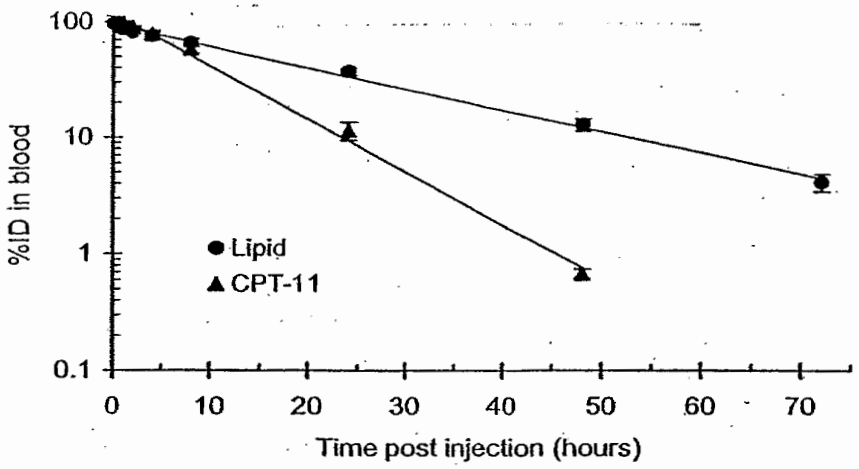

Figure 1.

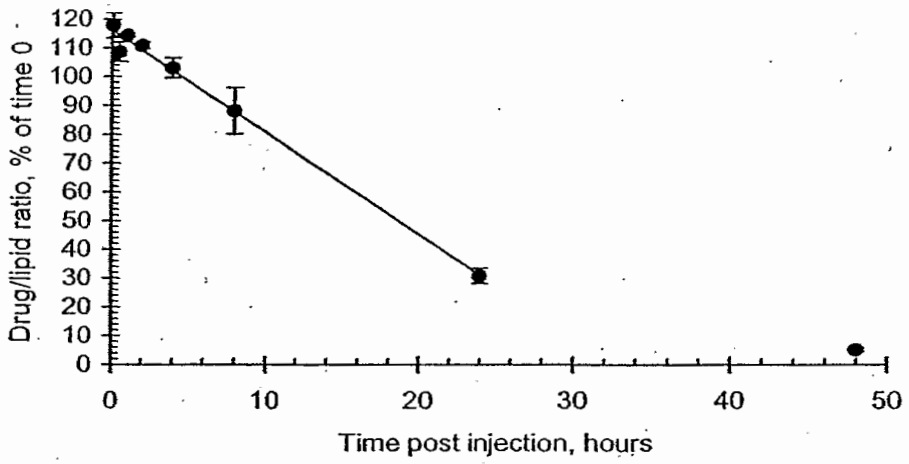

Figure 2.

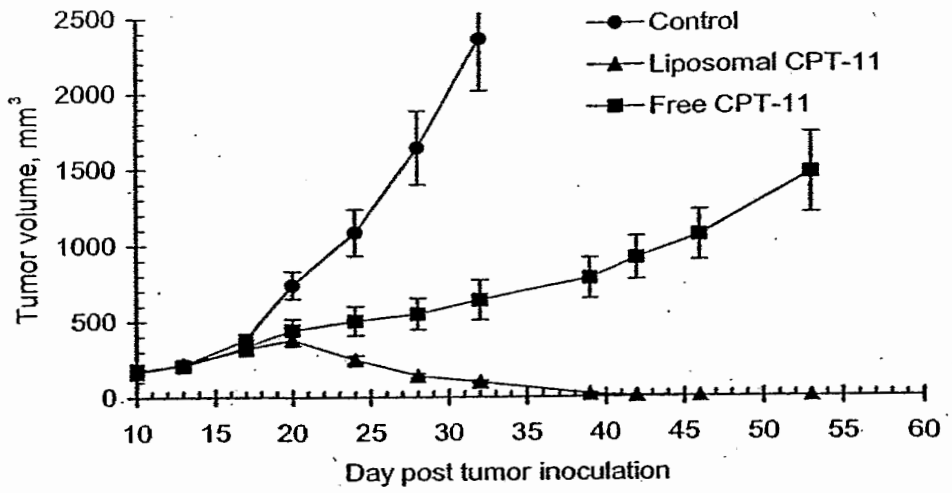

Figure 3

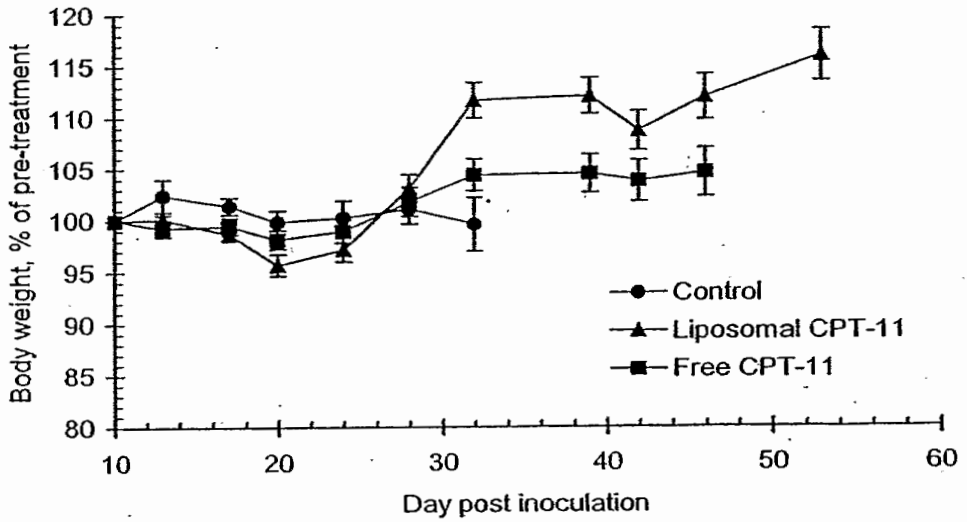

Figure 4

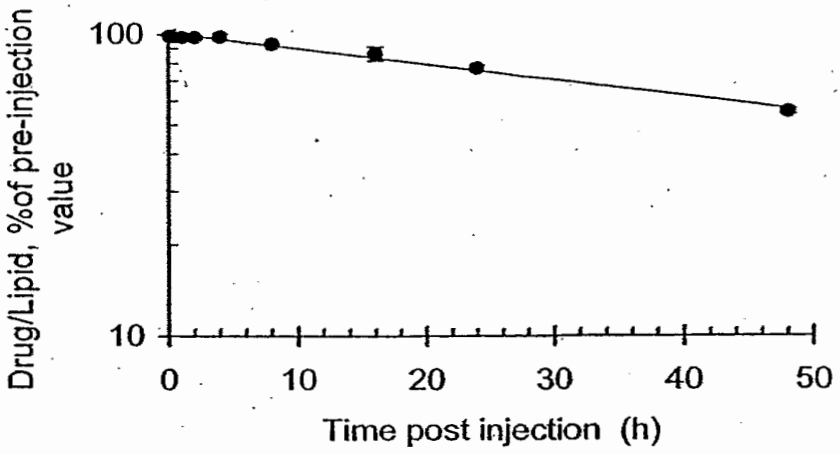

Figure 5

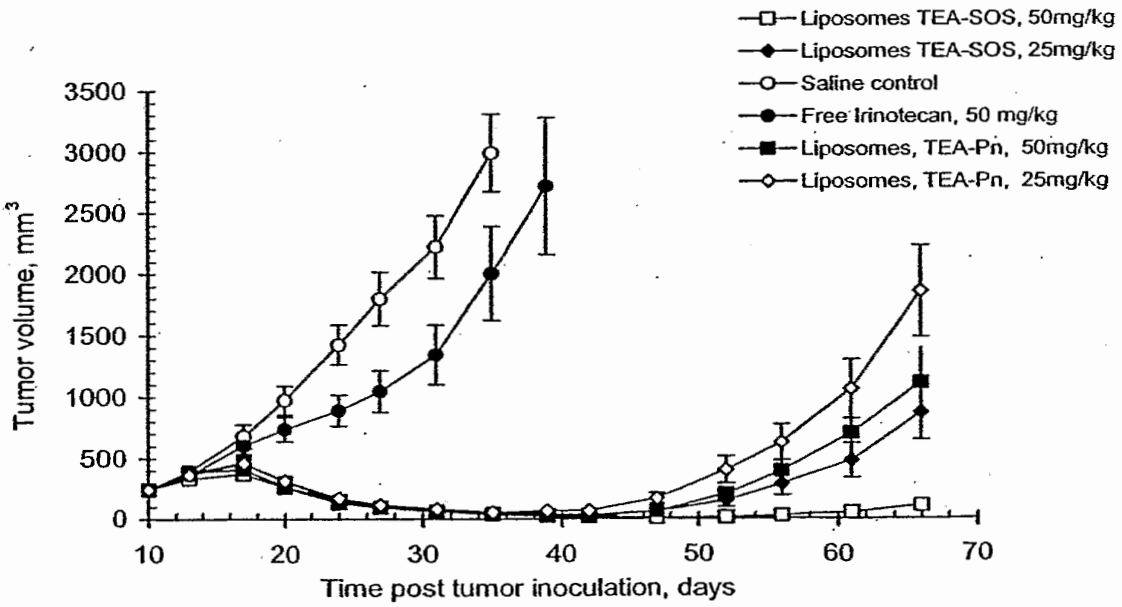

Figure 6

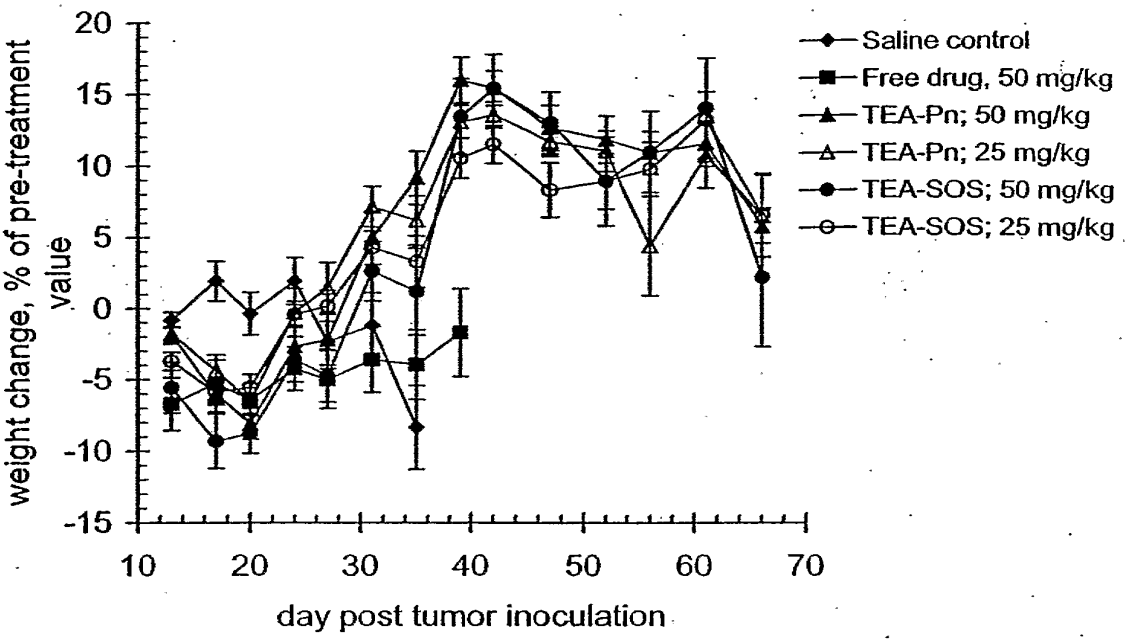
weight change, % of pre-treatment value
20
15
10
5
0
-5
-10
-15
10
20
30
40
50
60
70
day post tumor inoculation
Saline control
Free drug, 50 mg/kg
TEA-Pn; 50 mg/kg
TEA-Pn; 25 mg/kg
TEA-SOS; 50 mg/kg
TEA-SOS; 25 mg/kg

