/m^2 salt base) Q2W over 100 mg/m^2 Q3W.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Liposomal encapsulation extends the half-lives of irinotecan; however, the association of PK and the impact of liposomal encapsulation to efficacy or safety endpoints have never been reported from a large number of patients.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This study aimed to quantify plasma PK with liposomal irinotecan treatment to discern the differences between derived PK parameters C_{avg}, C_{max}, and $t_{uSN38>thr}$ and their impact on safety and efficacy, and to identify relevant baseline factors.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ These analyses identified that efficacy was associated with the average concentration of SN-38 and the duration of SN-38 above a threshold, while safety was associated with maximum concentrations.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

☑ This study provides an example of PK modification by liposomal encapsulation resulted in ability to differentiate aspects of PK associated to efficacy and safety. The results support the choice of the optimal dose regimen.

Liposomal formulations have been investigated as a drug delivery system to modulate the pharmacological properties of small molecules.[1] In cancer therapeutics, liposomal formulations can deposit in tumors through leaky vasculature by the enhanced permeability and retention effect (EPR), creating a local depot for drug release.[2] Nanoliposomal irinotecan (nal-IRI, MM-398, PEP02, BAX2398) is a liposomal formulation of irinotecan for intravenous injection designed to combine the properties of long plasma circulation and increased delivery of irinotecan to tumor lesions via the EPR effect. The clinical benefit of nal-IRI was demonstrated in a phase III study in patients with metastatic pancreatic cancer previously treated with a gemcitabine-based therapy (NAPOLI-1).[3] The results showed that nal-IRI in combination with 5-fluorouracil (5-FU) and leucovorin (LV) significantly increased median overall survival (OS) compared with a 5-FU/LV control arm (6.1 and 4.2 months, respectively), with an unstratified hazard ratio (HR) of 0.67 (P = 0.012). Additionally, the combination achieved a median progression-free survival (PFS) that approximately doubled that of the control arm (3.1 and 1.5 months, respectively; HR of 0.56; P = 0.0001). As neutropenia and diarrhea are side effects that are associated with irinotecan, further investigation with nal-IRI is warranted.[4–6]

The clinical pharmacokinetics (PK) of nal-IRI were previously compared with those of nonliposomal irinotecan (irinotecan HCl) in a phase II study in patients with gastric cancer.[7] Reanalysis of the data showed that compared with irinotecan HCl 300 mg/m^2 every 3 weeks (Q3W) (n = 27), nal-IRI 100 mg/m^2 Q3W (n = 37; free-base, equivalent to 120 mg/m^2 irinotecan hydrochloride trihydrate salt) had a total irinotecan (tIRI) maximum concentration (C_{max}) that was 13.4-times higher, a

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Received 25 July 2016; accepted 19 April 2017; advance online publication 26 April 2017. doi:10.1002/cpt.720

Table 1 Patient characteristics at baseline

Characteristics	Subgroup	*N* (%)[a]	Median (5th and 95th percentile)
Sex	Female	157 (44)	
	Male	196 (56)	
Race	Caucasian	182 (52)	
	Others	21 (6)	
	East Asian	150 (42)	
Liver metastasis (for NAPOLI-1 only)	No	87 (34)	
	Yes	171 (66)	
Study name	NAPOLI-1	258 (73)	
	Others	95 (27)	
UGT1A1*28 (for NAPOLI-1 only)	Non 7/7	244 (95)	
	7/7	14 (5)	
Treatment (for NAPOLI-1 only)	nal-IRI+5FU/LV	116 (45)	
	nal-IRI (mono)	142 (55)	
Tumor type at diagnosis	Colorectal cancer	18 (5)	
	Gastric & GEJ cancer	37 (10)	
	Metastatic pancreatic cancer	258 (73)	
	Solid tumor	40 (11)	
Initial dose, mg/m^2[b]	50 (60)	4 (1)	
	70 (80)	141 (40)	
	80 (90)	6 (2)	
	90 (100)	11 (3)	
	100 (120)	187 (53)	
	150 (180)	4 (1)	
Age, y		353	63 (39.8, 79.2)
Albumin, g/L		349	40 (29, 47)
ALT, U/L		352	25 (8.9, 96.3)
AST, U/L		352	29 (14.7, 81.9)
Bilirubin (umol/L)		352	7 (3, 19)
BSA, m^2		353	1.7 (1.3, 2.2)
CrCl, 10^{-3} L/s		352	1.36 (0.66, 2.53)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, body surface area; GEJ, gastroesophageal junction.
[a]Percent only included in baseline characteristics with subcategories. [b]Dose is given based on irinotecan free base with the original protocol dose (based on irinotecan hydrochloride trihydrate is in parentheses.

half-life ($t_{1/2}$) that was 2.0 times longer, and an area under the concentration-time curve ($AUC_{0-\infty}$) that was 46.2-times greater (reanalysis by calculating geometric means instead of arithmetic means and by reporting the actual values instead of dose-normalized values). The $t_{1/2}$ and $AUC_{0-\infty}$ of SN-38, the active metabolite of irinotecan, were also increased relative to nonliposomal irinotecan (3.0- and 1.4-times, respectively), while maintaining a 5.3-times lower SN-38 C_{max}. In a separate clinical trial, nal-IRI–mediated tumor delivery was evaluated in tumor biopsies from 13 patients collected 72 h after the administration of 70 mg/m^2 nal-IRI.[8] tIRI in the tumor was 0.5-times those in the plasma; however, the total SN-38 (tSN38) was 6-times higher in tumor than in plasma, and the ratio of tSN38:tIRI (a measure of the extent of conversion) was 8-times higher in tumor than in plasma.

The extended plasma PK of liposomal formulations provides an opportunity to dissect the differences between derived PK parameters, including average concentration (C_{avg}) and C_{max}, and