Figure 7

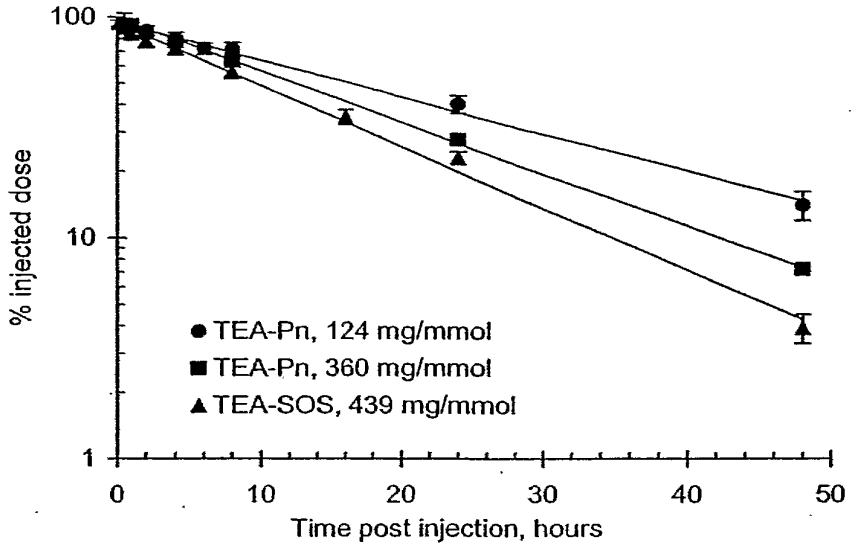

Figure 8A

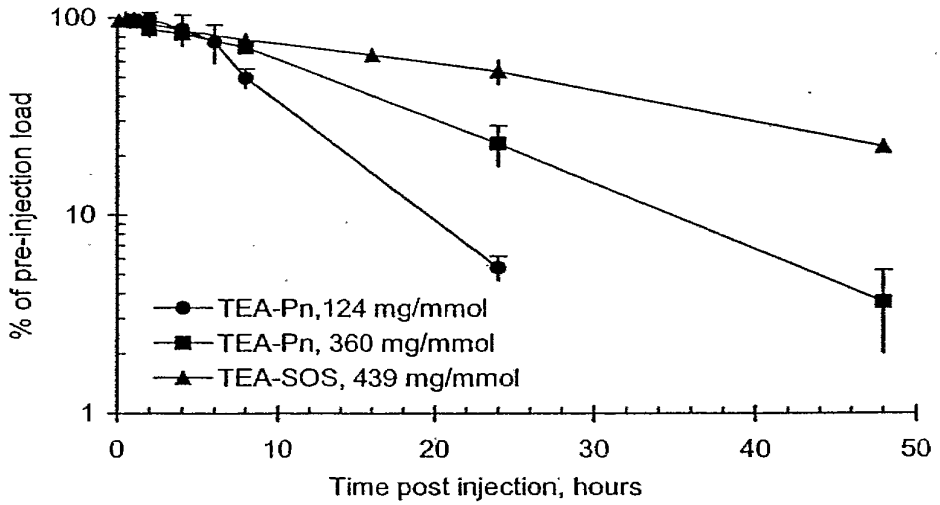

Figure 8B

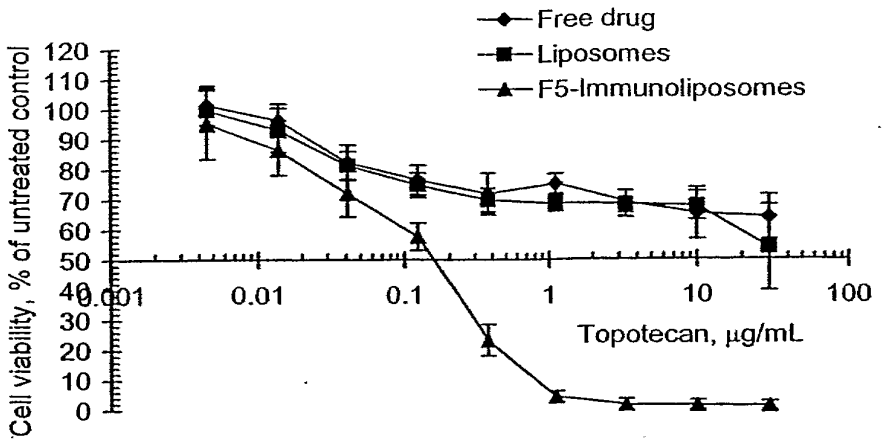

Figure 9

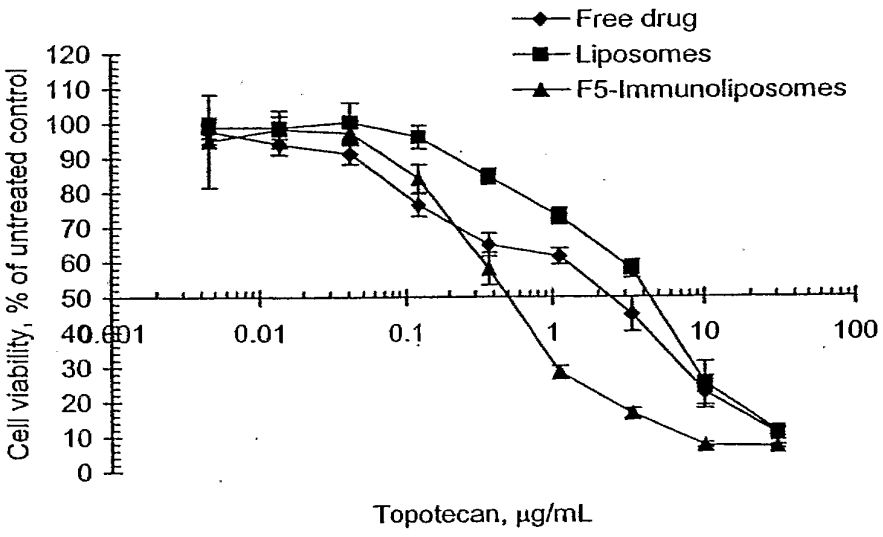

Figure 10

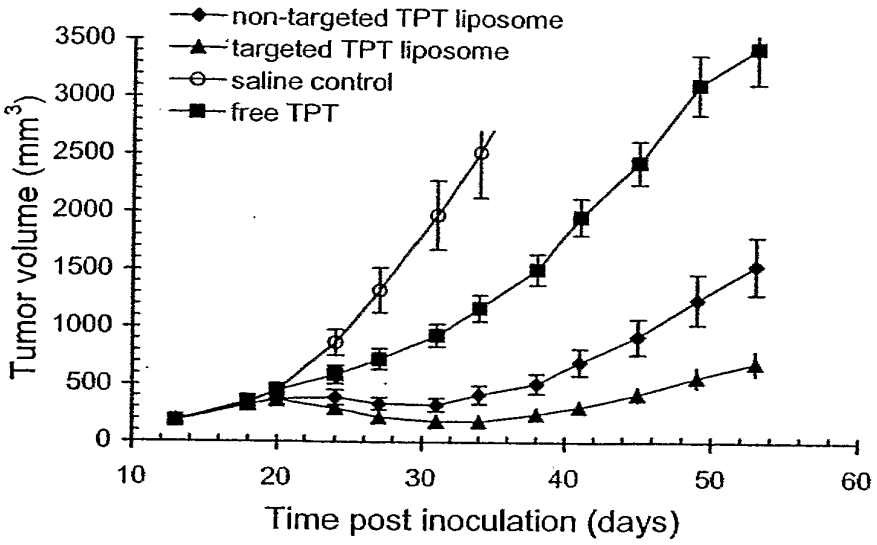

Figure 11

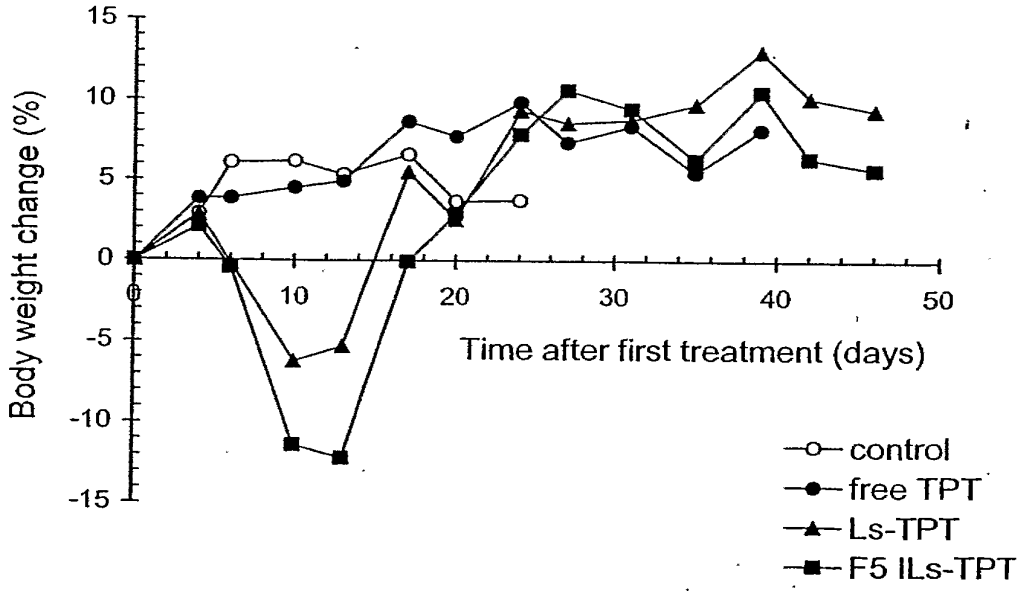

Figure 12

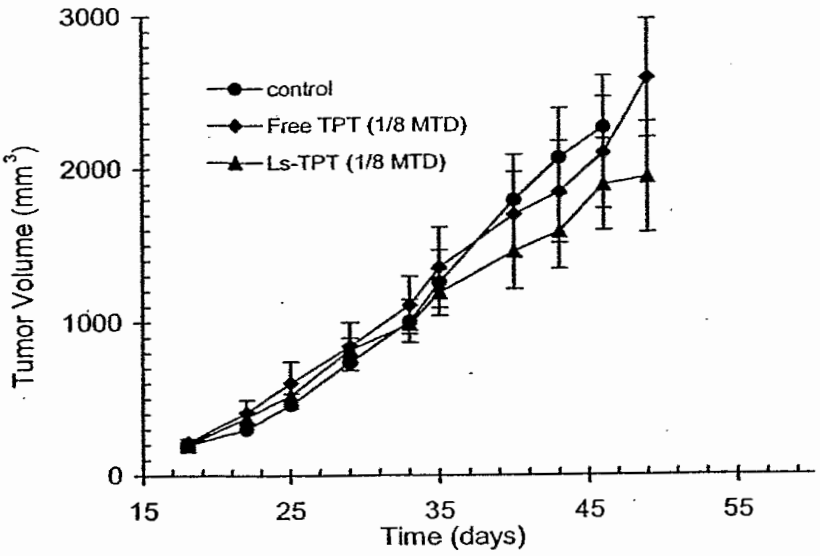

Figure 13A

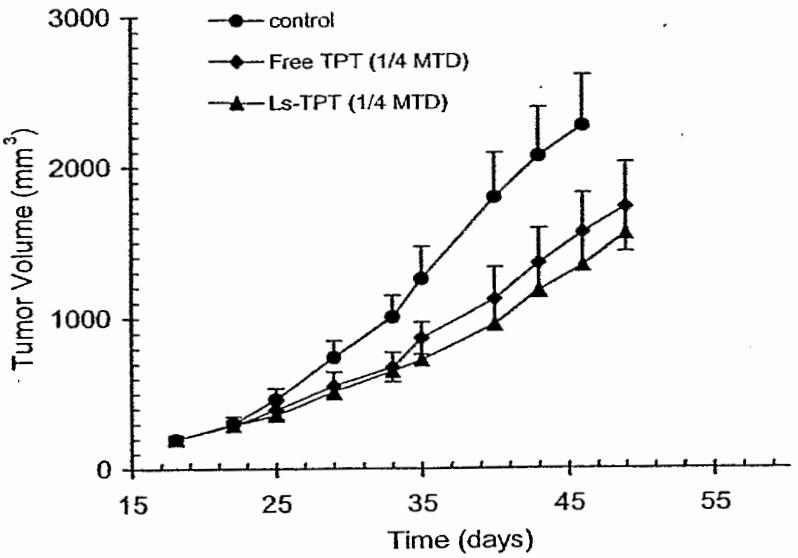

Figure 13B

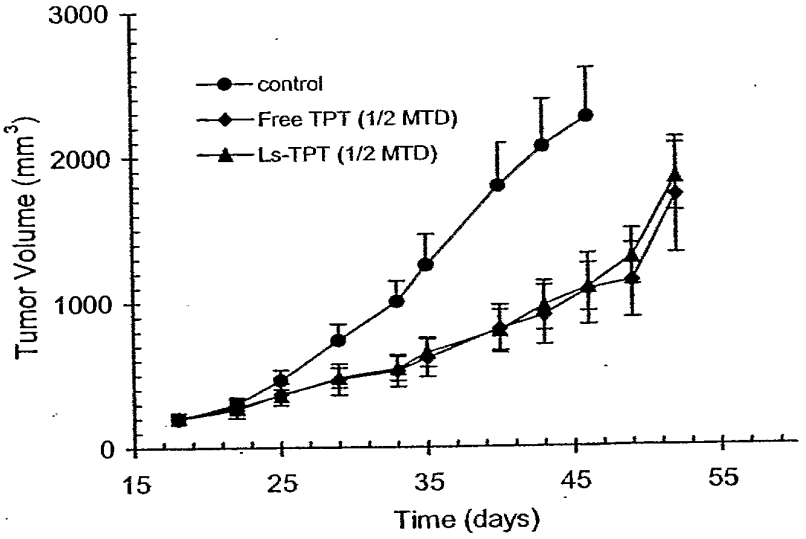

Figure 13C

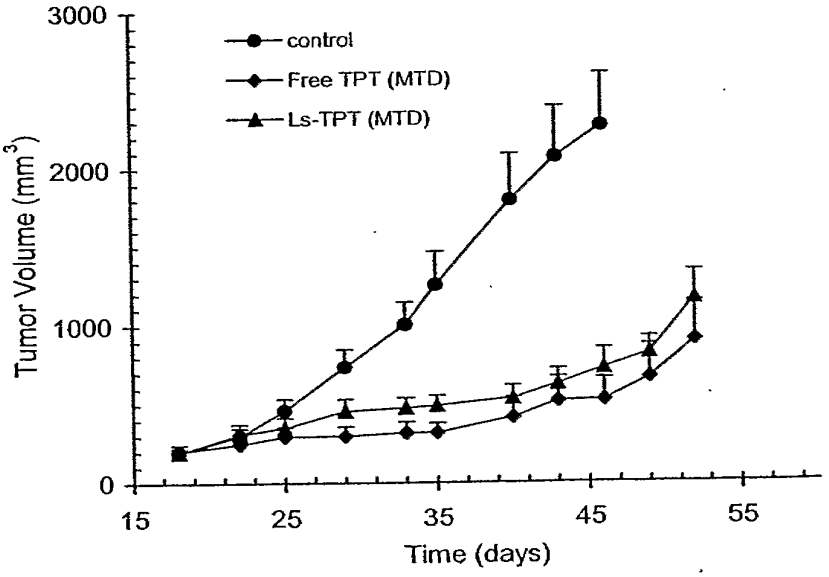

Figure 13D

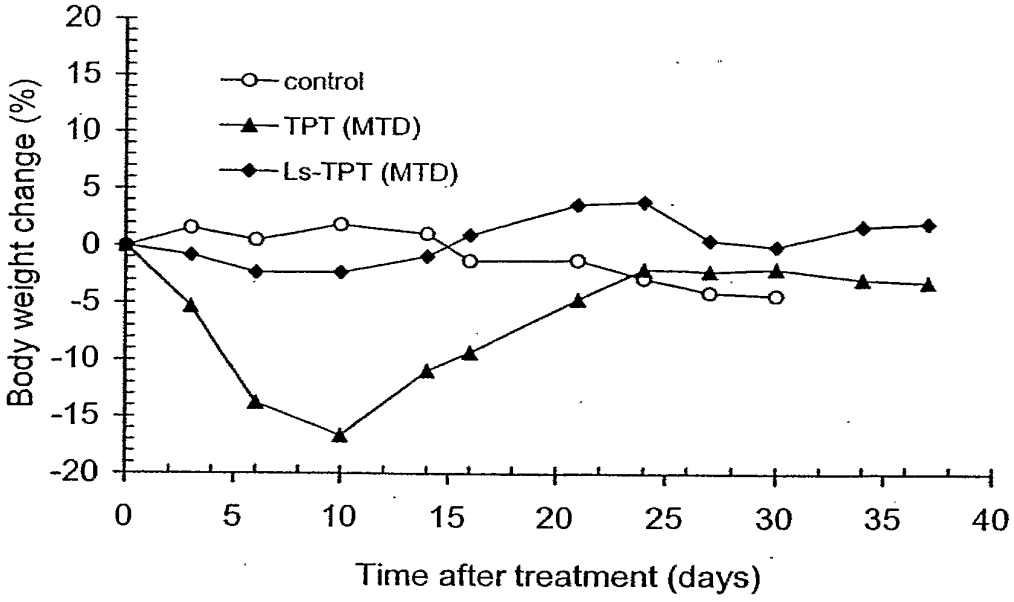

Figure 14

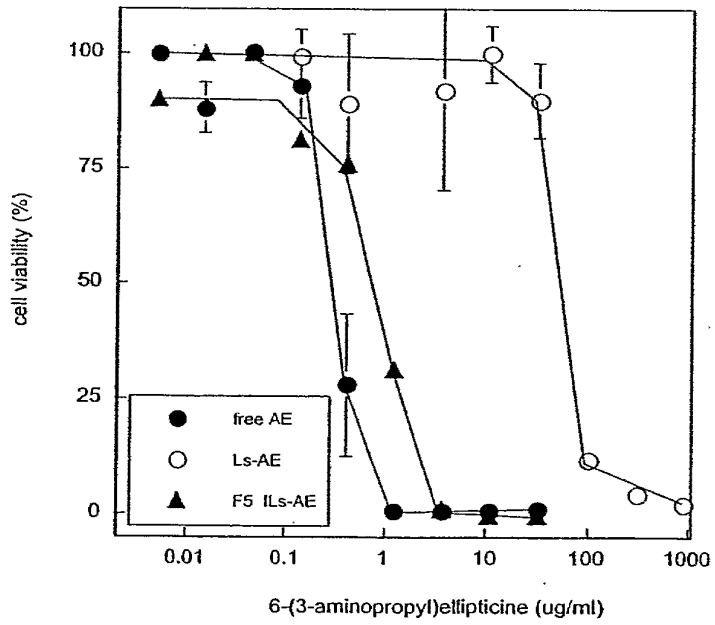

Figure 15

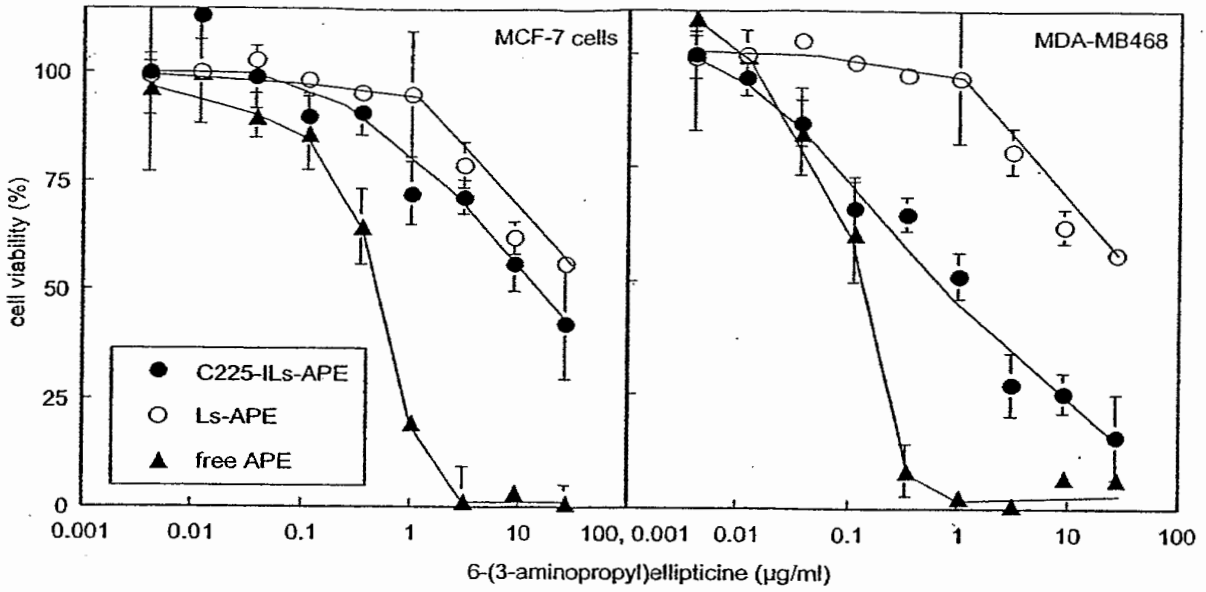

Figure 16

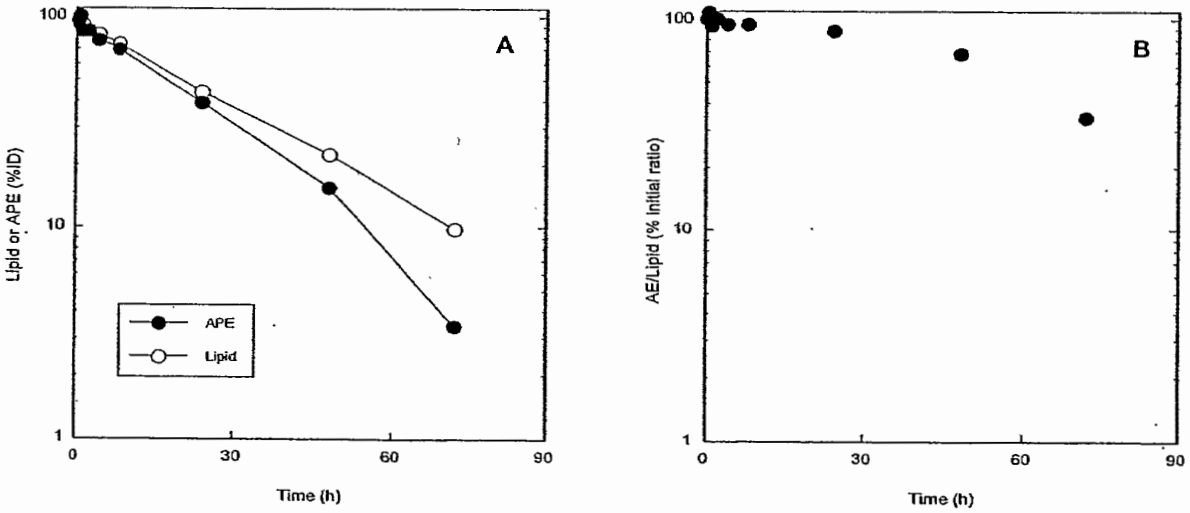

Figure 17

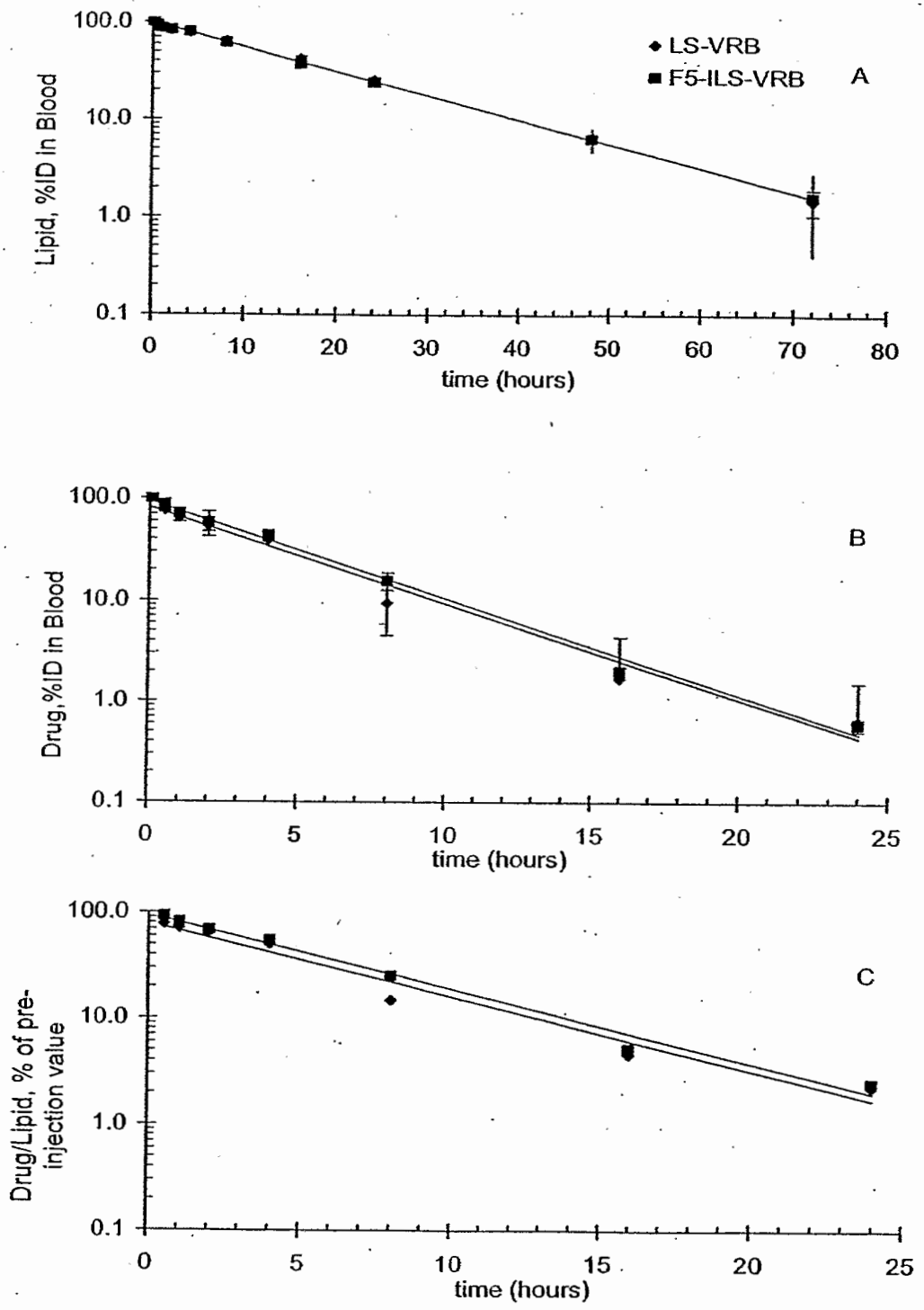
100.0
10.0
1.0
0.1
Lipid, %ID In Blood
LS-VRB
F5-ILS-VRB
A
0
10
20
30
40
50
60
70
80
time (hours)
100.0
10.0
1.0
0.1
Drug,%ID in Blood
B
0
5
10
15
20
25
time (hours)
100.0
10.0
1.0
0.1
Drug/Lipid, % of pre-injection value
C
0
5
10
15
20
25
time (hours)