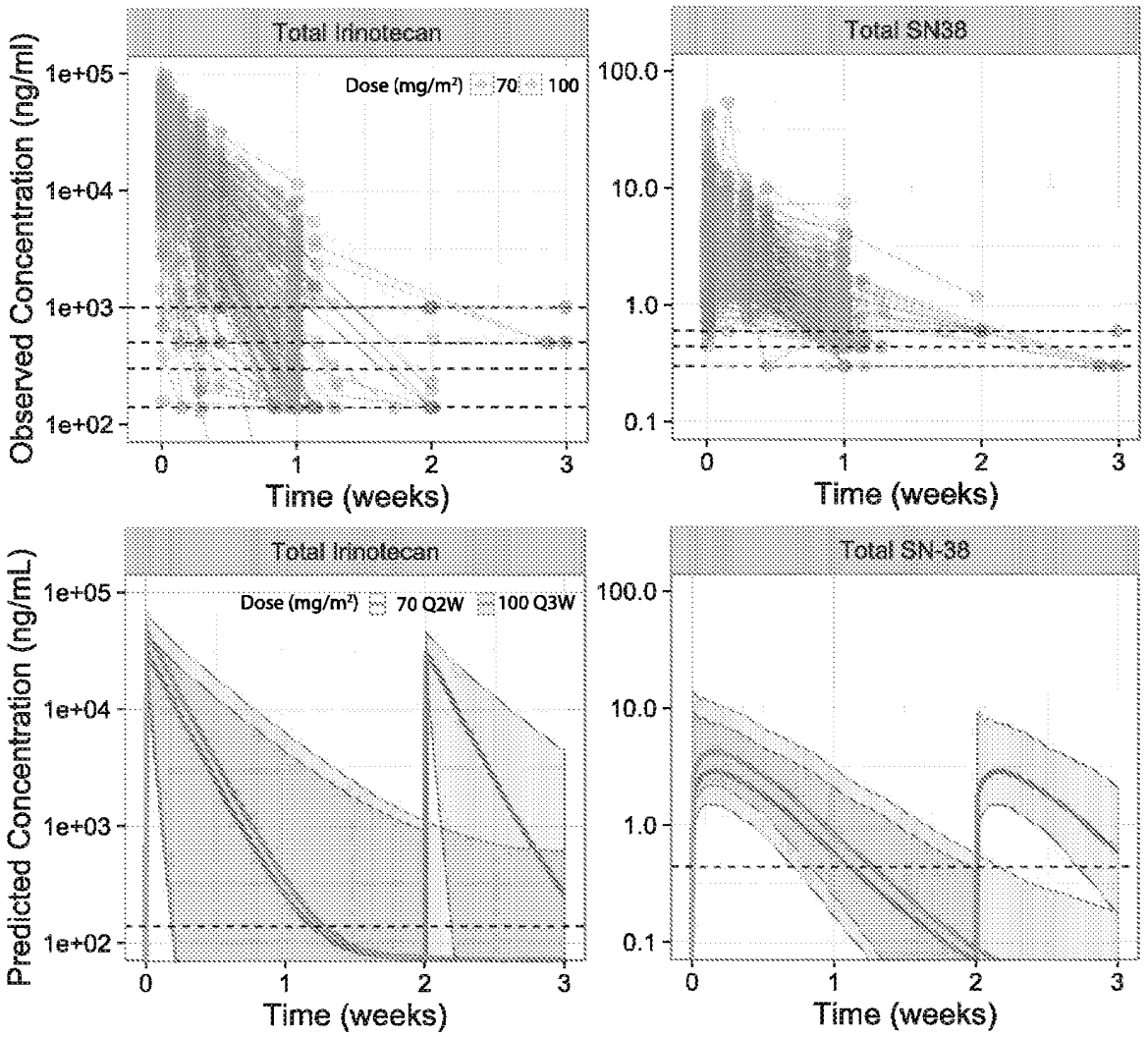

Figure 1 Observed and predicted typical plasma concentration profile of total irinotecan and SN-38 in patients administered nal-IRI 70 mg/m^2 Q2W or nal-IRI 100 mg/m^2 Q3W.

time above a threshold ($t_{uSN38>thr}$), and their association with efficacy and safety. With nonliposomal irinotecan, C_{avg} and C_{max} were highly correlated, and therefore, the dichotomization of the associations with efficacy and safety endpoints have been difficult to elucidate.[9] However, these parameters were less correlated with nal-IRI administration, and therefore, we aim to evaluate the association of these parameters with efficacy and safety in patients treated with nal-IRI.

RESULTS

Patients

Samples for PK measurements were collected during the first cycle of nal-IRI treatment in five phase I–II studies and one phase III study. Of the 368 treated patients from the six studies, 353 (96%) had samples analyzed for PK measurement, including 97% (258/266) of patients in the NAPOLI-1 study. Patient characteristics at baseline are listed in **Table 1**. Patients with hepatic or renal impairment were excluded from the enrollment; nevertheless, 20 patients were enrolled with bilirubin $\geq$1 mg/dL (19/20 had bilirubin 1–2 mg/dL; one patient had bilirubin >2 mg/dL). The majority (73%) was obtained from patients with metastatic pancreatic cancer. Most patients received an initial dose of 100 mg/m^2 (53%) or 70 mg/m^2 (39%). Most patients were either Caucasian (52%) or East Asian (42%).

PK parameter estimates

A total of 1,792 tIRI samples from 355 subjects and 1,765 tSN38 samples from 353 subjects were analyzed. Typical observed and predicted PK with 70 mg/m^2 and 100 mg/m^2 are shown in **Figure 1**. The final model sufficiently described the data, as evidenced by the comparison of the data and model fits (**Figure S3**) and by visual predictive checks for the overall data, and stratified by dose (**Figure S4–S5**). Final estimated parameters of IRI and SN-38 in the population PK models are listed in **Table S6** and **Table S7**, respectively. The tSN38 and uSN38 were highly correlated (Kendall τ of 0.81 and 0.70 for C_{avg} and C_{max}, respectively). The C_{max} and C_{avg} had lower correlation for tIRI and uSN38 (Kendall τ of 0.31 and 0.44, respectively).

Table 2 Summary of irinotecan and SN-38 pharmacokinetics parameters by nal-IRI dose regimen in NAPOLI-1

Analyte	Pharmacokinetic parameter[a]	70 (80) mg/m^2 Q2W[b]	100 (120) mg/m^2 Q3W[b]
Total irinotecan	C_{avg}, mg/L	1.19 (0.91–1.55)	1.66 (1.33–2.05)
	C_{max}, mg/L	26.6 (24.1–29.3)	41.5 (39.8–43.2)
	Clearance, L/week	13.3 (9.17, 22.8)	
	Volume, L	4.58 (4.14, 4.99)	
	First-phase $t_{1/2}$, h	38.2 (23.2–56.7)	
	Terminal $t_{1/2}$, h	12200 (3990–50200)	
Total SN-38	C_{avg}, ng/mL	0.721 (0.667–0.778)	0.870 (0.821–0.922)
	C_{max}, ng/mL	2.64 (2.47–2.83)	3.99 (3.77–4.23)
	Clearance, L/week	14.0 (12.7–14.6)	
	Terminal $t_{1/2}$, h	38.2 (36.5–41.9)	
Unencapsulated SN-38	C_{avg}, ng/mL	0.589 (0.543–0.639)	0.702 (0.661–0.745)
	C_{max}, ng/mL	2.07 (1.93–2.23)	3.05 (2.89–3.21)
	$t_{uSN38>thr}$, weeks (first 6 weeks, based on actual doses)	4.77 (4.59–4.95)	4.28 (4.12–4.44)
	$t_{uSN38>thr}$, weeks (first 6 weeks, based on simulated doses)	5.71 (5.64–5.79)	4.80 (4.69–4.92)

C_{avg}, average concentration; C_{max}, maximum concentration; $t_{uSN38>thr}$, time uSN38>threshold.
[a]For C_{avg}, C_{max}, and $t_{uSN38>thr}$, median values and 95% prediction intervals (representing interpatient variabilities) were obtained from NAPOLI1 patients; for Clearance, Volume, and $t_{1/2}$, median values and 95% confidence intervals (representing precision of parameter estimates) were obtained from bootstrapping. [b]Dose is given based on irinotecan free base with the original protocol dose (based on irinotecan hydrochloride trihydrate) is in parentheses.

The estimated PK parameters are provided in **Table 2**. The estimated initial and terminal $t_{1/2}$ of tIRI were 38.2 [95% confidence interval (CI) 23.2–56.7] and 12,200 (95% CI 3,990–50,200) h; the $t_{1/2}$ of SN-38 was 38.2 (36.5–41.9) h. The estimated terminal $t_{1/2}$ for tIRI should be treated with cautions because of the limited number of samples measured by assay with a lower limit of quantification. Compared to nal-IRI 100 mg/m^2 Q3W, nal-IRI 70 mg/m^2 Q2W was predicted to have similar tIRI and tSN38 C_{avg}, 1.5-fold lower tIRI and tSN38 C_{max}, and longer $t_{uSN38>thr}$ in the first 6 weeks. tIRI was approximately three orders of magnitude higher than tSN38. The estimated volume was 4.58 L, a value comparable to typical blood volume.

Exposure–efficacy relationships

In the nal-IRI+5FU/LV arm of NAPOLI-1, longer OS and PFS were associated with longer $t_{uSN38>thr}$ and higher C_{avg} of tIRI, tSN38, and uSN38, with the highest association observed for $t_{uSN38>thr}$. C_{max} of tIRI, tSN38, or uSN38 was not predictive of OS (P = 0.58–0.98). The relationship between OS and quartiles of $t_{uSN38>thr}$ for the nal-IRI 5-FU/LV and nal-IRI monotherapy arms are provided in **Figure 2** and **Figure S6,** respectively. Longer $t_{uSN38>thr}$ was associated with a higher probability of achieving objective response in the nal-IRI+5-FU/LV arm (**Figure S8**). This association was not observed in the nal-IRI monotherapy arm. The association between OS and uSN38 C_{avg} is provided in **Figure S7**, which also shows prolonged OS with higher uSN38 C_{avg} (uSN38 C_{avg} and $t_{uSN38>thr}$ is correlated with Kendall τ of 0.48).

Exposure–safety relationships

A total of 353 patients were included in the PK-safety analysis. Neutropenia was most strongly associated with uSN38 C_{max} (**Figure 3**). Higher uSN38 was associated with a higher probability of both incidence and severity of neutropenia. The association was observed in both grade ≥1 and grade ≥3 neutropenia. The association with neutropenia was more significant with the uSN38 than tSN38 (for example, for the incidence of neutropenia grade ≥3, the association P-values were <0.001 and 0.08 for uSN38 and for tSN38, respectively). The association between uSN38 and neutropenia was also greater for C_{max} than for C_{avg} (e.g., grade ≥3 neutropenia: P = <0.001 vs. P = 0.045 with uSN38 C_{max} and C_{avg}, respectively). In a multivariate logistic regression analysis of grade ≥3 neutropenia (**Table S8**), the association between uSN38 C_{max} and neutropenia was still significant (P = 0.00005) even after adding factors known to predict neutropenia (baseline ANC and 5-FU/LV coadministration). When baseline factors predictive of uSN38 were included (race, bilirubin, and body surface area, BSA), the association with uSN38 C_{max} was only borderline significant (P = 0.068).

Diarrhea was most strongly associated with tIRI C_{max} (**Figure 3**). Higher tIRI C_{max} was associated with a higher incidence and severity of diarrhea. The association was significant for grade ≥3 diarrhea but not for grade ≥1 diarrhea. The association between grade ≥3 diarrhea and tIRI was more significant for C_{max} (P = 0.001) than for C_{avg} (P = 0.019). The association between tIRI C_{max} and diarrhea was observed in each of the Caucasian and Asian subpopulations. In NAPOLI-1, this association was observed within the nal-IRI monotherapy arm, but not

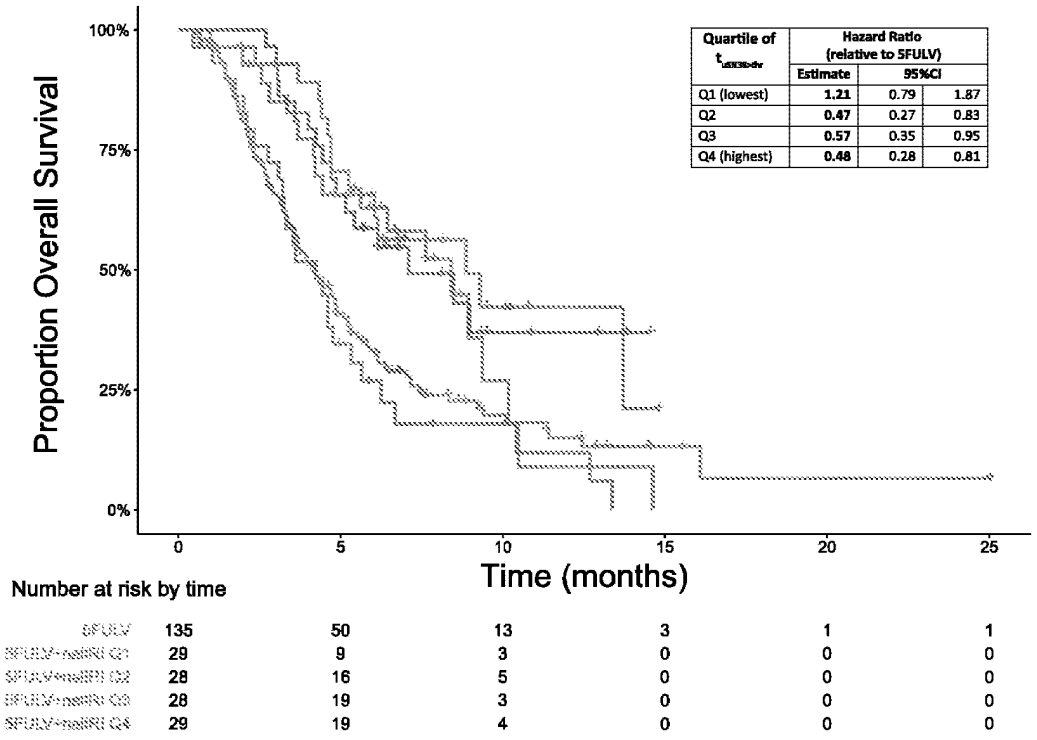

Figure 2 Kaplan–Meier plot of overall survival by quartiles of unencapsulated SN-38 (uSN38) time above threshold in the nal-IRI + 5-FU/LV arm of NAPOLI-1.

within the nal-IRI+5FU/LV arm. This was likely due to the absence of patients with high tIRI C_{max} in the nal-IRI+5FU/LV arm and lower nal-IRI dose. In a multivariate logistic regression analysis of grade ≥3 diarrhea (**Table S9**), the identified predictive factors were tIRI C_{max} and race (Caucasian vs. East Asian).

Analysis of the NAPOLI-1 safety data showed that compared with Caucasian patients, East Asian patients who received nal-IRI+5-FU/LV had a higher incidence of NCI CTCAE Grade 3 or 4 neutropenia (55% (18/33) vs. 18% (13/73), respectively), yet a lower incidence of Grade 3 or 4 diarrhea (3.0% (1/33) vs. 19.2% (14/73)), respectively.[20] Therefore, the differences in the observed rates of neutropenia and diarrhea by race can be explained by the racial differences in the C_{max} of tIRI and uSN38.

Baseline factors predictive of plasma PK

Baseline factors evaluated for associations to plasma PK include: BSA, demographics, hepatic and renal function, pharmacogenomics (UGT1A1*28), and extrinsic factors (**Table S3, Figures S9, S10**). The significant factors and the corresponding tIRI and uSN38 for nal-IRI 70 mg/m^2 are summarized in **Figure 4**.