Figure 18

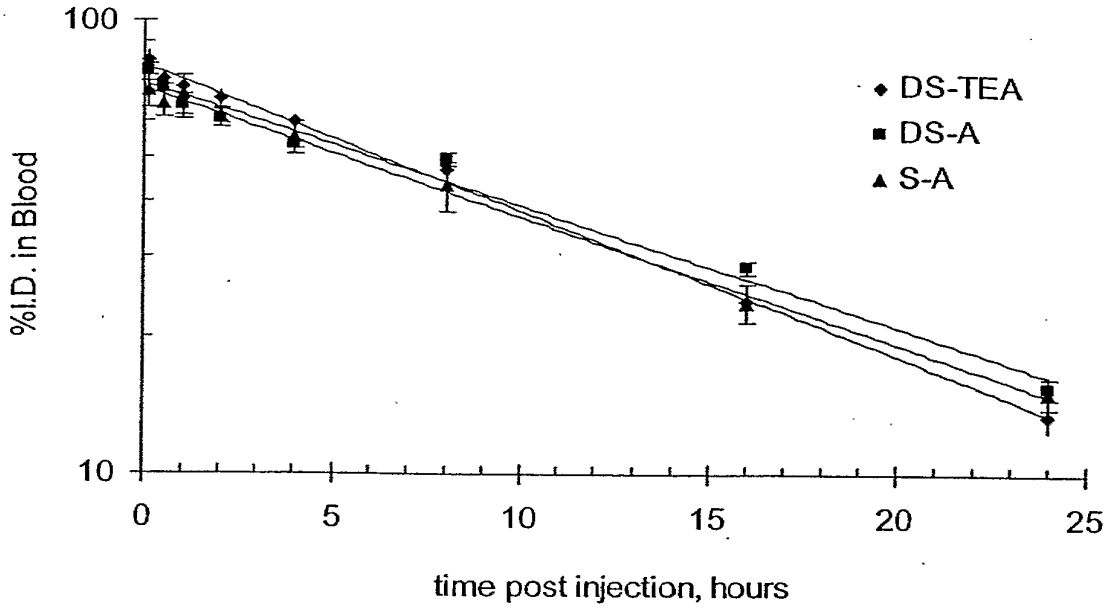

Figure 19

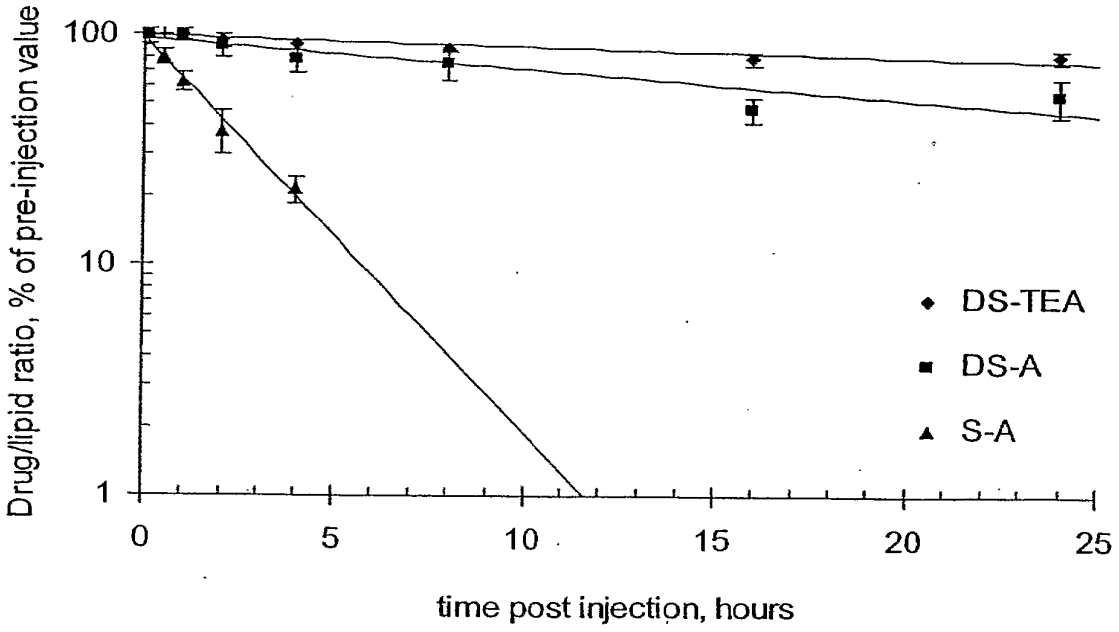

Figure 20

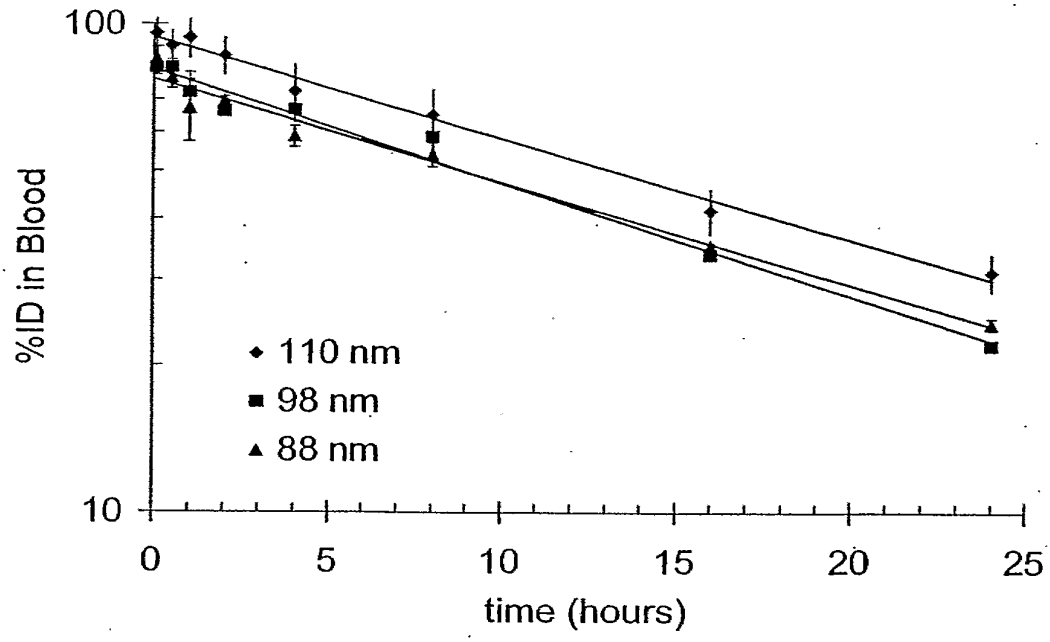

Figure 21

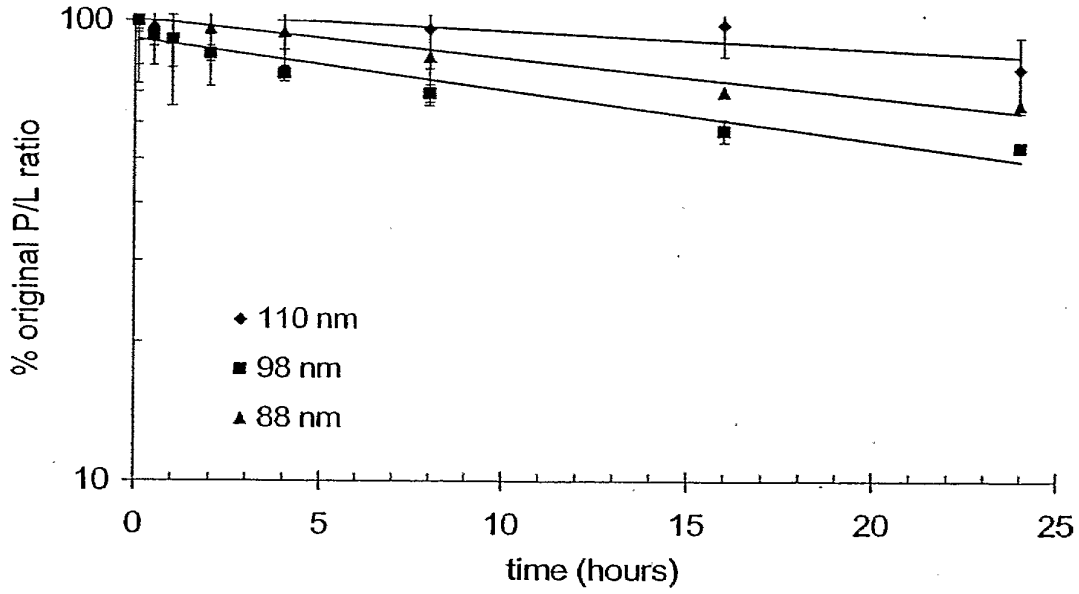

Figure 22

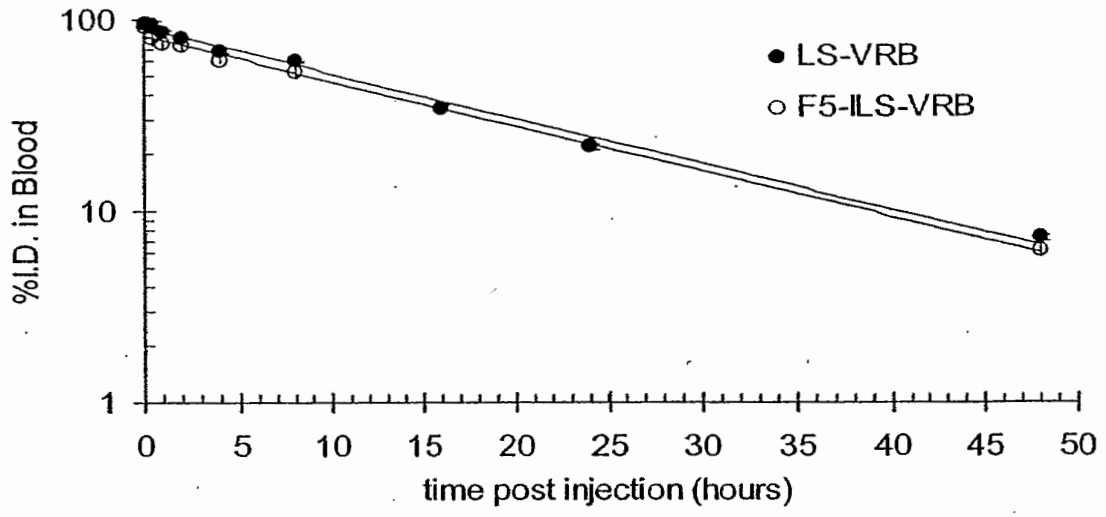

Figure 23

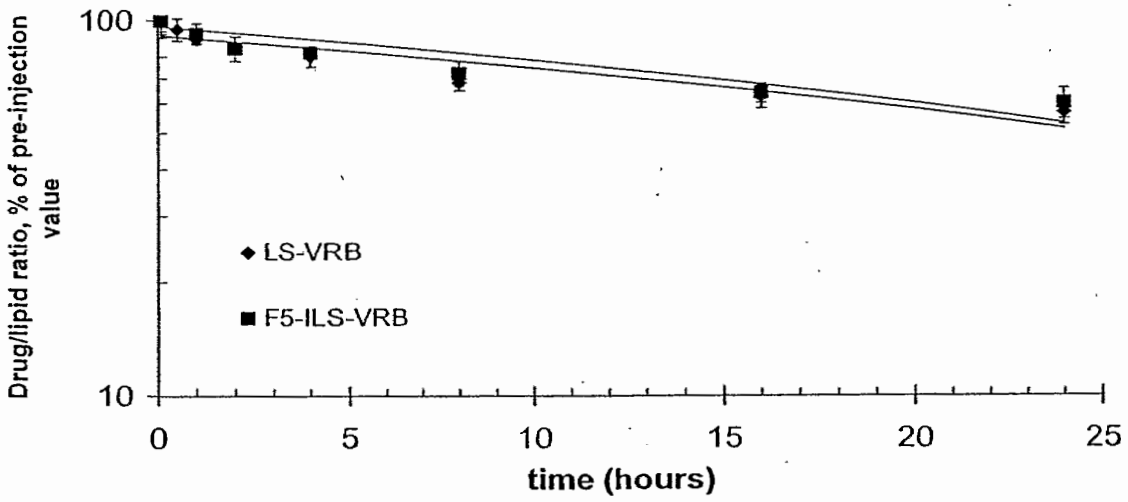

Figure 24

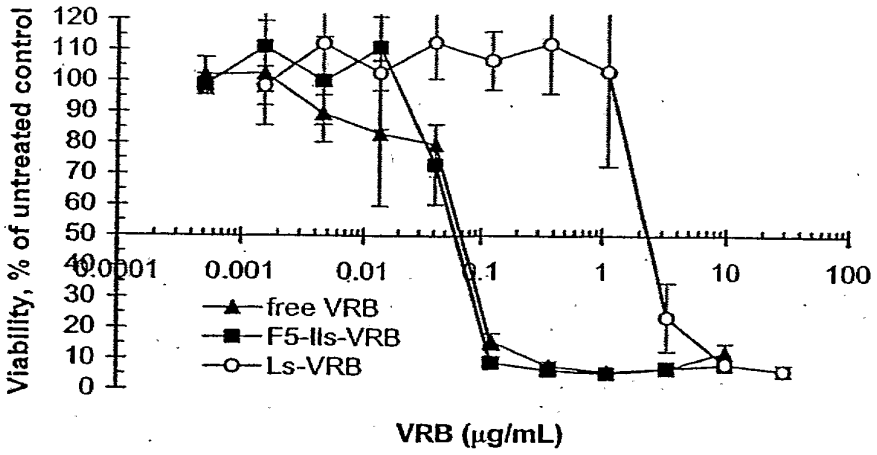

Figure 25

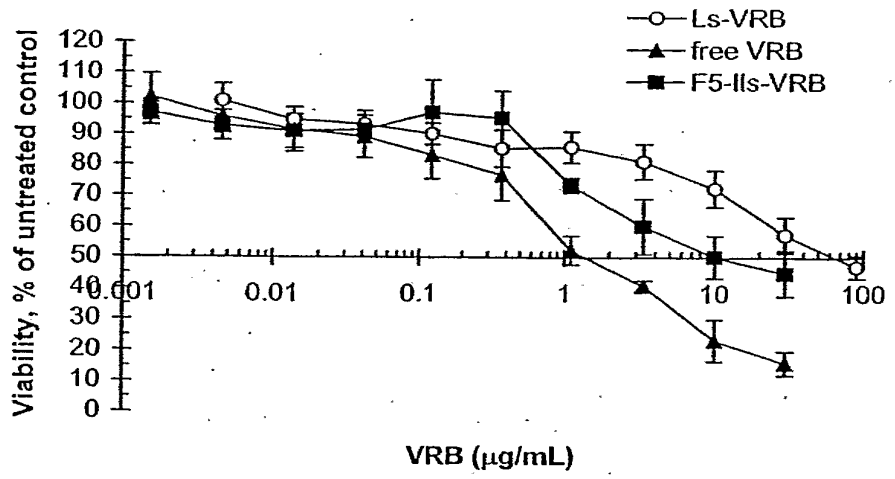

Figure 26

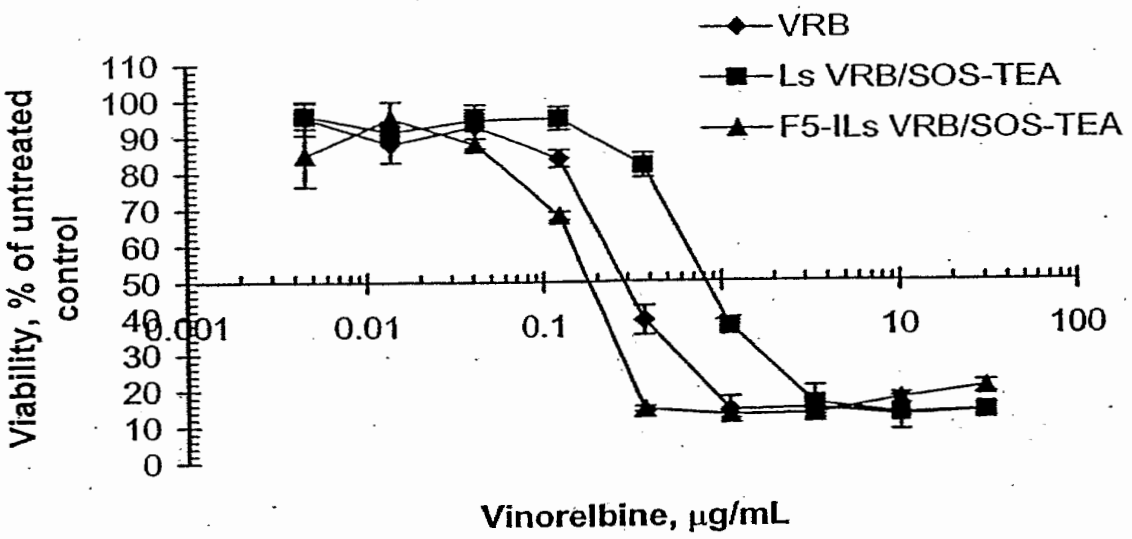

Figure 27

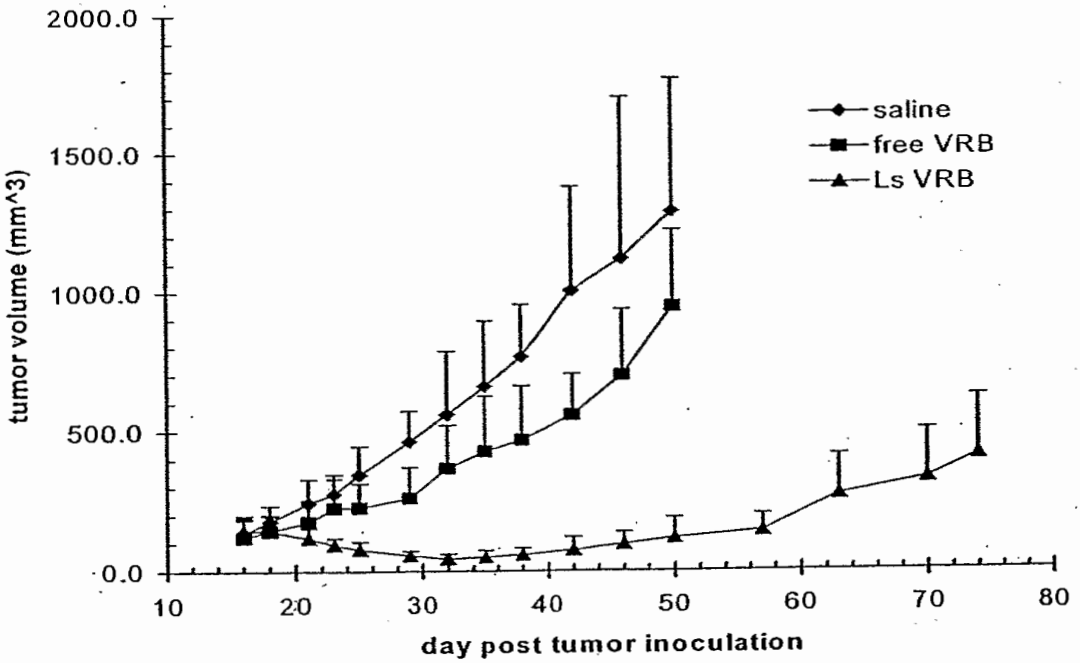

Figure 28

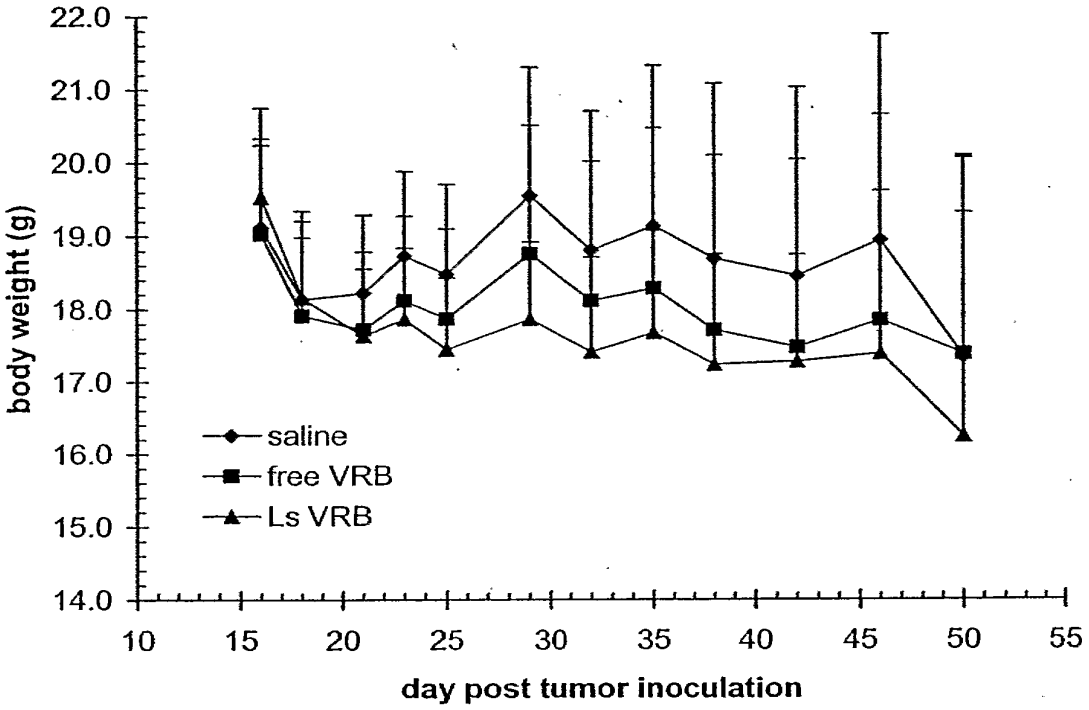
22.0
21.0
20.0
19.0
18.0
17.0
16.0
15.0
14.0
body weight (g)
saline
free VRB
Ls VRB
10
15
20
25
30
35
40
45
50
55
day post tumor inoculation

Figure 29

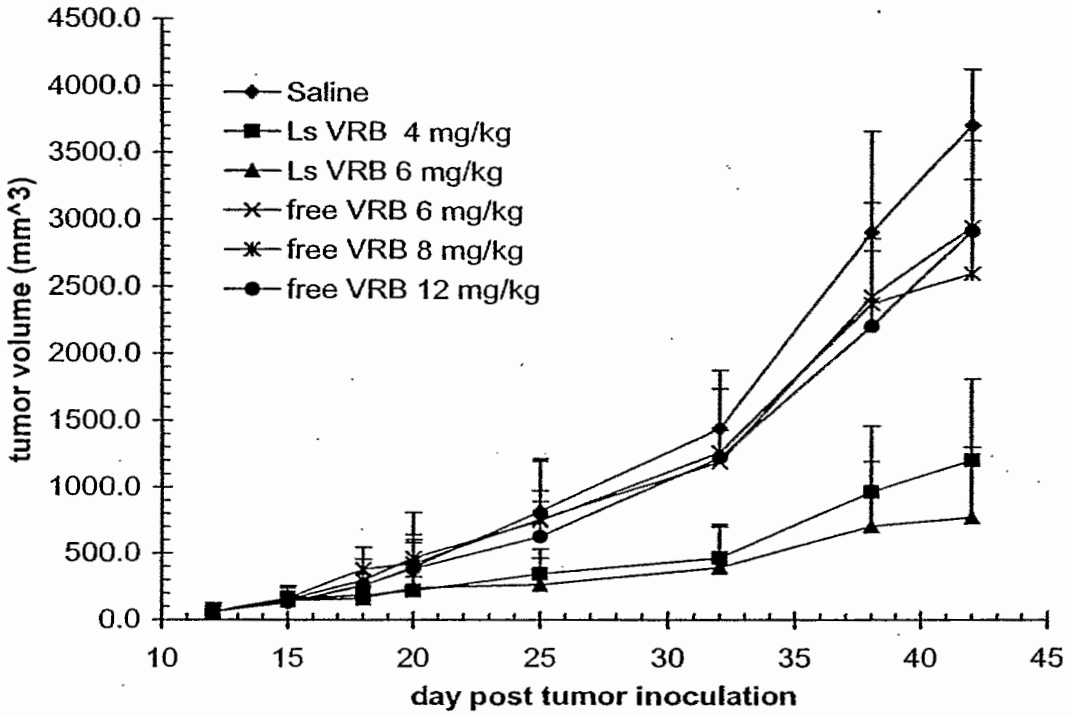
4500.0
4000.0
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
tumor volume (mm^3)
Saline
Ls VRB 4 mg/kg
Ls VRB 6 mg/kg
free VRB 6 mg/kg
free VRB 8 mg/kg
free VRB 12 mg/kg
10
15
20
25
30
35
40
45
day post tumor inoculation

Figure 30

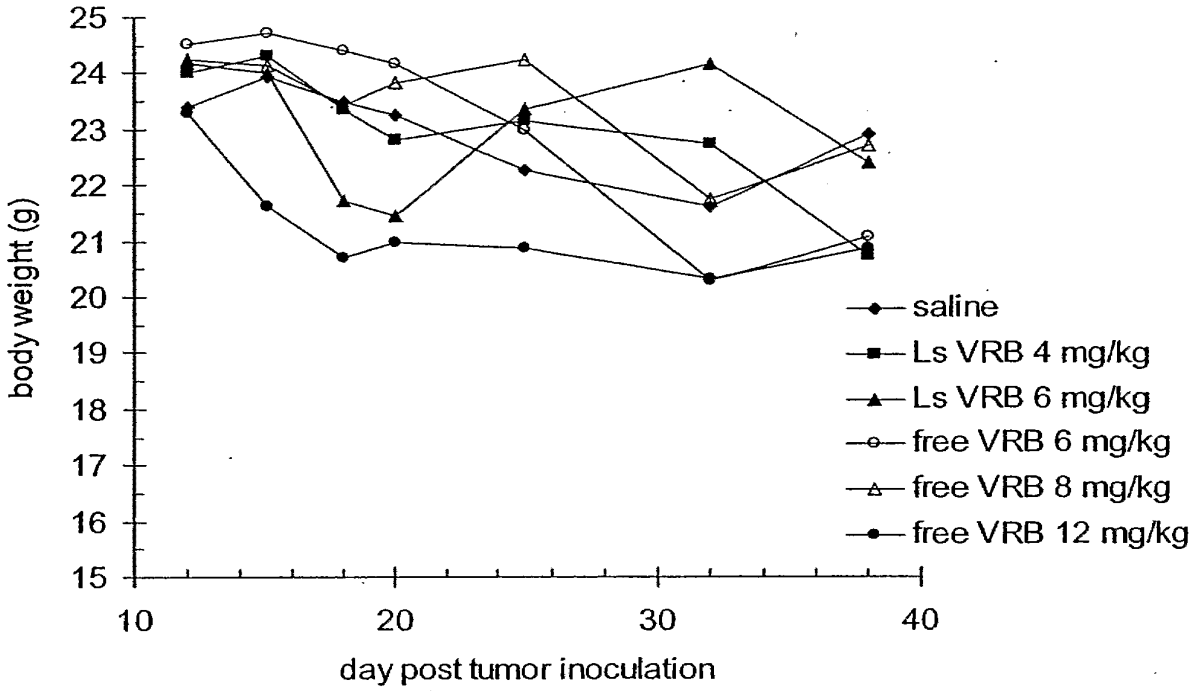
25
24
23
22
21
20
19
18
17
16
15
body weight (g)
10
20
30
40
day post tumor inoculation
saline
Ls VRB 4 mg/kg
Ls VRB 6 mg/kg
free VRB 6 mg/kg
free VRB 8 mg/kg
free VRB 12 mg/kg