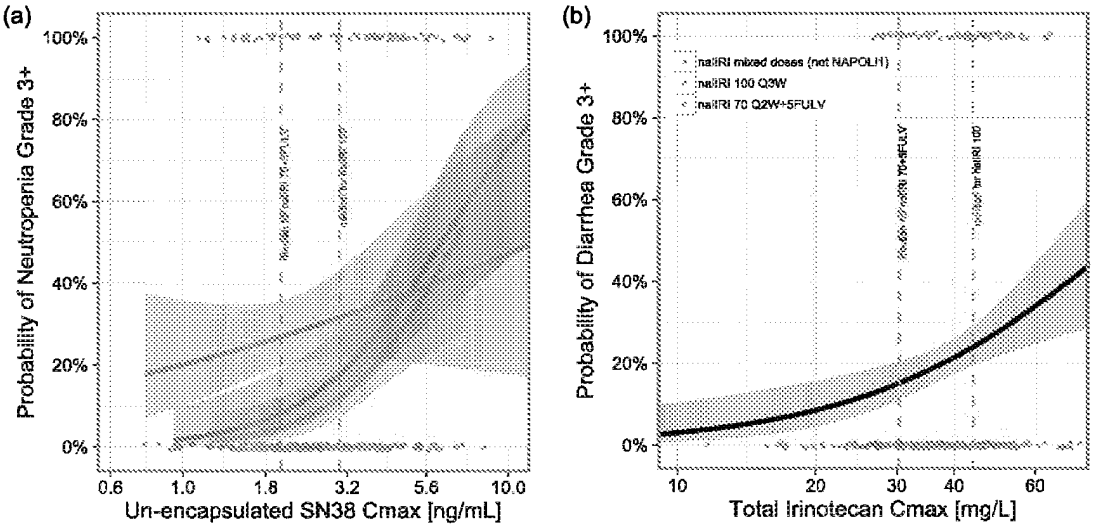

Figure 3 Incidence rates of neutropenia (**a**) and diarrhea (**b**) grade ≥3 by plasma PK in patients treated with nal-IRI.

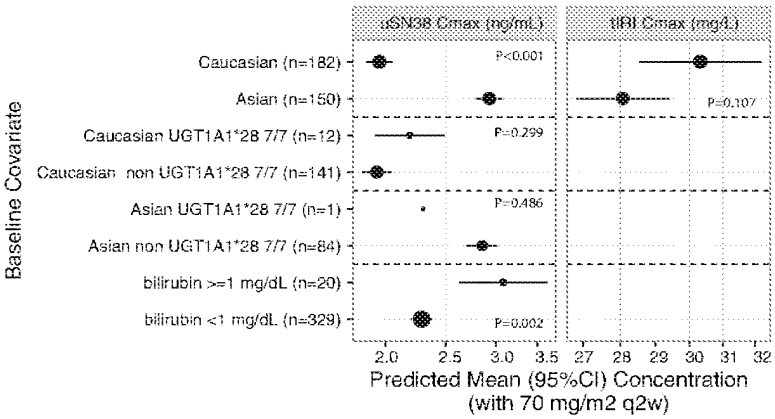

Figure 4 Selected baseline factors and associated plasma total irinotecan and unencapsulated SN-38 C_{max} with nal-IRI 70 mg/m^2.

Factors with significant association with tIRI PK were race and BSA. Factors with significant association with tSN38 were race, BSA, and bilirubin. Asians had lower tIRI and higher uSN38 compared with Caucasians (7% and 78% lower tIRI C_{max} and C_{avg}, 50% and 20% higher uSN38 C_{max} and C_{avg}; all $P \leq 0.001$). In the population PK model that accounted for multivariate analysis (including BSA), race remained a significant factor for both tIRI and tSN38 (**Tables S6, S7**). Comparison of BSA-based dosing to fixed dosing (70 mg/m^2 or an equivalent fixed dose of 116.7 mg) revealed that BSA-based dosing reduced variability of tIRI and uSN38 C_{max} (3% and 14% less interquartile range, **Table S10**). This result implies a benefit of BSA-based dosing in reducing the variability of tIRI and uSN38 C_{max}. While the number of patients with elevated bilirubin was small (n = 20), bilirubin was found to be a significant factor of tSN38: compared with patients with bilirubin <1 mg/dL, patients with bilirubin ≥1 mg/dL had a higher uSN38 C_{avg} (43% higher) and C_{max} (35% higher).

UGT1A1*28, a pharmacogenomic biomarker, was not a significant predictor of SN-38 with nal-IRI administration. In the population PK dataset, the prevalence of UGT1A1*28 7/7 homozygosity in Asians was low (2/129 (1.5%)). Compared with non-7/7 homozygous Caucasians, 7/7 homozygous Caucasians had similar uSN38 C_{max} if both were dosed at 70 mg/m^2 (**Figure 4**; 2.19 (95% CI 1.92–2.49, n = 12) and 1.94 (95% CI 1.84–2.05, n = 141) ng/mL; P = 0.30; geometric mean ratio: 1.13 (95% CI 0.90–1.42). In NAPOLI-1, the actual dose homozygous patients received were lower than the dose in nonhomozygous patients). The estimated SN-38 clearance in UGT1A1 7/7 homozygous was 1.000-times (0.0% difference) the clearance in non-7/7 (**Table S7**). A sensitivity analysis was performed to estimate the SN-38 clearance by more detailed categories of UGT1A1*28 alleles (separate evaluation for 6/6, 6/7, and 7/7; **Table S11**). The estimated clearance for UGT1A1*28 6/7 and UGT1A1*28 7/7 were within 0.0% and 2.7% of the clearance for UGT1A1*28 6/6. These results indicate that UGT1A1 is not a significant covariate to SN-38 clearance.

Other baseline factors evaluated were found not to have significant associations with tIRI or uSN38. Among measures of hepatic functions other than bilirubin, albumin had a weak association with tIRI, but not tSN38 nor uSN38. The direction of the albumin-tIRI association was the opposite of that expected in hepatic impairment and opposite of the observed diminished clearance reported in patients with hepatic impairment administered with nonliposomal irinotecan.[19] Because of the lack of association with the active metabolite SN-38, the effect of albumin is unlikely to be clinically relevant. Sex and creatinine clearance were not significantly associated with SN-38 after adjusting for BSA.

DISCUSSION

Similar to the liposomal formulation of doxorubicin, the liposomal formulation of irinotecan modifies the pharmacological properties of irinotecan, resulting in extended half-lives of plasma total irinotecan and SN-38. The extended plasma PK observed with nal-IRI provides a tool to distinguish C_{avg} and C_{max}, as evidenced by the low correlation between these parameters, and therefore is useful to evaluate pharmacological properties associated with efficacy and safety. The vastly different estimated volumes highlight the different disposition characteristics with liposomal formulation.[7]

In pancreatic cancer patients treated with nal-IRI+5-FU/LV, higher C_{avg} and longer $t_{uSN38>thr}$ was associated with longer OS and PFS and higher overall response rate. Conversely, C_{max} was not associated with OS. This is consistent with the hypothesis that dividing cells are sensitive to chemotherapy; thus, prolonged duration of chemotherapy drug exposures allow a greater number of tumor cells to be affected.[21] The observed association between C_{avg} and $t_{uSN38>thr}$ with efficacy indicates a strong association between plasma and tumor concentrations. This is consistent with the direct SN-38 measurements in biopsies during a phase I trial that demonstrated increased tumor SN-38 PK with nal-IRI administration.[8] Furthermore, the association between these two parameters and efficacy is consistent with the preclinical finding that showed a strong association between the *in vivo* activity of nal-IRI and the duration of SN-38 above a minimum inhibitory concentration.[17] This result indicates the potential benefit in extending duration of plasma and tumor exposure via liposomal encapsulation.

Neutropenia and diarrhea are the most prominent adverse events with nal-IRI treatment. For neutropenia, uSN38 was the analyte that had the highest association, with C_{max} exhibiting a stronger association than C_{avg}. The association between neutropenia and uSN38 C_{max} appeared to be robust and remained significant in the presence of known factors predictive of neutropenia (e.g., ANC and 5-FU coadministration). Diarrhea was associated with tIRI C_{max}, and as was seen with neutropenia, the association was stronger with C_{max} than C_{avg}. The dichotomization of the analytes associated with blood- and gut-related safety events are consistent with reports of differential metabolism occurring in the plasma and in the gut. In particular, it has been reported that SN-38G can be converted back to SN-38 in the gut via microflora, but this mechanism is absent in the plasma.[22,23] Because SN-38G in the plasma is observed at an ~10-times higher concentration than SN-38, the conversion in the gut may result in higher SN-38 concentrations in the gut compared with the plasma. While the ratio of SN-38 and SN-38G would depend on the activity of UGT1A enzymes, the sum of SN-38 and SN-38G—both in the gut and plasma—would increase as total drug exposure of irinotecan increased. As total drug exposure of nal-IRI is linearly proportional to plasma tIRI,[20] it can be hypothesized that plasma tIRI is a surrogate measurement of the sum of SN-38 and SN-38G in the gut lumen.

Among the baseline factors considered, race (Caucasian vs. East Asian) was the most significant predictive factor for both plasma total irinotecan and SN-38 PK following the administration of nal-IRI. Specifically, when compared with Caucasian patients, East Asian patients had lower tIRI and higher SN-38, and a lower corresponding risk for diarrhea and higher risk for neutropenia. The race-PK association has not been reported in patients receiving nonliposomal irinotecan. Therefore, the release kinetics of irinotecan from liposome may be linked to the race-related PK difference. The elimination of liposomal chemotherapy from circulation was hypothesized to follow two pathways: passive leakage from liposomes and active uptake by the mononuclear phagocyte system (MPS).[24] The first pathway, passive leakage, is likely to be dependent only on external factors such as manufacturing. Therefore, the second pathway, the uptake by MPS, is the hypothesized pathway that maybe affected by race and provides direction for future research in exploring pharmacogenomic factors.

The plasma SN-38 depends on both the incoming load of SN-38 and the activity of UGT1A enzymes. The activity of UGT1A enzymes can be assessed by either baseline bilirubin or by pharmacogenomics (UGT1A1*28). Liposomal encapsulation appears to reduce the incoming load of SN-38 by controlling the release of irinotecan. Hyperbilirubinemia, a surrogate of reduced UGT activity, has been shown to be predictive to plasma SN-38 and to neutropenia with administration of nonliposomal irinotecan.[19] In patients administered with nal-IRI described here, baseline bilirubin was also found to be a significant predictor of SN-38, and SN-38 concentrations were 44% higher in patients with hyperbilirubinemia. Because of the limited number of patients with bilirubin >1 mg/dL in the dataset, no nal-IRI dose recommendation is provided, and a lower starting dose may be warranted.

A consistent result is found by pharmacogenomics (UGT1A1*28). In patients treated with nonliposomal irinotecan, the associations between UGT1A1*28 7/7 homozygosity and hematological toxicity were observed only in patients treated with doses >150 mg/m^2; however, similar hematological toxicities were observed for both UGT1A1*28 homozygous and nonhomozygous patients with a lower dose of nonliposomal irinotecan of 100–125 mg/m^2 every week.[25] The association between UGT1A1*28 7/7 homozygosity and SN-38 concentrations are also dependent on the dose of nonliposomal irinotecan, with much higher SN-38 concentrations observed for 6/7 and 7/7 (compared to 6/6) when irinotecan was administered at a dose of 300 mg/m^2 than when it was administered at a dose of 15–75 mg/m^2 daily for 5 days for 2 consecutive weeks.[26,27] With nal-IRI treatment, SN-38 PK were similar across UGT1A1*28 polymorphisms. A likely mechanistic explanation is that the liposomal encapsulation protects the majority of irinotecan from being converted into SN-38 and, therefore, the slow release of irinotecan allows the lower load of SN-38 to be metabolized by UGT enzymes even in patients with reduced UGT enzyme activities (for example, UGT1A1*28 7/7 homozygous patients). Additional data in phase I–II studies in patients treated with nal-IRI tested for different UGT1A1 genotypes [UGT1A9*22 (*1b), UGT1A1G-3156A, UGT1A1*6, UGT1A1*27, UGT1A1T-3279G, and DPYD*2A] indicate that no difference in SN-38 concentrations was observed by UGT1A1 genotypes (in preparation). Because of the lack of precision in the comparison between homozygous and nonhomozygous patients (as evidenced by the wide 95% CI range of the ratio), the limited number of patients homozygous for the UGT1A1*28 allele treated with nal-IRI, and the lower starting nal-IRI dose used in NAPOLI-1 for these patients (50 mg/m^2), it is recommended that those known to be homozygous for the UGT1A1*28 allele be treated initially with 50 mg/m^2, which can be increased to 70 mg/m^2 if tolerated. However, UGT1A1*28 testing is not mandated.

In conclusion, the quantification of the plasma PK in patients treated with nal-IRI showed the benefit of the liposomal formulation in extending the half-lives of irinotecan and SN-38. The differential pharmacological parameters associated with efficacy and safety endpoints provide support to the selection of dose regimen for nal-IRI. Because efficacy is associated with C_{avg} and $t_{uSN38>thr}$, and safety is associated with C_{max}, a dose regimen of 70 mg/m^2 Q2W would result in improved safety while maintaining efficacy as compared to a dose regimen of 100 mg/m^2 Q3W. Therefore, these associations support the benefit in the current dosing of nal-IRI of 70 mg/m^2 Q2W.

METHODS

Patients and treatment

Data were prospectively collected from patients enrolled in six trials on a variety of tumor types, including colorectal, gastric, and pancreatic cancers (**Table S1**). Detailed eligibilities, methods, and clinical results of these studies have been described previously.[3,7,8,10–12] For example, the eligibility criteria in Study NAPOLI-1 included adequate bone marrow reserve (absolute neutrophil count (ANC) >1,500 cells/μL, platelet >10^6 cells/μL, hemoglobin >9 g/dL), adequate renal function (serum creatinine (SCr) ≤1.5 upper limit of normal (ULN)), and adequate liver

function (bilirubin ≤ULN, albumin ≥3.0 g/dL; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of ≤2.5 ULN or ≤5 ULN if liver metastases were present). The nal-IRI doses in these studies were calculated based on the equivalent doses of irinotecan hydrochloride trihydrate; in this report, the doses described are based on irinotecan as free-base (i.e., 70 mg/m^2 of irinotecan as the free-base is equivalent to 80 mg/m^2 of irinotecan as the hydrochloride trihydrate). The final population PK dataset consisted of 353 subjects. Two patients from NAPOLI-1 with tIRI but without tSN38 measurements were excluded from the analyses (**Table S2**).

PK data

PK sample collection during the first cycle consisted of intense sampling in early studies[7,8,10–12] and sparse sampling in the phase III study NAPOLI-1 (**Table S1**).[3] The analytes measured include tIRI (encapsulated plus unencapsulated irinotecan) and tSN38. In the first study, the levels of encapsulated irinotecan were found to be indistinguishable from tIRI[10]; therefore, only tIRI were measured in the subsequent studies. Samples collected after second dose administrations were excluded because of suspected inaccuracy in the sampling times.