Figure 31

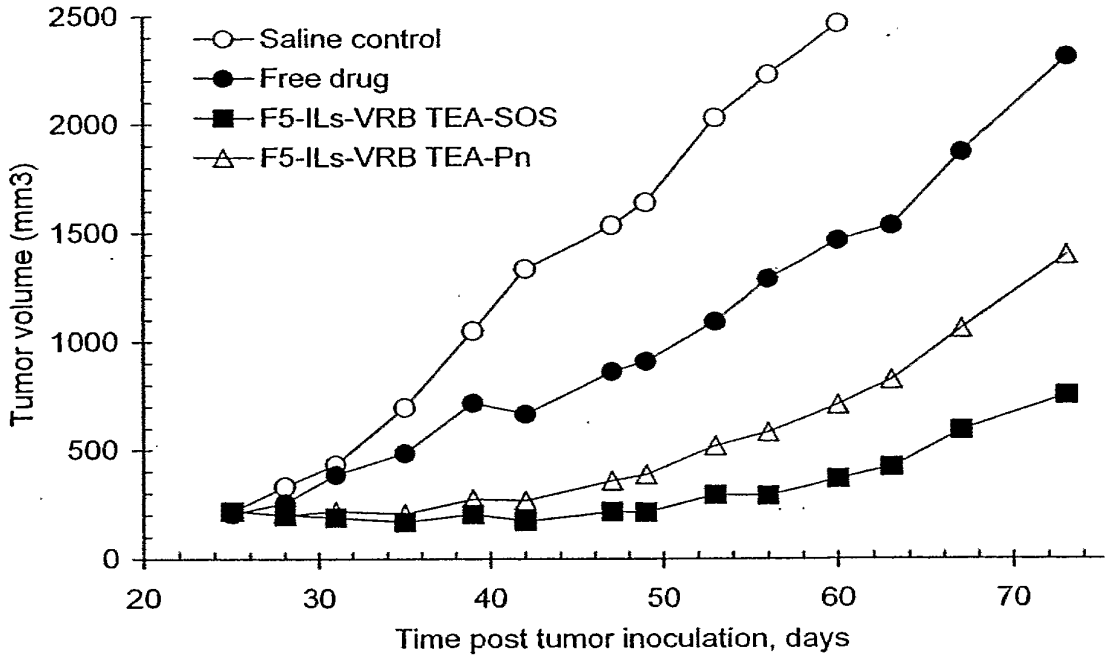

Figure 32

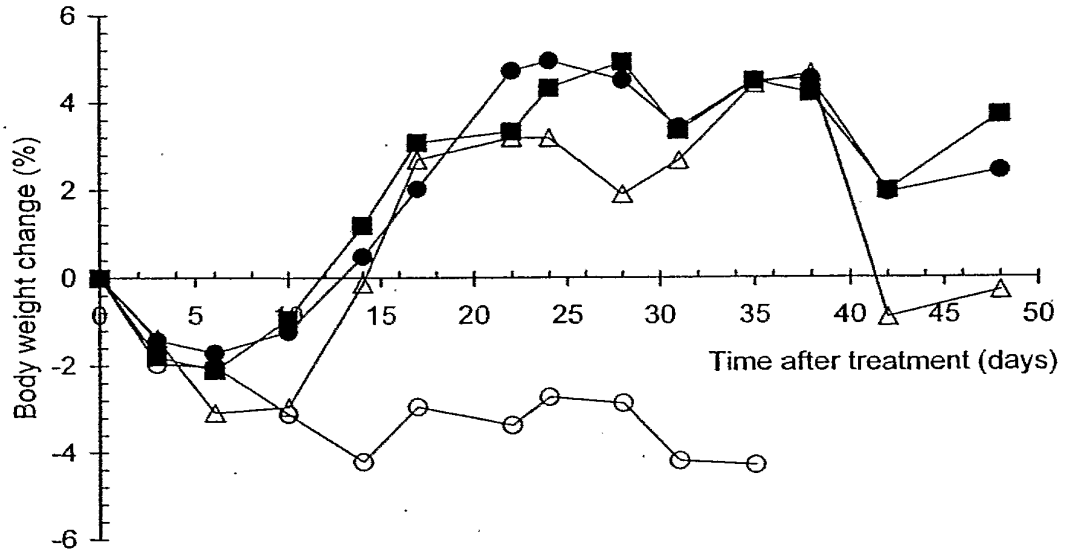

Figure 33

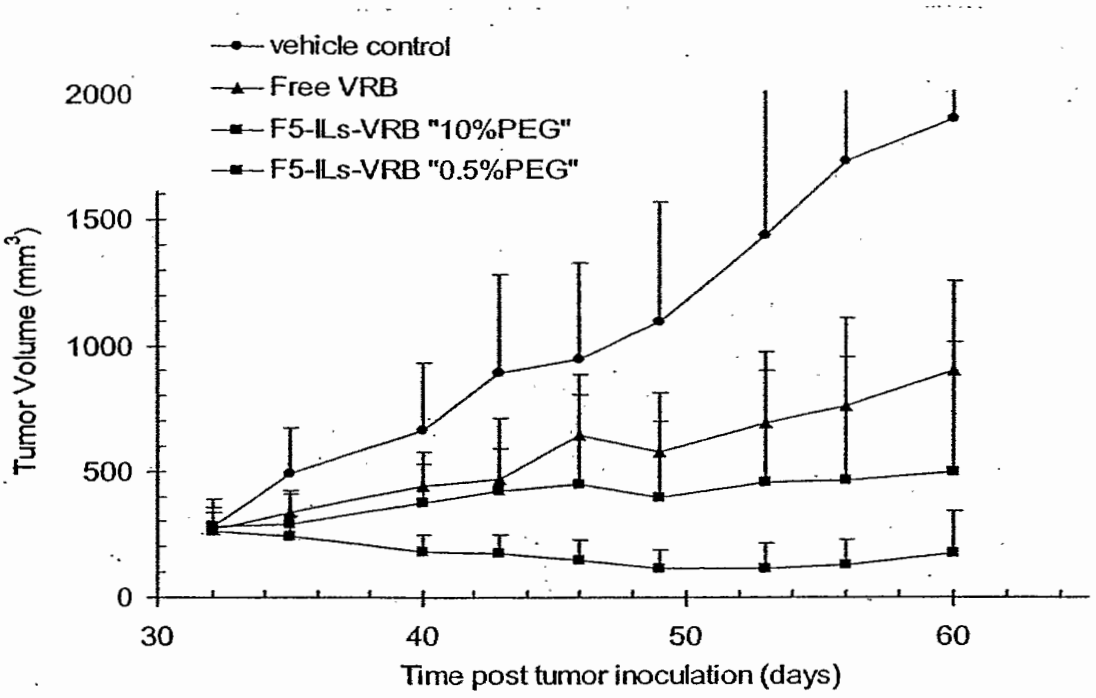

Figure 34

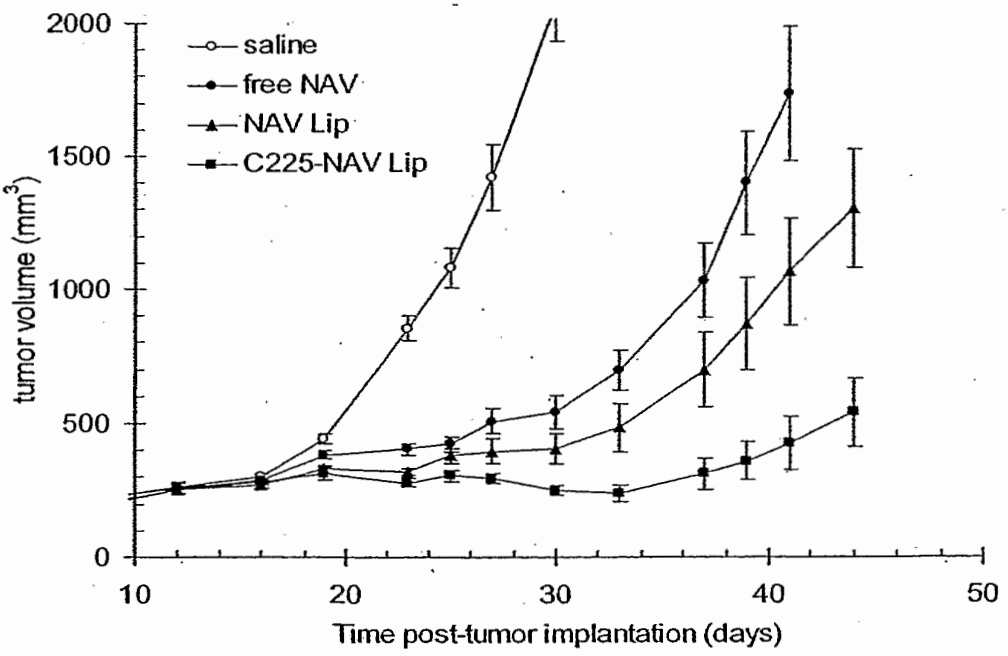

Figure 35

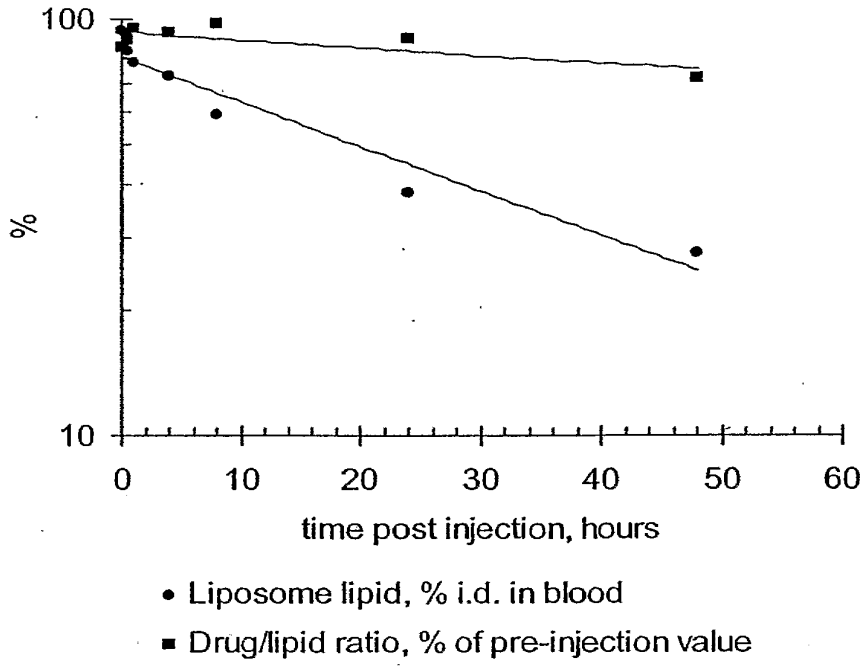
100
%
10
0
10
20
30
40
50
60
time post injection, hours
• Liposome lipid, % i.d. in blood
▪ Drug/lipid ratio, % of pre-injection value

Figure 36

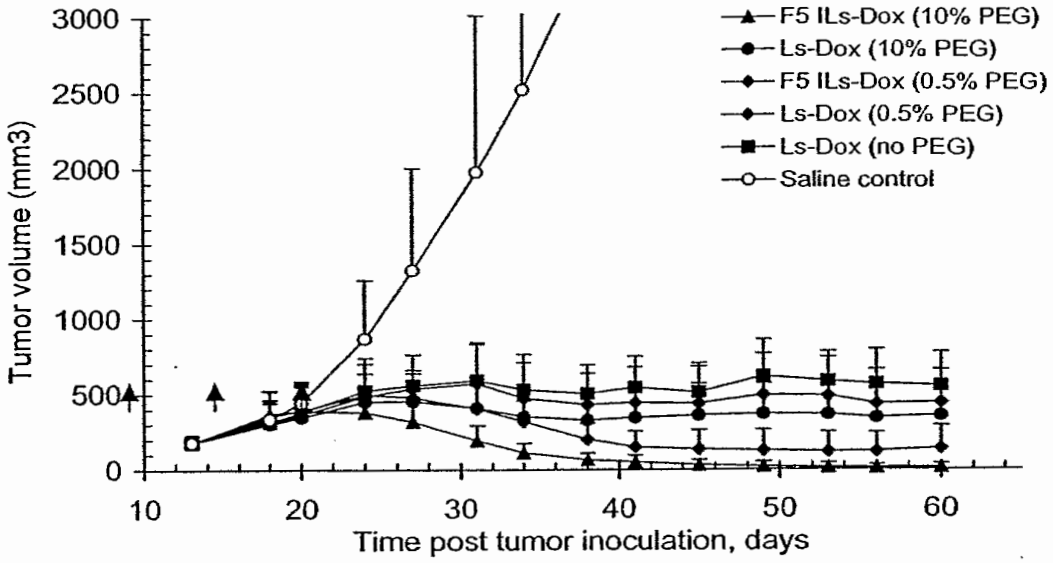

Figure 37

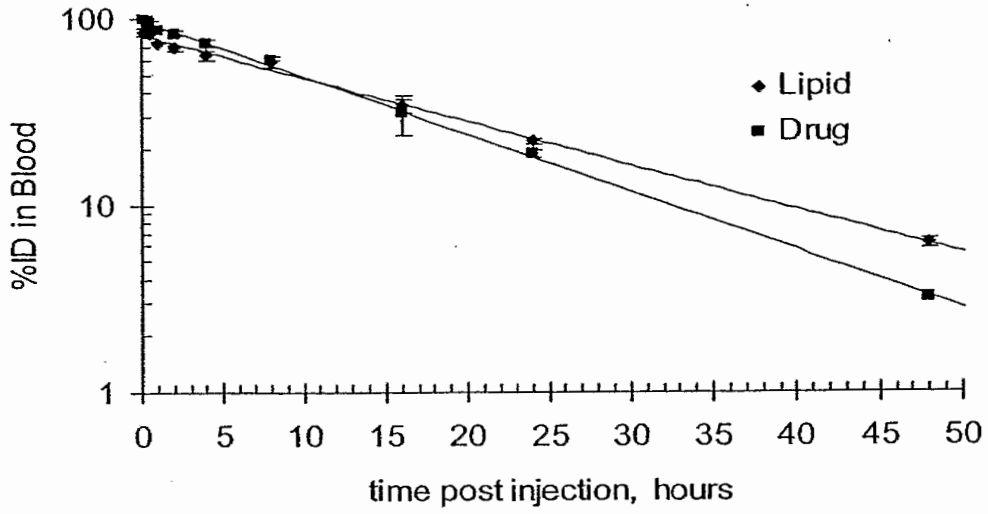