Covariate analysis was conducted using a full covariate approach.[13] Baseline information to predict plasma PK included body size (BSA), demographics, hepatic and renal function, pharmacogenomics, and extrinsic factors such as product manufacturing site and coadministration with 5-FU. Laboratory measurements (ALT, AST, bilirubin, creatinine clearance, and albumin) were log-transformed (log-normal distributions were observed) (**Table S3**). Liver metastasis status was only available from NAPOLI-1; therefore, the values for the other studies were imputed to "No"; the effect of this imputation was evaluated in a sensitivity analysis. The estimated clearance of IRI was added as a covariate to the SN-38 input flux. Mechanistically, increased clearance of IRI was hypothesized to generate more release of unencapsulated irinotecan that would be available for conversion to SN-38 (clearance of nal-IRI likely results in broken liposome and release of irinotecan).

Population PK modeling analysis methods

Modeling assumptions. Nonlinear mixed effect modeling (NONMEM) was used to analyze the PK data of tIRI and tSN38 in patients administered nal-IRI. To account for measured values below the detection limit, the M3 method[15] was implemented with concentrations in log-transformed values using the Laplacian estimation method.[16]

A diagram of the PK models of tIRI and tSN38 is shown in **Figure S1**. The final model of tIRI was a two-compartment model with first-order elimination, and the tSN38 depends on the tIRI model. tSN38 was represented as a sum of unencapsulated SN-38 (uSN38) and encapsulated SN-38 (eSN38), with eSN38 as a time-invariant fraction of tIRI, and uSN38 as a one-compartment model with first-order production rate representing the process of release of irinotecan and its conversion to SN-38. The existence of eSN38 was supported by *in vitro* measurements and by the observation of delayed metabolism of SN-38 with nal-IRI administration. In study PEP0206,[7] the delayed appearance of SN-38G relative to the appearance of SN-38 was observed after nal-IRI administration, in contrast to the immediate appearance of SN-38G and SN-38 after nonliposomal irinotecan administration (**Figure S2**). This observation supports the hypothesis that only the uSN38 is bioavailable for glucuronidation. The fraction of eSN38 in tIRI was estimated to be 0.01%, which is comparable to the *in vitro* measurement of 0.015% and is below the specification limit of irinotecan manufacturing.[14] The inclusion of the uSN38 and eSN38 improved the model fitting (**Table S4**).

Simulation analysis methods. Simulations from *post-hoc* estimates were used to derive PK parameters for the first cycle of nal-IRI, including the C_{avg} and C_{max} for tIRI, tSN38, and uSN38, as well as $t_{uSN38>thr}$ in the first 6 weeks. In a preclinical study, nal-IRI activity was strongly associated with $t_{uSN38>thr}$.[17] The threshold of 0.03 ng/mL was chosen based on the median IC_{50} of SN-38 in *in vitro* pancreatic cell lines (different choices of threshold of 0.02–0.3 ng/mL resulted in similar OS concordance indices). For the evaluation of baseline covariates associated with PK, simulations were based on 70 mg/m^2 Q2W. For the evaluation of fixed- and BSA-based dosing, simulations were based on 70 mg/m^2 Q2W, or 116.7 mg Q2W (equivalent dose for a subject with median BSA).

Exposure–efficacy analysis methods. PK efficacy analysis was performed for each treatment arm of NAPOLI-1. The associations between PK parameters and survival endpoints were measured using the concordance index.[18] The selection of PK parameters was based on the magnitude of the concordance index and the (positive) direction of the association.

Exposure–safety analysis methods. The safety dataset included patients from all six clinical studies (**Table S1**) and was evaluated for diarrhea and neutropenia. Specialized grouping based on individual MedDRA v. 14.1 terms was used for diarrhea and neutropenia (**Table S5**) to establish systematic reporting. The reported AEs included any grade and grade ≥3 according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) 4.0.

Software. All data preparation and presentation was performed using SAS v. 9.3 or later (SAS Institute, Cary, NC) and R v. 3.0.2 (Vienna, Austria). PK analysis was performed using NONMEM v. 7.3, with FOCEI and Laplacian estimation method. Package Perl Speaks NONMEM (PSN) v. 3.7.6 was used for interface to NONMEM and for assessing models. R package Xpose4 v. 4.5.0 was used to display diagnostics.

Additional Supporting Information may be found in the online version of this article.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work. This study was funded by Merrimack Pharmaceuticals. We thank Li-Tzong Chen, Stephan Klinz, Bart Hendriks, Marc Pipas, Eliel Bayever, and Peter Laivins for contributions to the design and the interpretation of the analysis. Medical writing support (funded by Merrimack Pharmaceuticals) was provided by Beth Kamp.

CONFLICT OF INTEREST

Drs. Adiwijaya, J Kim, Fitzgerald, Belanger, and Molnar are employees of Merrimack Pharmaceuticals. Drs. Lang, Csöszi, Cubillo, Chen, JS Kim, Rau, and Gallego Plazas report no conflicts of interest. Dr. Wong reports being a member of a Baxalta advisory board. Dr. Park reports receiving honoraria from Celgene, serving as a consultant for Celgene and Agios Pharmaceuticals, Inc., and receiving research funding from Celgene and AstraZeneca. Dr. Melichar reports receiving honoraria for speeches and advisory roles from Lilly, Sanofi, Roche, Novartis, Pfizer, Janssen, Astellas, BMS, MSD, GSK, Merck, and Amgen. Dr. Ma reports receiving funding for clinical trials and honorarium for advisory boards from Merrimack Pharmaceuticals.

AUTHOR CONTRIBUTIONS

B.A., J.K., J.B.F., I.M., and W.W.M. wrote the article; B.A., J.K., J.B.F., B.B., and W.W.M. designed the research; B.A., J.K., I.L., T.C., A.C., J-S.C., M.W., J.O.P., J.S.K., K-M.R., B.M., J.G.P., and W.W.M. performed the research; B.A., J.K., J.B.F., B.B., and W.W.M. analyzed the data.

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Effect of Baseline Carbohydrate Antigen 19-9 (CA19-9) Level on Overall Survival (OS) in NAPOLI-1 Trial: a Phase 3 Study of MM-398 (nal-IRI), with or without 5-Fluorouracil and Leucovorin (5-FU/LV), versus 5-FU/LV in Metastatic Pancreatic Cancer (mPAC) Previously Treated with Gemcitabine-based Therapy

Background: CA19-9 has been shown to correlate with response to therapy and OS in patients with mPAC. NAPOLI-1, a randomized phase 3 study evaluated nal-IRI, a nanoliposomal formulation of irinotecan, with or without 5-FU/LV, versus 5-FU/LV in patients with mPAC previously treated with gemcitabine-based therapy. nal-IRI + 5-FU/LV significantly improved OS (primary endpoint) versus 5-FU/LV (6.1 vs 4.2 months; unstratified hazard ratio (HR) = 0.67; $P = 0.012$). CA19-9 response (≥50% decline from baseline) was superior with nal-IRI + 5-FU/LV compared with 5-FU/LV (29% vs 9%; $P = 0.0006$). nal-IRI arms did not show a statistical improvement in survival.

Methods: Patients with a recorded baseline CA19-9 measurement were divided into quartiles to evaluate the treatment effect pattern of CA19-9 from nal-IRI + 5-FU/LV and 5-FU/LV arms. Quartile ranges were based on 404 available CA19-9 values from randomized patients (N = 417). Unstratified Cox proportional hazards regression was used to estimate HRs and corresponding 95% CIs. Effect of baseline CA19-9 on time to response, progression-free survival, and response will be presented.

Results: Of patients randomized to receive nal-IRI + 5-FU/LV (n = 117) or 5-FU/LV enrolled contemporaneously (n = 119), 218 received study drug and had a baseline CA19-9 measurement. Results show a greater treatment effect on OS with higher CA19-9 level relative to 5-FU/LV.

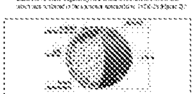
	CA19-9 (U/mL) quartile			
	Q1 CA19-9 < 59	Q2 59≤ CA19-9 < 582	Q3 582≤ CA19-9 < 2,879	Q4 CA19-9 ≥ 2,879
Median OS, months (n in each group)				
nal-IRI + 5-FU/LV (n = 115)	7.6 (n = 27)	6.7 (n = 25)	6.1 (n = 27)	3.6 (n = 26)
5-FU/LV (n = 103)	7.2 (n = 31)	5.3 (n = 23)	3.6 (n = 21)	1.9 (n = 28)
HR (95% CI)	1.12 (0.57-2.22)	0.74 (0.37-1.48)	0.43 (0.22-0.84)	0.35 (0.19-0.64)

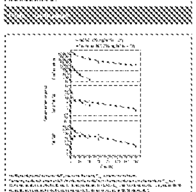
Conclusions: In patients with mPAC previously treated with gemcitabine-based therapy, nal-IRI + 5-FU/LV significantly improved OS supported by progression-free survival and objective response rate. The CA19-9 serum level can provide important information with regard to OS.

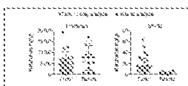
Poster #226

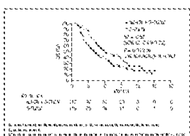
Effect of Baseline Carbohydrate Antigen 19-9 (CA19-9) Level on Overall Survival (OS) in NAPOLI-1 Trial: a Phase 3 Study of MM-398 (nal-IRI), with or without 5-Fluorouracil and Leucovorin (5-FU/LV), versus 5-FU/LV in Metastatic Pancreatic Cancer (mPAC) Previously Treated with Gemcitabine-based Therapy

BACKGROUND



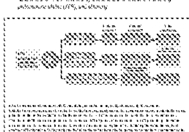




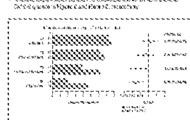


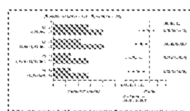
OBJECTIVE

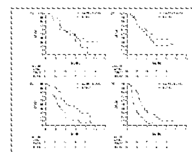
NAPOLI-1 METHODS

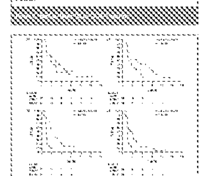


RESULTS









CONCLUSIONS

POSTER PRESENTED AT THE GASTROINTESTINAL CANCERS SYMPOSIUM OF THE AMERICAN SOCIETY OF CLINICAL ONCOLOGY; JANUARY 21-23, 2016; SAN FRANCISCO, CALIFORNIA.

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

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Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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	8	7846440	B2	2010-12-07	Schoeberl et al.	

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	15809815
Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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	1	PCT/US2013/046914: International Preliminary Report on Patentability dated December 23, 2014, 7 pages.
	2	PCT/US2013/046914: PCT International Search Report mailed September 2, 2013, 3 pages.
	3	PCT/US2013/075513: PCT International Preliminary Report on Patentability issued June 16, 2015, 7 pages.
	4	PCT/US2013/075513: PCT International Search Report mailed June 6, 2014, 2 pages.
	5	PCT/US2014/062007: PCT International Preliminary Report on Patentability issued April 26, 2016, 10 pages.
	6	PCT/US2014/062007: PCT International Search Report mailed January 9, 2015, 3 pages.
	7	PCT/US2015/064491: PCT International Preliminary Report on Patentability issued June 13, 2017, 7 pages.
	8	PCT/US2015/064491: PCT International Search Report mailed February 19, 2016, 4 pages.
	9	PCT/US2016/047814: International Search Report mailed November 17, 2016, 3 pages.
	10	PCT/US2016/047814: PCT International Preliminary Report on Patentability issued February 20, 2018, 6 pages.
	11	PCT/US2016/047827: International Search Report mailed November 17, 2016, 3 pages.

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	12	PCT/US2016/047827: PCT International Preliminary Report on Patentability issued February 20, 2018, 6 pages.
	13	Pfizer Background Document on the UGT1A1 Polymorphisms and Irinotecan Toxicity: ACPS November 3, 2004 Advisory Committee Meeting, 19 pages.
	14	RAMANATHAN R, et al., "Correlation between Ferumoxytol Uptake in Tumor Lesions by MRI and Response to Nanoliposomal Irinotecan in Patients with Advanced Solid Tumors: A Pilot Study," Clin Cancer Res. 23(14):3638-48 (2017).
	15	RAMANATHAN R, et al., "Lesion Characterization with Ferumoxytol MRI in Patients with Advanced Solid Tumors and Correlation with Treatment Response to MM-398, Nanoliposomal Irinotecan (nal-IRI)." Poster presented at EORTC-NCI-AACR International Conference on Molecular Targets and Cancer Therapeutics on November 20, 2014, 7 pages.
	16	RAMANATHAN R, et al., "Lesion Characterization with Ferumoxytol MRI in Patients with Advanced Solid Tumors and Correlation with Treatment Response to MM-398, Nanoliposomal Irinotecan (nal-IRI)," Abstract no. 261. Eur. J. Cancer, 50:87 (2014).
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	18	SACHDEV J, et al., "A Phase 1 Study in Patients with Metastatic Breast Cancer to Evaluate the Feasibility of Magnetic Resonance Imaging with Ferumoxytol as a Potential Biomarker for Response to Treatment with Irinotecan Liposome Injection (nal-IRI, MM-398)." Poster presented at 38th Annual San Antonio Breast Cancer Symposium on December 8, 2015, 10 pages.
	19	SACHDEV J, et al., "Characterization of Metastatic Breast Cancer Lesions with Ferumoxytol MRI and Clinical Response to MM-398, Nanoliposomal Irinotecan (nal-IRI), in 3 Subjects." Poster presented at San Antonio Breast Cancer Symposium 2014, 8 pages.
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	21	SAITO R, et al., "Distribution of Liposomes into Brain and Rat Brain Tumor Models by Convection-Enhanced Delivery Monitored with Magnetic Resonance Imaging," Cancer Res. 64(7):2572-9 (2004).
	22	SAITO R, et al., "Gadolinium-loaded Liposomes Allow for Real-Time Magnetic Resonance Imaging of Convection-Enhanced Delivery in the Primate Brain," Exp Neurol. 196(2):381-9 (2005).