Figure 38

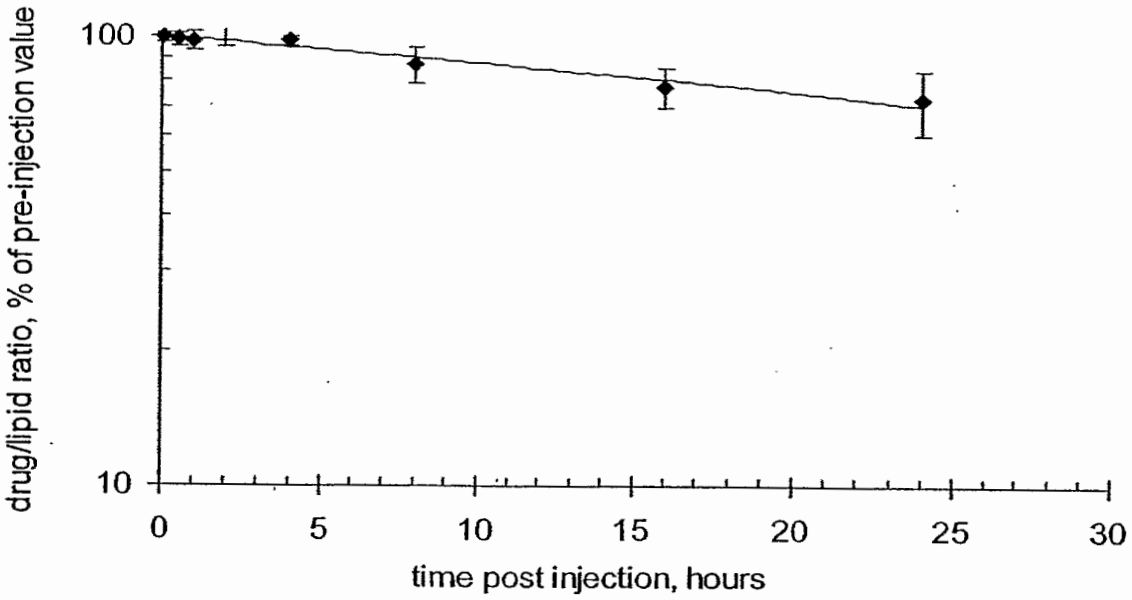

Figure 39

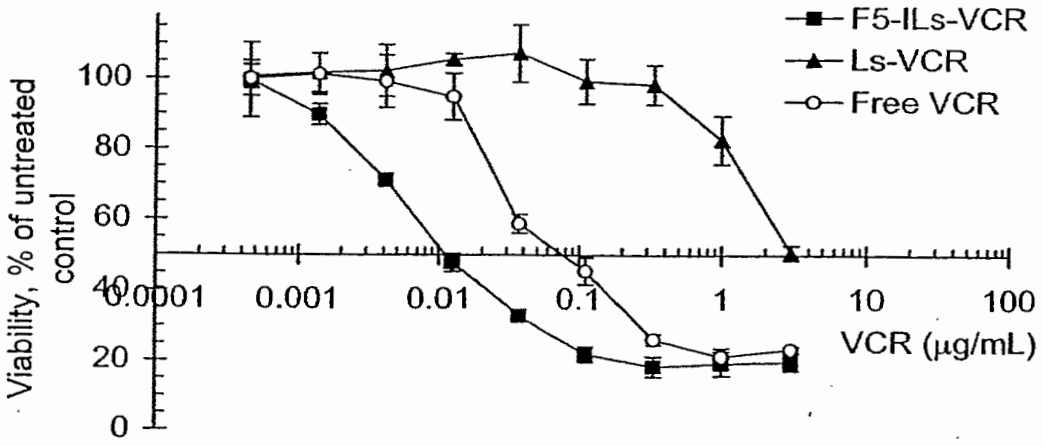

Figure 40

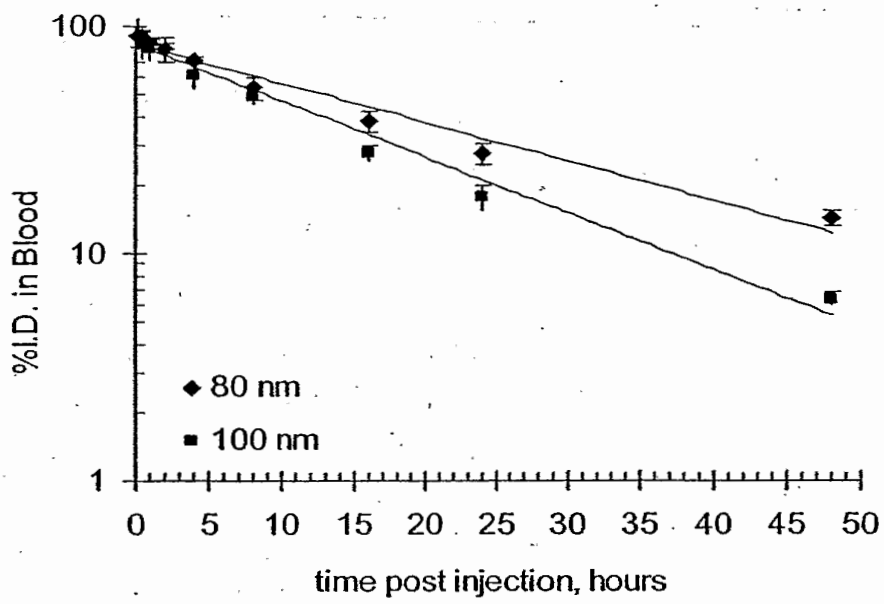

Figure 41

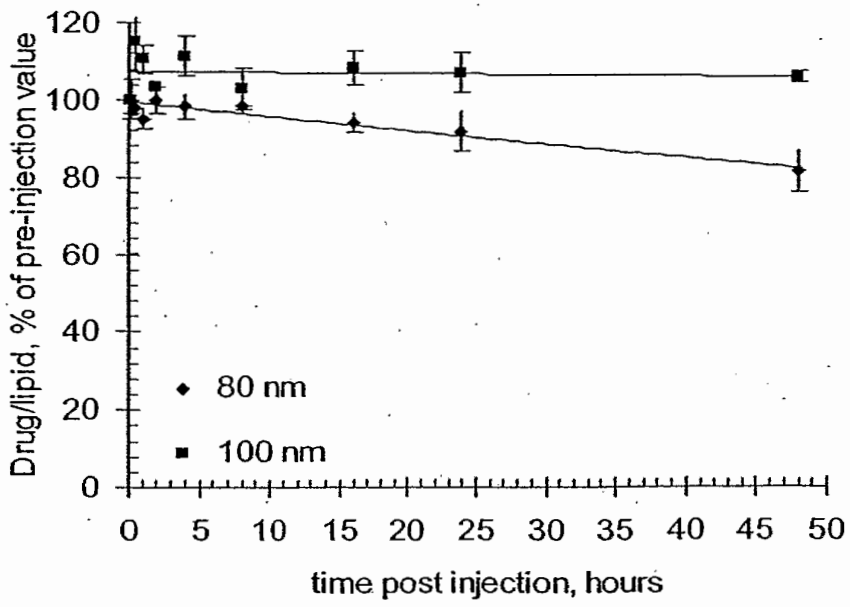

Figure 42

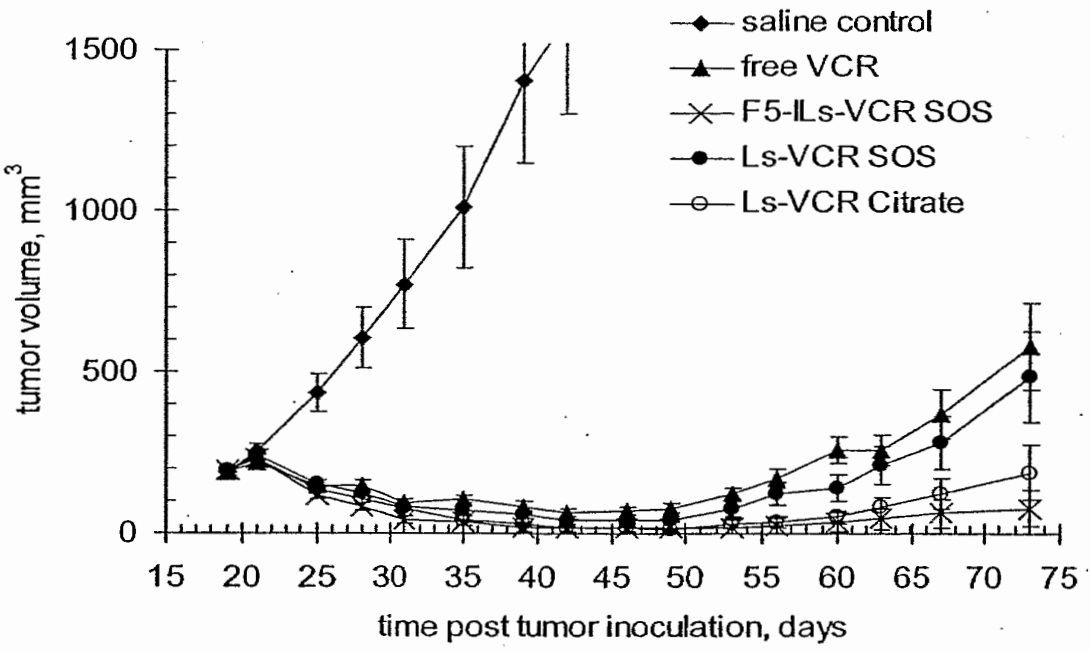

Figure 43

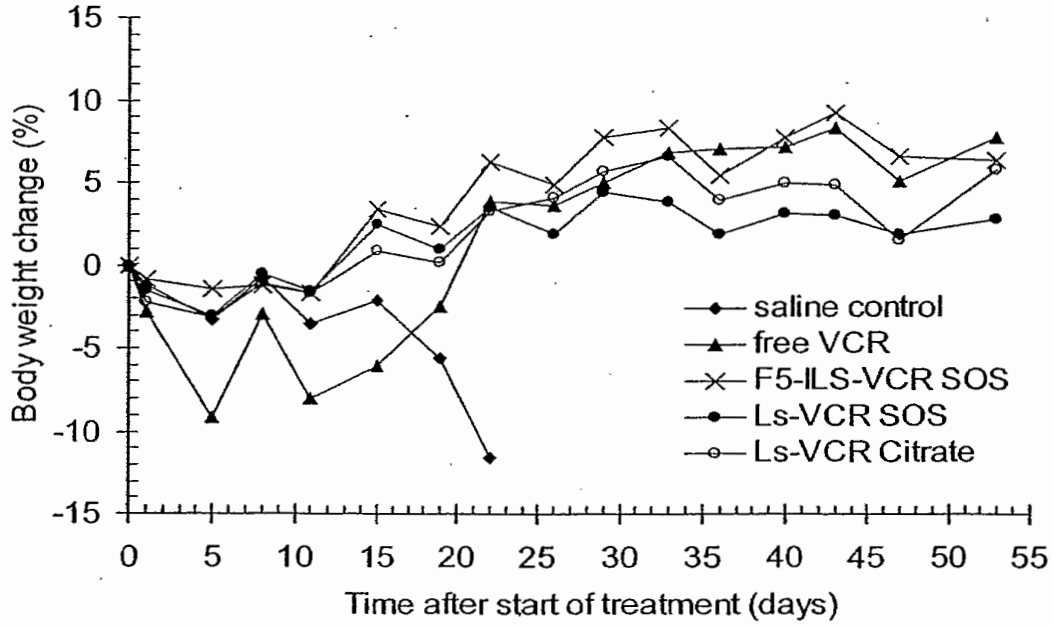

Figure 44

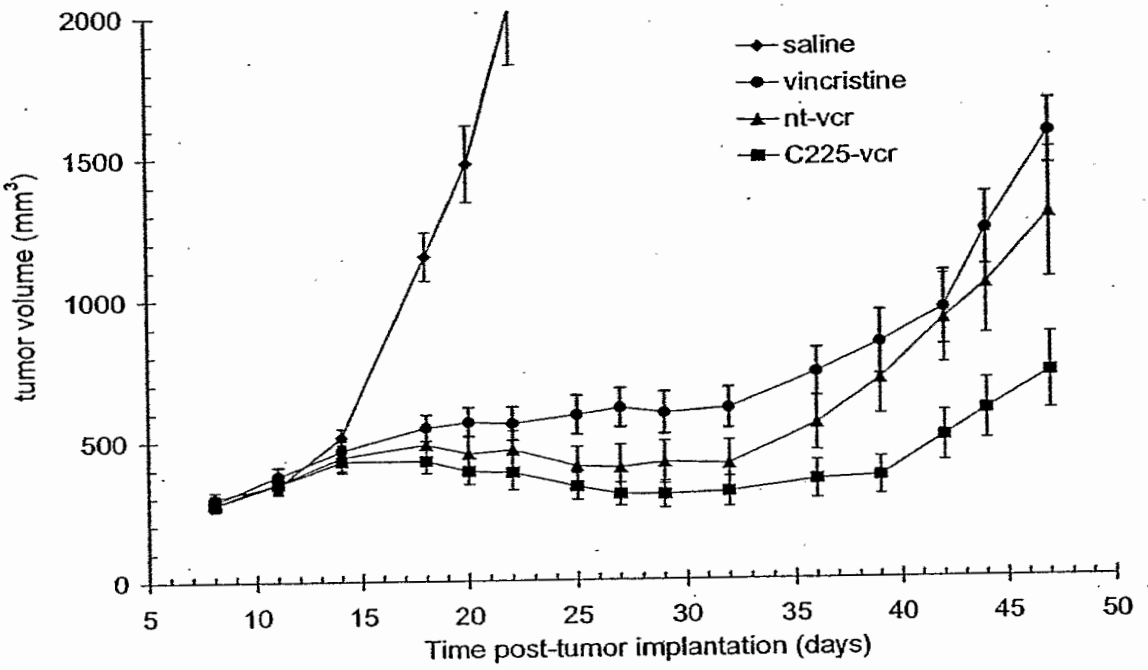
2000
1500
1000
500
0
tumor volume (mm3)
5
10
15
20
25
30
35
40
45
50
Time post-tumor implantation (days)
saline
vincristine
nt-vcr
C225-vcr