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	23	SAITO R, et al., "Tissue Affinity of the Infusate Affects the Distribution Volume During Convection-Enhanced Delivery into Rodent Brains: Implications for Local Drug Delivery," J Neurosci Methods. 154(1-2):225-32 (2006).	
	24	TAHARA M, et al., "The Use of Olaparib (AZD2281) Potentiates SN-38 Cytotoxicity in Colon Cancer Cells by Indirect Inhibition of Rad51-Mediated Repair of DNA Double-Strand Breaks," Mol Cancer Ther. 13(5):1170-80 (2014).	
	25	TARDI P, et al., "Drug Ratio-Dependent Antitumor Activity of Irinotecan and Cisplatin Combinations In Vitro and In Vivo," Mol Cancer Ther. 8(8):2266-75 (2009).	
	26	TENTORI L, et al., "Influence of MLH1 on Colon Cancer Sensitivity to Poly(ADP-ribose) Polymerase Inhibitor Combined with Irinotecan," Int J Oncol. 43(1):210-8 (2013).	
	27	U.S. Patent Application No. 14/964,571: 2019-06-12 Final Office Action, 15 pages.	
	28	U.S. Patent Application No. 15/664,976: 2019-05-21 Nonfinal Office Action, 11 pages.	
	29	U.S. Patent Application No. 15/768,352: 2019-02-14 Non-Final Office Action, 15 pages.	
	30	U.S. Patent Application No. 15/768,352: 2019-06-03 Examiner Interview Summary, 5 pages.	
	31	U.S. Patent Application No. 15/768,352: 2019-06-12 Notice of Allowance including Examiner's Reasons for Allowance and Examiner Interview Summary, 21 pages.	
	32	U.S. Patent Application No. 15/768,352: 2019-07-12 Examiner Interview Summary, 4 pages.	
	33	U.S. Patent Application No. 15/768,352: 2019-08-28 Notice of Allowance including Examiner's Reasons for Allowance and Examiner Interview Summary, 16 pages.	

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	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

	34	U.S. Patent Application No. 15/809,815: 2019-07-08 Non-Final Office Action, 13 pages.
	35	U.S. Patent Application No. 15/896,389: 2019-07-18 Nonfinal Office Action, 24 pages.
	36	U.S. Patent Application No. 15/896,436: 2019-07-05 Nonfinal Office Action, 18 pages.
	37	U.S. Patent Application No. 16/012,351: 2019-03-08 Non-Final Office Action, 13 pages.
	38	U.S. Patent Application No. 16/012,372: 2019-03-08 Non-Final Office Action, 8 pages.
	39	U.S. Patent Application No. 16/036,885: 2019-09-03 Non-Final Office Action, 15 pages.
	40	VENTURA M, et al., "Imaging-Based Assessment of the Treatment Efficacy of Nanoliposomal Irinotecan (nal-IRI) in a Triple Negative Breast Cancer Model of Spontaneous Metastasis." Poster presented at Annual World Molecular Imaging Congress, Sept 7-10, 2016, 8 pages.
	41	VON PAWEL J, et al., "Randomized Phase III Trial of Amrubicin Versus Topotecan as Second-Line Treatment for Patients with Small-Cell Lung Cancer," J Clin Oncol. 32(35):4012-9 and appendix (1 page) (2014).
	42	VON PAWEL J, et al., "Topotecan Versus Cyclophosphamide, Doxorubicin, and Vincristine for the Treatment of Recurrent Small-Cell Lung Cancer," J Clin Oncol. 17(2):658-67 (1999).
	43	WÄHLBY C, et al., "Sequential Immunofluorescence Staining and Image Analysis for Detection of Large Numbers of Antigens in Individual Cell Nuclei," Cytometry. 47(1):32-41 (2002).
	44	ZANDER S, et al., "EZN-2208 (PEG-SN38) Overcomes ABCG2-Mediated Topotecan Resistance in BRCA1-Deficient Mouse Mammary Tumors," PLoS One. 7(9):345248 (2012), pages 1-9.

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	Art Unit	1612
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	45	ZHANG Y, et al., "Poly(ADP-ribose) Polymerase and XPF-ERCC1 Participate in Distinct Pathways for the Repair of Topoisomerase I-Induced DNA Damage in Mammalian Cells," Nucleic Acids Res. 39(9):3607-20 (2011).	
	46	ZHAO M, et al., "Clinical Observation of Irinotecan or Topotecan as Second-Line Chemotherapy on Treating 43 Patients with Small-Cell Lung Cancer," Chin Oncol. 21(2):156-8 (2011), text in Chinese with Tables 1-3 and Figure 1 in English.	
	47	ZHENG J, et al., "[18F]FAZA-PET Detection of Hypoxia Changes following Anti-cancer Therapy." Poster presented at Annual World Molecular Imaging Congress, September 18-21, 2013, 7 pages.	
	48	ZHENG J, et al., "Longitudinal Tumor Hypoxia Imaging with [18F[FAZA-PET Provides Early Prediction of Nanoliposomal Irinotecan (nal-IRI) Treatment Activity," EJNMMI Res 5(1):57, 10 pages (2015).	
	49	ZNOJEK P, et al., "Preferential Potentiation of Topoisomerase I Poison Cytotoxicity by PARP Inhibition in S Phase," Br J Cancer. 111(7):1319-26 (2014).	

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	Application Number	15809815
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	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Mary R. Henninger/	Date (YYYY-MM-DD)	2020-01-09
Name/Print	Mary R. Henninger	Registration Number	56992

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	15809815
Filing Date:	10-Nov-2017
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Filer:	Mary Rucker Henninger/Richard King
Attorney Docket Number:	263266-421428

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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		Total in USD ($)		**240**

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EFS ID:	38246482
Application Number:	15809815
International Application Number:	
Confirmation Number:	5137
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Customer Number:	153749
Filer:	Mary Rucker Henninger/Richard King
Filer Authorized By:	Mary Rucker Henninger
Attorney Docket Number:	263266-421428
Receipt Date:	09-JAN-2020
Filing Date:	10-NOV-2017
Time Stamp:	10:54:53
Application Type:	Utility under 35 USC 111(a)

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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New International Application Filed with the USPTO as a Receiving Office

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Inventors:	Group Art Unit: 1612
Eliel BAYEVER et al.	Examiner: Celeste A. Roney
Application No.: 15/809,815	Confirmation No.: 5137
Filed: November 10, 2017	
Title: Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin	

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INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. § 1.97(c)

Pursuant to 37 C.F.R. §§ 1.56 and 1.97(c), Applicant brings to the attention of the Examiner the documents listed on the enclosed IDS Form PTO/SB/08. This Information Disclosure Statement is being filed after the mailing of an Office Action on the merits, but to Applicant's knowledge, prior to the mailing of a Final Office Action, *ex parte Quayle* Action, or Notice of Allowance. This Information Disclosure Statement is accompanied by $240, as required by 37 C.F.R. §1.97(c).

Copies of the listed foreign patent documents and non-patent literature documents are enclosed.

Applicant respectfully requests that the Examiner consider the listed documents and indicate that they were considered by making appropriate notations on the attached form.

This submission does not represent that a search has been made or that no better art exists and does not constitute an admission that each or all of the listed documents are material or

constitute "prior art." If the Examiner applies any of the documents as prior art against any claim in the application and Applicant determines that the cited documents do not constitute "prior art" under United States law, Applicant reserves the right to present to the U.S. Patent and Trademark Office the relevant facts and law regarding the appropriate status of such documents.

Applicant further reserves the right to take appropriate action to establish the patentability of the claimed invention over the listed documents, should one or more of the documents be applied against the claims of the present application.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account 506488.

Respectfully submitted,

MCNEILL BAUR PLLC.

Dated: January 9, 2020

By: /Mary R. Henninger/
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