Figure 45

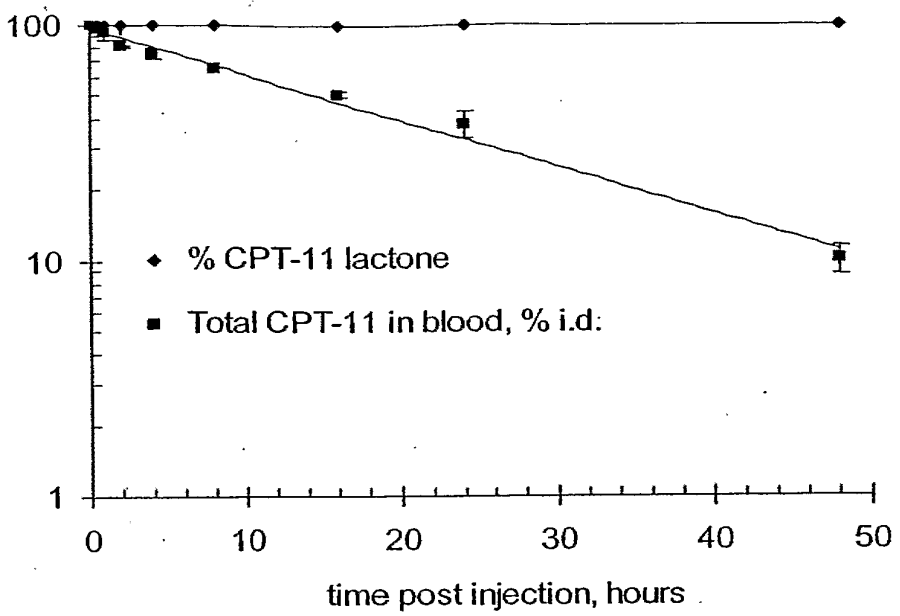

Figure 46

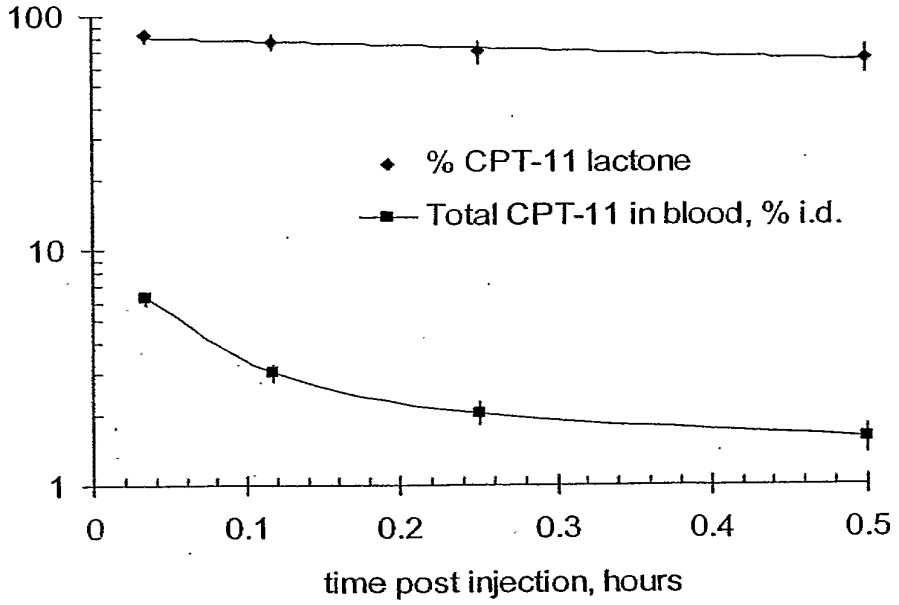

Figure 47

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US05/15349

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 9/127
US CL : 424/450
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)
U.S. : 424/450

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,785,987 A (HOPE et al) 28 July 1998 (28.07.1998) col. 4, line 50 through col. 5, line 36, col. 9, line 26 through col. 14, line 60 and Examples.	1-7, 9-12, 40-42, 57-62, 143-148 and 153 --- 8, 13-22, 43-53, 149-152 and 154-155
X --- Y	US 6,110,491 A (KIRPOTIN) 29 August 2000 (29.08.2000) col. 6, line 18 and Examples.	40-42 and 58-62 --- 1-22, 43-51, 57, 79-91 and 149-152

☐ 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

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

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Date of the actual completion of the international search
27 July 2005 (27.07.2005)

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/15349

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 23-39,54-56,63-78,92-142 and 156-159
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

Continuation of B. FIELDS SEARCHED Item 3:
West:
Search terms: liposome, triethylammonium salt, trimethylammonium salt, ammonium salt

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JOURNAL OF LIPOSOME RESEARCH, 12(1&2), 1–3 (2002)

THE NEXT GENERATION OF LIPOSOME DELIVERY SYSTEMS: RECENT EXPERIENCE WITH TUMOR-TARGETED, STERICALLY-STABILIZED IMMUNOLIPOSOMES AND ACTIVE-LOADING GRADIENTS

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ABSTRACT

Three topics are discussed. Enhanced anti-tumor efficacy of targeted doxorubicin-containing sterically-stabilized liposomes using an anti-β_1 integrin Fab' ligand. Use of tumor targeting with an internalizing ligand to improve the efficacy of a non-leaky cisplatin-containing sterically-stabilized liposome formulation. Formulation variables (remote-loading with dextran ammonium sulfate, rigid lipid bilayer) used to optimize in vivo performance of a liposomal camptothecin analog.

Conventional liposome formulations represent the 'first generation' of liposome delivery systems, the introduction of sterically-stabilized liposomes exhibiting prolonged in vivo circulation times and 'passive' targeting launched the 'second generation', and sterically-stabilized immunoliposomes provided a 'third generation' improvement. In addition, the ability to actively load suitable drugs into liposomes using chemical gradients allowed the creation of formulations with high drug-to-lipid ratios.[1,2]

These improvements have enabled liposome formulations to meet criteria for successful products: adequate drug loading in a stable vehicle, prolonged in vivo half-life enabling extravasation at sites having compromised capillary beds (tumor) and active delivery to target cells.[2] The following examples illustrate the challenges encountered working with specific drugs and targeting systems.

Remote-Loaded, Sterically-Stabilized Immunoliposomes Containing Doxorubicin

DOXIL® (doxorubicin HCl) liposomes were converted into immunoliposomes by inserting the targeting ligand covalently bound to maleimide PEG-DSPE (in the form of micelles). The ligand used was an anti-β_1 integrin Fab'; the β_1 integrins are expressed on the majority of human non-small cell lung carcinomas. When a lung tumor xenograft model in SCID mice was treated with these immunoliposomes, significant suppression of tumor growth and metastatic spread was demonstrated versus controls.[3]

Passively-Loaded, Sterically-Stabilized Immunoliposomes Containing Cisplatin

A sterically-stabilized liposome formulation encapsulating cisplatin exhibited enhanced activity compared to cisplatin alone in a variety of preclinical murine tumor models. However, this formulation lacked efficacy in pilot clinical studies. Using a microdialysis technique to evaluate unbound platinum in the extracellular fluid of a mouse tumor model revealed that the liposomes were entering the tumor but releasing less platinum into the extracellular fluid than was detected on dosing with cisplatin alone. This formulation was clearly retaining cisplatin too avidly after reaching the tumor site, consequently too little active drug reached tumor cells. The formulation was converted into an immunoliposome and tested in the murine lung tumor xenograft model described above. The targeted cisplatin-liposomes were significantly more effective than conventional liposomes against both primary and metastatic tumors. The ability of the targeting ligand to facilitate internalization of bound liposomes allowed intracellular delivery of the liposome payload and enhanced the efficacy of this formulation.

Remote-Loading and Formulation Variables Affecting Performance

The following example illustrates tailoring a sterically-stabilized formulation to optimize its drug release rate profile. Initial attempts to formulate a

camptothecin analog into the DOXIL® (doxorubicin HCl) liposome matrix resulted in inferior pharmacokinetic and efficacy performance. Re-formulating; creating a more rigid bilayer (DSPC/MPEG-DSPE = 95 : 5 versus HSPC/MPEG-DSPE/CH = 56 : 5 : 39 molar ratio) and providing a more powerful active-loading battery (100 mg/mL dextran ammonium sulfate versus 250 mM ammonium sulfate) resulted in a formulation having the desired circulation half-life together with promising efficacy in a mouse xenograft tumor model. Physical/chemical properties must be taken into account when optimizing drug delivery.

REFERENCES

1. Allen, T.M.; Hansen, C.B.; Stuart, D.D. Targeted Sterically Stabilized Liposomal Drug Delivery. In *Medical Applications of Liposomes*, Lasic, D., Papahadjopoulos, D., Eds.; Elsevier Science B.V., New York, 1998; pp. 297–323.
2. Barenholz, Y. Liposome Application: Problems and Prospects. Current Opinion in Colloid and Interface Science **2001**, *6*, 66–77.
3. Sugano, M.; Egilmez, N.K.; Yokota, S.J.; Chen, F.-A.; Harding, J.; Huang, S.K.; Bankert, R.B. Antibody Targeting of Doxorubicin-Loaded Liposomes Suppresses the Growth and Metastatic Spread of Established Human Lung Tumor Xenografts in Severe Combined Immunodeficient Mice. Cancer Research **2000**, *60*, 6942–6949.

A Phase I/II Study of Trifluridine/Tipiracil (TAS-102) in Combination with Nanoliposomal Irinotecan (NAL-IRI) in advanced GI cancers

Olatunji B. Alese, Walid L. Shaib, Christina Wu, Mehmet Akce, Bassel F. El-Rayes

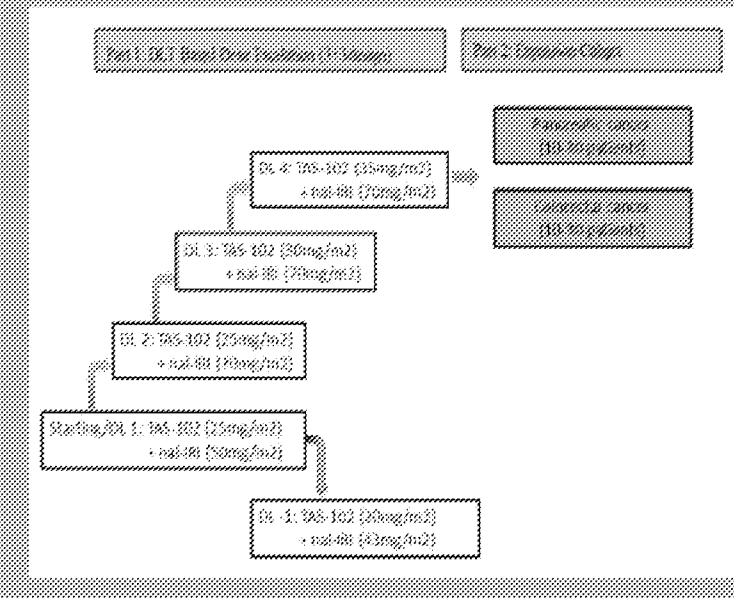
Winship Cancer Institute of Emory University, Atlanta, GA

Introduction

- Each year, an estimated 300,000 people are diagnosed with GI cancers, with over 150, 000 people dying annually.
- CRC and pancreatic cancers are the second and fourth leading causes of cancer death in both sexes the US
- Trifluridine/tipiracil (FTD/TPI, TAS-102) is a combination of a nucleoside analogue and a thymidine phosphorylase inhibitor. TAS-102 has shown activity in 5FU-resistant colorectal cancer (CRC).
- Nano liposomal-Irinotecan (Nal-IRI, Onivyde) achieves higher intra-tumor concentrations than irinotecan (142-fold) and its major metabolite, SN-38 (9-fold), resulting in superior anti-tumor activity compared to free irinotecan in multiple tumor xenografts.
- Clinical trials have established the activity of Nal-IRI combined with 5FU in pancreatic cancer.
- The combination of Nal-IRI with the more potent nucleoside analogue TAS-102 may result in a more effective systemic therapy regimen in CRC and pancreatic cancer.
- The aim of this study is to define the recommended phase II dose (RP2D) of the combination and evaluate the activity in pancreatic and CRC.

Eligibility

- Age ≥18 years
- Dose escalation phase (I): patients with stage IV or locally advanced unresectable GI adenocarcinomas (gastric, cholangiocarcinoma, pancreatic, colorectal)
- Dose expansion phase (II):
  - Arm A - 20 patients with pancreatic adenocarcinoma
  - Arm B - 20 patients with colorectal adenocarcinoma
- ECOG performance status 0-1
- Adequate organ function
- Recovered from the effects of any prior surgery, radiotherapy or other cancer therapy
- Measurable disease per RECIST 1.1.



Methods

- The trial design for phase I part is standard 3+3.
- TAS-102 is administered orally in four dose levels at BID on days 1-5, with Nal-IRI at corresponding dose levels as IV infusion on day 1, in 14-day cycles.
- After recommended phase II doses are established, an expansion phase will follow as in schema above
- The primary endpoint of the phase II portion is overall response rate.
- Simon's two-stage design will be used for each arm of the phase II component. In the first stage, 10 patients will be accrued. If there are fewer than 1 responder, the cohort will be stopped. Otherwise, 10 additional patients will be accrued for a total of 20.
- Enrollment to the escalation phase I part of the study started in February 2018.

Trial Endpoints

Primary Objectives

- Phase I: Determine the recommended phase II dose for the combination of TAS-102 and nal-IRI.

Hypothesis: The combination of a TAS102 with nal-IRI is safe with no significant added toxicity.

- Phase II: Evaluate the activity of the combination of TAS102 and nal-IRI in previously treated patients with metastatic colorectal cancer and pancreatic cancer.

Hypothesis: The longer half-life and increased tumor levels of nal-IRI would allow for more overlap with TAS102 and increase the antitumor effect.

Secondary Objectives

- Define the toxicity profile of the combination of TAS-102 and nal-IRI. The additional patients enrolled on the expansion cohorts will help confirm the toxicity profile.
- Evaluate the activity of the combination of TAS-102 and nal-IRI in previously treated patients with metastatic colorectal cancer and pancreatic cancer. Specifically, we will measure response rate, response duration and progression free survival. This will only be preliminary data since this is a secondary objective.

Sponsor: Winship Cancer Institute of Emory University
Registration: clinical-trials.gov: NCT03368963

Journal of Clinical Oncology
An American Society of Clinical Oncology Journal

GASTROINTESTINAL (NONCOLORECTAL) CANCER

A phase I/II study of trifluridine/tipiracil (TAS-102) in combination with nanoliposomal irinotecan (NAL-IRI) in advanced GI cancers.

Olatunji B. Alese, Walid Labib Shaib, Mehmet Akce, Christina Wu, Bassel F. El-Rayes

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Winship Cancer Institute, Atlanta, GA; Winship Cancer Institute of Emory University, Atlanta, GA; Ohio State University Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, Columbus, OH; Department of Hematology and Medical Oncology, Winship Cancer Institute of Emory University, Atlanta, GA

Abstract Disclosures

Abstract

COMPANION ARTICLES

No companion articles

ARTICLE CITATION

DOI: 10.1200/JCO.2018.36.15_suppl.
Journal of Clinical Oncology 36, no. 15_suppl

Published online June 01, 2018.

TPS4155

Background: Trifluridine/tipiracil (FTD/TPI, also known as TAS-102) is a combination of a nucleoside analogue and a thymidine phosphorylase inhibitor. TAS-102 has shown activity in 5FU-resistant colorectal cancer (CRC). Nano liposomal-Irinotecan (Nal-IRI) achieve higher intra-tumor concentrations than irinotecan (142-fold) and its major metabolite, SN-38 (9-fold), resulting in superior anti-tumor activity compared to free irinotecan in multiple tumor xenografts. Clinical trials have established activity of Nal-IRI combined with 5FU in pancreatic cancer. The combination of Nal-IRI with the more potent nucleoside analogue TAS-102 may result in a more effective systemic therapy regimen in CRC and pancreatic cancer. The aim of this study is to define the recommended phase II dose (RP2D) of the combination and evaluate the activity in pancreatic cancer and CRC. **Methods:** Eligible patients for the phase I trial include stage IV or locally advanced unresectable gastrointestinal adenocarcinomas, who have failed at least one prior therapy; age ≥18 years, ECOG PS 0-1 and measurable disease per RECIST 1.1. The trial design is standard 3+3. TAS-102 is administered orally in four dose levels of 25, 25, 30, 35mg/m^2 BID on days 1-5, with Nal-IRI at corresponding dose levels of 50, 70, 70, 70mg/m^2 IV on day 1 , in 14-day cycles. After recommended phase II doses are established, an expansion phase will enroll 20 patients with pancreatic adenocarcinoma (Arm A) and 20 patients with colorectal adenocarcinoma (Arm B). These patients must have either locally advanced unresectable or metastatic disease, and have failed at least one prior therapy that must not have included irinotecan. The primary endpoint of the phase II portion is overall response rate. Simon's two-stage design will be used for each arm of the phase II component. In the first stage, 10 patients will be accrued. If there are fewer than 1 responder, the cohort will be

stopped. Otherwise, 10 additional patients will be accrued for a total of 20. Enrollment to the escalation phase I part of the study started in February 2018. Clinical trial information: NCT03368963.

Treatment of HR+, HER2−, Node-Positive, High-Risk, Early Breast Cancer (monarchE)
Johnston et al.

Updated Analysis From KEYNOTE-189: Pembrolizumab or Placebo Plus Pemetrexed and Platinum for Previously Untreated Metastatic Nonsquamous Non–Small-Cell Lung Cancer
Gadgeel et al.

British Journal of Cancer (2008) 98, 1312–1319

www.bjcancer.com

A pharmacokinetic and pharmacodynamic study on metronomic irinotecan in metastatic colorectal cancer patients

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The pharmacokinetics (PK) and pharmacodynamics (PD) of metronomic irinotecan have not been studied in cancer patients. The aim of the study is to investigate the PK/PD profile of irinotecan/SN-38 administered by metronomic schedule. Twenty chemotherapy-refractory or chemotherapy-resistant patients with metastatic colorectal carcinoma were enrolled. Irinotecan was infused continuously as follows: irinotecan 1.4 mg m^{-2} day^{-1} ($n=7$), 2.8 mg m^{-2} day^{-1} ($n=5$) and 4.2 mg m^{-2} day^{-1} ($n=8$). Drug levels were examined by HPLC, whereas ELISAs and real-time RT-PCR were used, respectively, for the measurement of plasma levels and gene expression in peripheral blood mononuclear cells of vascular endothelial growth factor/thrombospondin-1. Pharmacokinetic analysis demonstrated that the steady-state levels (C_{ss}) of SN-38 were between 1 and 3.3 ng ml^{-1}. From a PD point of view, higher thrombospondin-1 (TSP-1) plasma levels (153.4 ± 30.1 and 130.4 ± 9.2% at day 49 *vs* pretreatment values at 1.4 and 2.8 mg m^{-2} day^{-1} dose levels, respectively) and increased gene expression in PBMC were found during the metronomic irinotecan infusion, especially at the lower doses. Four patients (20%) obtained a stable disease (median 3.9 months) despite progressing during previous standard irinotecan schedule. Toxicities > grade 1 were not observed. Metronomic irinotecan administration is very well tolerated and induces an increase of gene expression and plasma concentration of TSP-1 at low plasma SN-38 concentrations.
British Journal of Cancer (2008) **98**, 1312–1319. doi:10.1038/sj.bjc.6604311 www.bjcancer.com
Published online 25 March 2008

Keywords: metronomic chemotherapy; angiogenesis; irinotecan; colon cancer; clinical study; thrombospondin-1

Chemotherapy administration using long-term, continuous and low-dose schedule has been recently introduced in the therapy of solid tumours. Colleoni and co-workers have used the metronomic/antiangiogenic strategy, mainly based on the use of daily oral cyclophosphamide (CTX) in combination with low-dose methotrexate given 2 days/week, in clinical trials for the treatment of metastatic breast cancer patients, and reported promising clinical activity in the absence of serious adverse events (Colleoni *et al*, 2002, 2006; Orlando *et al*, 2006a, b). Moreover, the low-toxicity profile (Kerbel and Kamen, 2004) and the low costs (Bocci *et al*, 2005b) of the metronomic CTX regimens enhanced the quality of life of patients and suggested immediate potential use in various clinical settings. Glode *et al* (2003) and Vogt and co-workers (Vogt *et al*, 2003; Coras *et al*, 2004) studied a metronomic chemotherapy schedule based on alkylating agents (CTX and trofosfamide, respectively) in combination with drugs thought to have some antiangiogenic effects (i.e., dexamethasone, rofecoxib and pioglitazone), demonstrating efficacy as a salvage therapy in the treatment of patients with hormone-refractory prostate carcinoma (Glode *et al*, 2003) or palliative treatment of patients with advanced malignant vascular tumours (Vogt *et al*, 2003) and endemic Kaposi sarcoma (Coras *et al*, 2004). More recently, the metronomic administration of CTX or vinblastine (Stempak *et al*, 2006) was studied in paediatric cancer patients, while temozolomide (Baruchel *et al*, 2006; Stempak *et al*, 2006) was administered in children with recurrent/refractory brain tumours without severe toxicities and with positive results. Continuous oral thalidomide and celecoxib with alternating oral etoposide and CTX have been also studied in paediatric cancer patients (Kieran *et al*, 2005). Garcia *et al* (2006) have recently reported encouraging phase II trial results of metronomic cyclophosphamide, administered daily, in combination with bevacizumab given every 2 weeks, for treatment of recurrent ovarian cancer.

As stated above, preclinical and clinical experiences on metronomic chemotherapy are so far almost mostly focused on low-dose CTX; therefore, studies on other chemotherapeutic drugs are urgently needed. Moreover, rational and less empirical approaches to the clinical development of new metronomic schedules could help setting a more rigorous standard into this evolving and growing field of chemotherapy.

Drugs affecting pathological angiogenesis represent a new and promising approach to metastatic colorectal cancer (mCRC), as shown by the results of the bevacizumab-based phase III clinical studies (Hurwitz *et al*, 2005). Further evaluations of antiangiogenic

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Received 21 November 2007; revised 29 January 2008; accepted 8 February 2008; published online 25 March 2008

regimens in preclinical and clinical studies have considerable potential to improve prognosis and quality of life of patients with mCRC. Over the last few years, the introduction of new chemotherapeutic drugs such as irinotecan has resulted in improved prognosis of patients with mCRC (Holen and Saltz, 2001). Despite abundant information about the pharmacology of irinotecan (Di Paolo *et al*, 2006), and its active metabolite SN-38, on cancer cells using different therapeutic approaches, no data are currently available about clinical effects of metronomic irinotecan administration.

Based on this background, we decided to perform a pharmacokinetic/pharmacodynamic (PK/PD) study in 20 patients with mCRC, heavily pretreated with both irinotecan- and oxaliplatin-based chemotherapy, to investigate the (i) PK of metronomic irinotecan/SN-38 at different dose levels; and (ii) changes in antiangiogenic (thrombospondin-1 (TSP-1)) and proangiogenic factors (vascular endothelial growth factor (VEGF)) during treatments.

MATERIALS AND METHODS

Patient selection

The study was approved by the local ethics committee and registered in the European Clinical Trial Database EudraCT (http://eudract.emea.europa.eu; EudraCT registration number 2006-001045-34), and patients were informed of the investigational nature of the study and provided their written informed consent. Patients with a confirmed histological diagnosis of mCRC with no operable disease were studied (Table 1). Other main eligibility criteria included (1) previous chemotherapy with fluoropyrimidines, oxaliplatin, irinotecan; (2) measurable disease progressing during (refractory patients) or within 3 months (resistant patients) from the end of the treatments; (3) age $\leqslant 75$ years; (4) ECOG performance status of $\leqslant 2$; (5) adequate bone marrow, renal and liver function (leukocyte count $\geqslant 3000\,mm^{-3}$, platelet count $\geqslant 100\,000\,mm^{-3}$, serum creatinine $\leqslant 1.3\,mg\,dl^{-1}$, serum bilirubin $\leqslant 1.5\,mg\,dl^{-1}$, AST and ALT $\leqslant 2.5$ times normal values); (6) life expectancy of more than 3 months. Exclusion criteria were as follows: brain metastasis, symptomatic cardiac disease, recent history of myocardial infarction, active infections and inflammatory bowel disease.

Table 1 Patient characteristics

n	20
Median age (years) (range)	71 (51–79)
Gender (male/female)	11/9
ECOG (0/1/2)	8/11/1
Primary tumour site (n)	
Colon	16
Rectum	4
Metastatic sites (n)	
Liver	16
Lungs	13
Peritoneum	4
Lymph nodes	3
Others	3
No. of metastatic sites (n)	
Single	7
Multiple	13
Previous chemotherapy (%)	
Irinotecan based	100
Fluoropyrimidine based	100
Oxaliplatin based	100
Cetuximab+irinotecan	35
No. of previous cancer treatments for advanced disease (%)	
Two	55
More than two	45
Irinotecan dose ($mg\,m^{-2}\,day^{-1}$) (n)	
1.4	7
2.8	5
4.2	8
Median CEA ($ng\,ml^{-1}$) (range)	62.3 (0.8–5104)
Median body surface area (m^2) (range)	1.77 (1.64–2)
TSP-1 pretreatment levels ($ng\,ml^{-1}$) (mean ± s.d.)	213.9 ± 110.08
VEGF pretreatment levels ($pg\,ml^{-1}$) (mean ± s.d.)	158.6 ± 83.5

CEA, cardino-embryonic antigen; ECOG, eastern cooperative oncology group; *n* = no. of patients; TSP-1 = thrombospondin-1; VEGF = vascular endothelial growth factor.

Treatment schedule and doses

Irinotecan was administered as continuous i.v. infusion. Its administration required the implant of a central venous catheter and the use of an external programmable pump (Deltec CADD-Plus, St Paul, MN, USA). Every week external volumetric pumps were refilled with the weekly dose of irinotecan dissolved in NaCl 0.9%. Under these conditions, irinotecan is stable for extended periods of time (Li and Koda, 2002). To define the optimal metronomic dose of irinotecan, we calculated the weekly dose intensity (DI) of irinotecan when administered with the schedule proposed by Herben *et al* (1999) ($39.4\,mg\,m^{-2}$; irinotecan is given 3 out of 4 weeks continuously) that corresponded to a daily dose of irinotecan of $5.6\,mg\,m^{-2}$; therefore, we chose three different dose levels of irinotecan, starting from a reduction of 75%, followed by the 50 and 25% with respect to the calculated dose of $5.6\,mg\,m^{-2}\,day^{-1}$ previously reported. The doses of irinotecan administered by metronomic schedule for each group of patients were as follows:

(1) $1.4\,mg\,m^{-2}\,day^{-1}$ (DI = $9.8\,mg\,m^{-2}\,week^{-1}$, number of patients = 7)
(2) $2.8\,mg\,m^{-2}\,day^{-1}$ (DI = $19.6\,mg\,m^{-2}\,week^{-1}$, number of patients = 5)
(3) $4.2\,mg\,m^{-2}\,day^{-1}$ (DI = $29.4\,mg\,m^{-2}\,week^{-1}$, number of patients = 8)

Neither antiemetic premedication nor prophylactic treatment with granulocyte colony-stimulating factor (G-CSF) was administered. The patients continued the treatment until progression of disease or patient's consent withdrawal.

Clinical assessment, toxicity and response criteria

Pretreatment evaluation included history and physical examination, performance status assessment, complete blood cell with differential and platelet counts, complete blood profile, tumour markers, urinalysis, ECG, chest X-ray or computed tomography scan, abdominal computed tomography scan and/or sonogram, and any other appropriate diagnostic procedure to evaluate metastatic sites. During treatment, a physical examination, a complete blood cell count, blood profile, urinalysis and toxicity evaluation were performed every week, immediately before pump refilling. Sites of metastatic disease were radiologically re-evaluated every 2 months, according to the RECIST criteria (Therasse *et al*, 2000). A chest X-ray and/or an abdominal sonogram were repeated at least every 6 months if there was no evidence of lung or abdominal disease, respectively. Toxicities were scored according to the standard NCI Common Terminology Criteria for Adverse Events, version 3.0. Duration of responses was calculated from the first day of treatment to the date of first observation of progressive disease or last examination.

Pharmacokinetics of metronomic irinotecan, SN-38 and SN-38 glucuronide

The PK analysis of irinotecan and its main metabolites SN-38 and SN-38 glucuronide (SN-38glu) was performed as previously described (Masi *et al*, 2004) with minor modifications. Blood samples (4 ml each) for drug assays were taken from an indwelling i.v. cannula placed in an antecubital vein at baseline, 30 min, 1 h, and at days 7, 14, 21, 28, 35, 42, 49, 56 and 63 after the beginning of irinotecan i.v. infusion; at day 63, at the end of the infusion and before refilling the pump, the blood sampling was performed after 30 min, 2 and 6 h. Blood was collected in heparinised tubes (Vacutainer tubes; Becton Dickinson Vacutainer System) and then centrifuged (10 min, 4000 r.p.m., 4°C) to separate plasma, which was stored at −20°C and assayed within 1 week. Briefly, concentration of irinotecan and SN-38 was evaluated after extraction of 1 ml of plasma with methanol containing 0.1% HCl (10 N); the samples were then centrifuged and the clear supernatant was evaporated to dryness under nitrogen flow in a thermostated bath at 45°C. The resulting pellet was reconstituted in methanol acidified with 0.1% HCl (10 N) and eluted through a μBondapack C_{18} stationary phase column (300 × 3.9 mm, 10 μm; Waters, Milford, MA, USA) by KH_2PO_4 (0.1 M)/acetonitrile (60 : 40, v/v; pH 6.0) containing sodium heptansulphonate 3 mmol l^{-1}. The chromatographic system LC Module 1 Plus (Waters) was equipped with a Model 474 scanning fluorescence detector with excitation and emission wavelengths set at 375 and 525 nm, respectively. Data analysis was performed using Millennium 2.1 software (Waters). The SN-38glu plasma concentration was measured after incubation of plasma samples with β-glucuronidase (10 U μl^{-1} of plasma) at 37°C for 2 h before extraction. The difference between peak areas corresponding to SN-38 in β-glucuronidase-treated *vs* untreated samples corresponded to the plasma levels of SN-38glu. Standard calibration curves were generated on each day of analysis by adding irinotecan and SN-38 to 1 ml of blank plasma obtained from healthy donors, resulting in final concentrations that ranged from 12 500 to 0.8 ng ml^{-1} and 2500 to 0.16 ng ml^{-1} for irinotecan and SN-38, respectively. The range of linearity of the HPLC method was from 0.8 to 12 500 ng ml^{-1} for irinotecan and 2500 to 0.16 ng ml^{-1} for SN-38.

Individual plasma concentration profiles of irinotecan and its catabolites were fitted according to a two-compartment open model by means of the WinNonlin 5.1 computer software (Pharsight Corporation, Mountain View, CA, USA) The AUC of irinotecan (CPT-11), SN-38 and SN-38glu from 0 to 63.25 days was calculated by the log-linear trapezoidal method until the last sampling time. Peak plasma concentration (C_{max}) was obtained by visual inspection of the concentration *vs* time profile of irinotecan and metabolites, whereas steady-state concentrations (C_{ss}) were calculated as mean values of plasma levels at days 7, 14, 21, 28, 35, 42, 49, 56 and 63 of infusion. Finally, the clearance of irinotecan (CL) was determined as the infusion rate of the drug divided by its C_{ss} value. The relative extent of metabolic conversion (REC) of irinotecan was calculated as $AUC_{SN\text{-}38}/AUC_{CPT\text{-}11}$, whereas the drug metabolic ratio (MR) was obtained as $(AUC_{SN\text{-}38} + AUC_{SN\text{-}38glu})/AUC_{CPT\text{-}11}$. The glucuronidation ratio (GR) was calculated as the $AUC_{SN\text{-}38glu}/AUC_{SN\text{-}38}$ to obtain an indirect estimate of the activity of glucuronidation of the active metabolite SN-38, and the biliary index (BI) was evaluated as $(AUC_{CPT\text{-}11}) \times (AUC_{SN\text{-}38}/AUC_{SN\text{-}38glu})$.

Assessment of human *VEGF* and *TSP-1* gene expression and plasma levels

Before drug administration and at days 7, 14, 21, 28, 35, 42, 49, 56 and 63, 10 ml of blood was drawn from the antecubital vein of patients. Peripheral blood mononuclear cells (PBMC) were collected as described (Bocci *et al*, 2006) and the cell suspension was centrifuged at 1000 r.p.m. for 10 min and washed with PBS; the resulting pellet was immediately frozen in liquid nitrogen and stored at −80°C. Briefly, RNA (1 μg) was reverse transcribed (Bocci *et al*, 2005a) and the resulting cDNA was diluted (2 : 3) and then amplified by QRT-PCR with the Applied Biosystems 7900HT sequence detection system. Vascular endothelial growth factor- and TSP-1-validated primers were purchased from Applied Biosystems (Assay ID Hs00170236_m1 and Hs00173626_m1, respectively). The PCR thermal cycling conditions and optimisation of primer concentrations were followed as *per* the manufacturer's instructions. Amplifications were normalised to GAPDH and the quantitation of gene expression was performed using the $\Delta\Delta C_t$ calculation; the amounts of VEGF and TSP-1, normalised to the endogenous control and relative to the calibrator (PBMC sample at day 0), are given as $2^{-\Delta\Delta C_t}$. The data are presented as the percentage of $2^{-\Delta\Delta C_t}$ before the beginning of irinotecan infusion.

Plasma samples obtained at the same days of PBMC collection were assessed for TSP-1 and VEGF levels using the commercially available ELISA and EIA kits. Each sample was assayed for human VEGF and TSP-1 concentrations by the ELISA Kit Quantikine® (R&D Systems, USA) and by the ChemiKine™ Human TSP-1 EIA Kit (Chemicon, Temecula, CA, USA), respectively. Measurements were performed by the microplate reader Multiskan Spectrum (Thermo Labsystems, Milan, Italy) set to 450 nm (with a wavelength correction set to 540 nm) for the VEGF kit and 490 nm for TSP-1 kit. The data are presented as the percentage of the VEGF and TSP-1 plasma levels at day 0.

Statistical analysis

Since the study was exploratory in nature, no statistical hypothesis testing has been performed. Moreover, the sample size (20 patients) has been judged to be adequate based on the suggestions of the entropy-based approach to sample size in translational clinical trials (Piantadosi, 2005). The analysis by ANOVA, followed by the Student–Newman–Keuls test, was used to assess the statistical differences of data. *P*-values lower than 0.05 were considered significant. Statistical analyses were performed using the GraphPad Prism software package version 4.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Patients and toxicity

As outlined in Table 1, 20 patients with advanced mCRC entered the study. Median age was 71 years (range, 51–79 years), ECOG performance status was 0–1 in 18 patients and 2 in one. As reported, the entire study population was heavily pretreated and irinotecan refractory or resistant. Of note, 35% of patients had also received a cetuximab-based therapy. Overall, 188 weeks of irinotecan were administered by metronomic schedule with a median of 9 weeks per patient (range, 2–20 weeks). The end of treatment was due to disease progression in all patients.

All patients were assessable for toxicities. Toxicities were very uncommon. In particular, we did not observe toxicities higher than grade 1. Three (15%) and five patients (25%) experienced, respectively, a transient grade 1 diarrhoea and nausea, resolved without interrupting the treatment. No haematological toxicities were observed.

Antitumour activity and survival

All 20 patients were assessable for response to treatment. Four patients treated at 1.4 mg m^{-2} day^{-1} (1 patient), 2.4 mg m^{-2} day^{-1} (2 patients) and 4.2 mg m^{-2} day^{-1} (1 patient) obtained a stabilisation of disease that lasted a median period of 3.9 months (range, 3–5 months). In the remaining 16 patients, disease progression

was observed at the first clinical evaluation. After a median follow-up of 20 months, median progression-free survival (PFS) was 2.07 months (95% CI: 1.99–2.14) and median overall survival (OS) was 8.4 months (95% CI: 5.1–11.7); curves estimated by the Kaplan–Meier method from the first day of treatment are reported in Figure 1.

Pharmacokinetics of metronomic irinotecan, SN-38 and SN-38glu

Main PK parameters of irinotecan and its metabolites are reported in Table 2, whereas the plasma profiles of irinotecan, SN-38 and SN-38glu at the different infusion schedules are shown in Figure 2A–C, respectively. Pharmacokinetic analysis demonstrated that the C_{ss} of irinotecan 1.4, 2.8 and 4.2 mg m^{-2} day^{-1} were 143.1 ± 56.8, 231.6 ± 101.4 and 390 ± 171 ng ml^{-1}, respectively, whereas those of SN-38 were 1.00 ± 0.52, 2.29 ± 0.87 and 3.33 ± 0.96 ng ml^{-1}, respectively, and resulted statistically different among them ($P<0.05$). Moreover, the C_{ss} of SN-38glu were, as expected, higher than the ones of SN-38. Pharmacokinetic analysis of irinotecan showed an increased metabolism of the parent drug into the active metabolite SN-38 when higher doses were administered (Figure 2B). As expected, irinotecan AUC value was higher at the 4.2 dose with respect to the 2.8 and 1.4 doses, even though it was not statistically significant, whereas mean AUC value of SN-38 was significantly lower at 1.4 dose than at 2.8 and 4.2 doses ($P<0.05$) (Table 2). The comparison of PK of irinotecan at different doses did not reveal any significant difference among C_{max} and $t_{1/2}\beta$ values (Table 2). Instead, significant differences were found for C_{max} values of SN-38 and SN-38glu at different irinotecan doses. Further analysis demonstrated that the higher doses led to an increase in BI, REC and MR values, even if only the increase of BI was statistically significant ($P<0.05$). Instead, GR value did not significantly increase after administration of higher doses of irinotecan (Table 2).

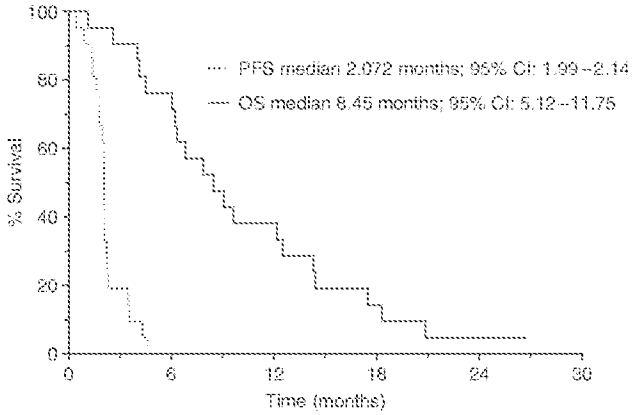

Figure 1 Actuarial PFS and OS curves calculated by the Kaplan–Meier method from the first day of metronomic irinotecan chemotherapy.

Changes in TSP-1 and VEGF plasma and gene expression in PBMC

To compare the variations of plasma TSP-1 and VEGF before and during the metronomic treatments, graphs were drawn to show the concentrations as a percentage of the concentration at day 0 of individual patients before the starting of irinotecan infusion. Figure 3A shows that TSP-1 levels markedly increased in treated patients and remain consistently higher for more than 8 weeks.

Table 2 Pharmacokinetic parameters of irinotecan, SN-38 and SN-38glu at the doses of irinotecan 1.4, 2.8 and 4.2 mg m^{-2} day^{-1} in 20 patients

	Mean ± s.d.		
	1.4 mg m^{-2} day^{-1} ($n=7$)	**2.8 mg m^{-2} day^{-1} ($n=5$)**	**4.2 mg m^{-2} day^{-1} ($n=8$)**
Irinotecan			
AUC (day ng ml^{-1})	8714.7 ± 1564.3	13877.7 ± 3035.2	23051.6 ± 5002.3
CL (ml day^{-1} m^{-2})	154.32 ± 28.4	170.31 ± 44.2	146.11 ± 25.3
$t_{1/2}\beta$ (h)	15.9 ± 5.1	20.2 ± 6.2	14.6 ± 3.2
C_{max} (ng ml^{-1})	277.6 ± 125.3	382.9 ± 261.8	484.1 ± 243.1
C_{ss} (ng ml^{-1})	143.1 ± 56.8	231.6 ± 101.4	390.0 ± 171.0[a,b]
T_{max} (day)	35	35	28
SN-38			
AUC (day ng ml^{-1})	59.43 ± 7.47	136.21 ± 10.61[c]	200.48 ± 12.26[a,b]
$t_{1/2}\beta$ (h)	18.9 ± 4.3	22.8 ± 6.7	19.9 ± 7.2
C_{max} (ng ml^{-1})	1.62 ± 0.45	2.61 ± 1.07	4.03 ± 2.19[a]
C_{ss} (ng ml^{-1})	1.00 ± 0.52	2.29 ± 0.87[c]	3.33 ± 0.96[a,b]
T_{max} (day)	42	35	35
SN-38glu			
AUC (day ng ml^{-1})	100.94 ± 8.82	268.86 ± 14.52[c]	430.10 ± 24.34[a,b]
$t_{1/2}\beta$ (h)	22.31 ± 5.1	17.4 ± 5.6	21.33 ± 6.8
C_{max} (ng ml^{-1})	2.24 ± 0.58	5.59 ± 1.91[c]	8.45 ± 2.54[a,b]
C_{ss} (ng ml^{-1})	1.63 ± 0.53	4.42 ± 1.38[c]	7.20 ± 1.59[a,b]
T_{max} (day)	49	42	42
BI	5130.1 ± 745	7030.7 ± 537[c]	10744.9 ± 892[a,b]
REC	0.0068 ± 0.0048	0.0098 ± 0.0035	0.0087 ± 0.0025
MR	0.0184 ± 0.0104	0.0292 ± 0.0183	0.0274 ± 0.0116
GR	1.698 ± 0.206	1.974 ± 0.402	2.145 ± 0.623

AUC = area under the time/concentration curve; BI = biliary index; C_{max} = maximal plasma concentration; C_{ss} = steady-state concentration; GR = glucuronidation ratio; MR = metabolic ratio; REC = relative extent of conversion; SN-38glu = SN-38 glucuronide; $t_{1/2}\beta$ = terminal half-life; T_{max} = time to peak. [a]$P<0.05$ 4.2 *vs* 1.4. [b]$P<0.05$ 4.2 *vs* 2.8. [c]$P<0.05$ 2.8 *vs* 1.4.

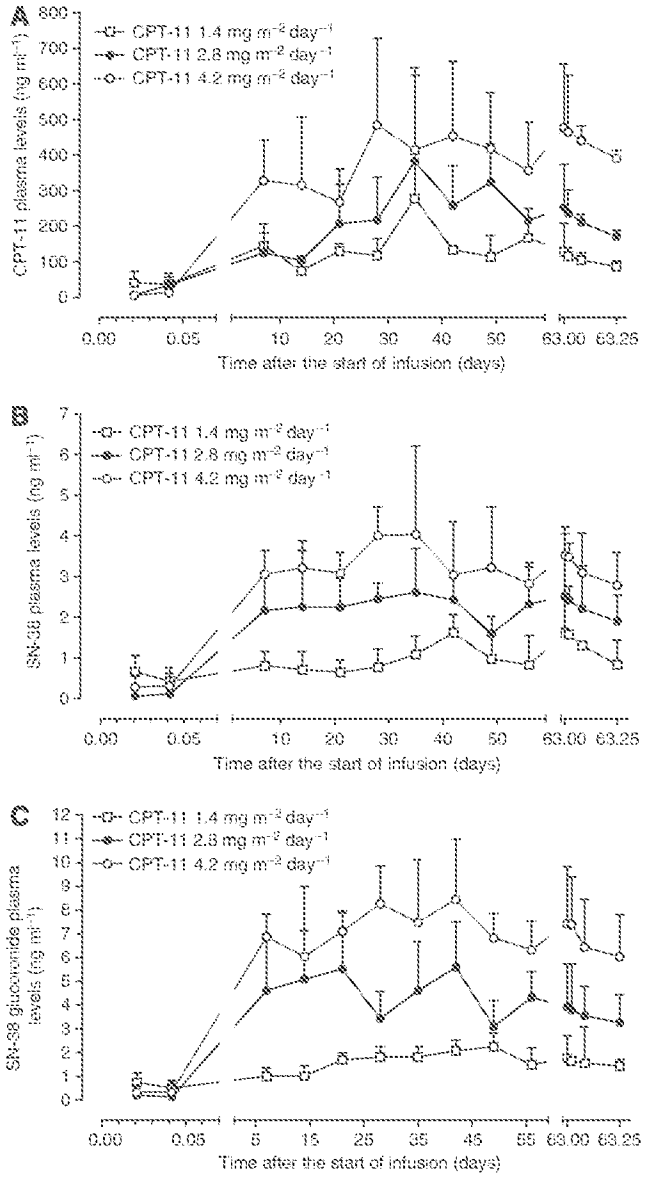

Figure 2 Plasma levels of irinotecan (CPT-11) (**A**), SN-38 (**B**) and SN-38 glucuronide (**C**) in 20 mCRC patients receiving an i.v. continuous infusion of CPT-11 at three different dose levels. Symbols and bars represent mean and s.d.

However, differences were noted for TSP-1 concentrations among the three dose levels: the average TSP-1 increase was higher at the lower doses of 1.4 and 2.8 mg m^{-2} day^{-1} (Figure 3A) reaching maximum increments at day 49 of 153.4 ± 30.9 and 130.5 ± 9.3%, respectively, *vs* 100% of day 0. In contrast, TSP-1 concentrations in patients treated with CPT-11 at 4.2 mg m^{-2} day^{-1} returned to the baseline values after an initial increase (Figure 3A). Figure 3B shows the results of the different profiles of VEGF plasma levels in the treated patients. Mean plasma VEGF levels, although with a high variability, increased in the first 3 weeks of treatment, whereas after day 28 they returned to baseline levels (Figure 3B). However, differences were noticed among the three dose levels: at the lowest dose (1.4 mg m^{-2} day^{-1}) VEGF concentrations decreased (at day 56, 77.9 ± 18 *vs* 100% of day 0), whereas at the highest dose (4.2 mg m^{-2} day^{-1}) they increased (at day 56, 126.3 ± 67 *vs* 100% of day 0) during the CPT-11 infusion. Interestingly, only at lower metronomic doses, there was a simultaneous increase in TSP-1 levels and a decrease of VEGF concentrations, which would suggest a shift to an antiangiogenic state.

Figure 3C and D shows TSP-1 and VEGF gene expression profiles in PBMC, a normal cell compartment. Differences were found in TSP-1 gene expression among the three dose levels. Indeed, the irinotecan 1.4 mg m^{-2} day^{-1} determined a marked increase in TSP-1 gene expression in PBMC at least until day 42 (222.4 ± 106.9 *vs* 100% of day 0), whereas at the other doses a marked increase was seen only after day 35 (Figure 3C). In contrast, VEGF expression profile in PBMC was similar to baseline, with the exception of an initial increase (at day 14, 172.6 ± 73.3 *vs* 100% of day 0 for 1.4 mg m^{-2} day^{-1} dose).

DISCUSSION

The present study described, for the first time, the PK of metronomic irinotecan and demonstrated a marked increase in TSP-1 plasma concentrations and gene expression (already at lower irinotecan dose levels), suggesting a possible use of this PD marker in irinotecan-based metronomic treatment strategies. Moreover, low-dose metronomic irinotecan was not toxic and potentially active in heavily pretreated, refractory or resistant population of mCRC patients.

Based on promising antiangiogenic and antitumour preclinical results (Bocci *et al*, 2007), we decided to test the metronomic irinotecan schedule in the clinic, specifically in patients with mCRC resistant to irinotecan standard doses. The 'ethical condition' to evaluate the clinical effect of an irinotecan metronomic treatment in this patient population depended on the fact that there was no evidence that a third/fourth line of chemotherapy could produce an improvement in terms of clinical benefit or efficacy in patients with mCRC already treated with both oxaliplatin- and irinotecan-based chemotherapy. The results of several phase II clinical studies showed that a third/fourth line of fluoropyrimidine-based chemotherapy in this setting of patients generally produces a poor response rate (around or less than 10%), with a median PFS between 2 and 3 months and with a median OS of approximately 6–9 months (Chong *et al*, 2005; McCollum *et al*, 2006; Scartozzi *et al*, 2006). Besides the weak antitumour activity, these treatments are limited by a substantial toxicity ⩾grade 3 (NCI scale) reported in about 10–15% of all patients. The role of the targeted therapy in these setting of patients has also been evaluated. Chen *et al* (2006) have recently published the results of a large multicentre trial of bevacizumab in combination with 5-fluorouracil/leucovorin in patients with mCRC pretreated with both oxaliplatin- and irinotecan-based chemotherapy. The results have shown a response rate of 4%, a median PFS of 3.5 months and a median OS of 9 months. Of note, adverse events ⩾grade 3 were reported in 47% of all treated patients.

More promising results seem to come from the use of the monoclonal antibodies against the epidermal growth factor receptor cetuximab and panitumumab in this setting of patients. Jonker *et al* (2007) have evaluated, in a phase III clinical study, the role of cetuximab in patients with mCRC pretreated with an oxaliplatin- and irinotecan-based chemotherapy, showing an improvement in terms of OS of cetuximab plus best supportive care (BSC) respect to BSC alone (median OS of 6.1 *vs* 4.6 months, $P = 0.0046$). Van Cutsem *et al* (2007) in a similar setting of patients have compared panitumumab plus BSC to BSC alone, showing that panitumumab prolonged the PFS (HR: 0.54; 95% CI: 0.44–0.66, $P < 0.0001$), with no difference in terms of OS. Despite the promising results, some issues could limit the use of these therapies in this setting of patients, such as the low impact on survival, the high cost of the drugs and the significant higher rate of grade 3–4 adverse events with respect to BSC. Thus, the study of metronomic chemotherapy could be of interest in heavily

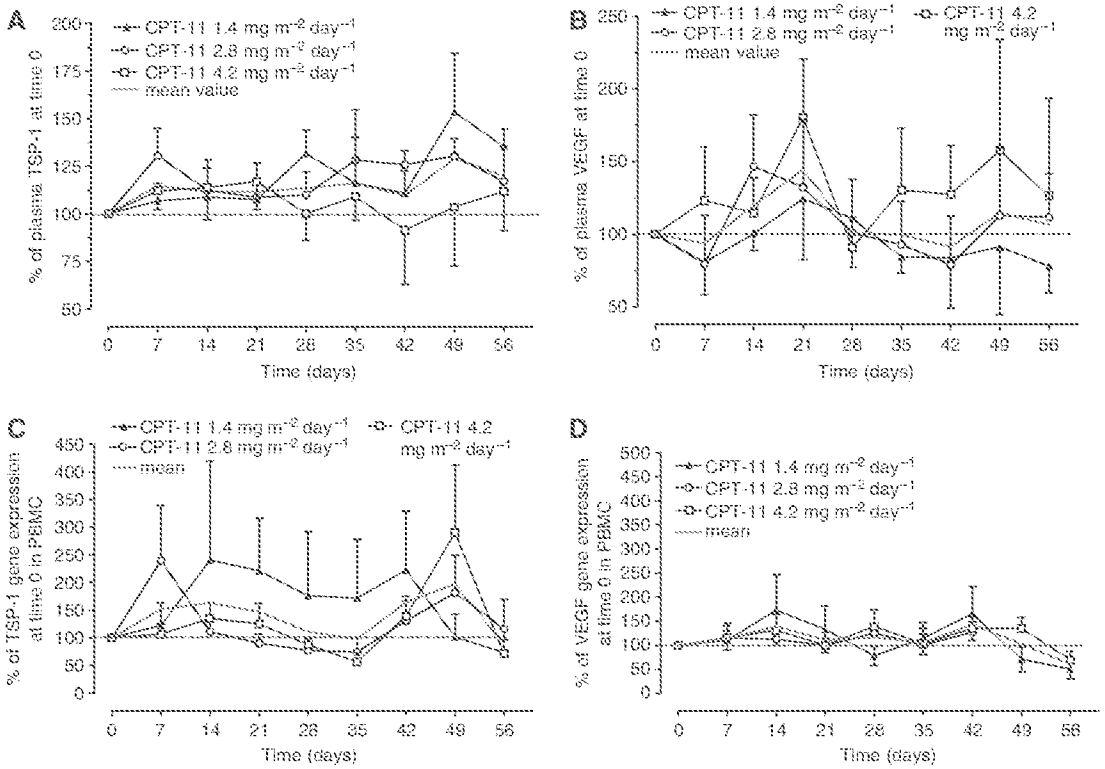

Figure 3 TSP-1 (**A**) and VEGF (**B**) plasma concentrations and TSP-1 (**C**) and VEGF (**D**) gene expression in PBMC of patients administered with three different metronomic irinotecan (CPT-11) doses. Symbols and bars represent mean and s.d. The data are presented as a percentage of the concentration at day 0 (before the beginning of CPT-11 infusion) of each individual patient or as the percentage of $2^{-\Delta\Delta C_T}$ at day 0 of each single patient.

pretreated mCRC. A recent study has evaluated the role of a combination metronomic treatment with CTX, vinblastine and rofecoxib in patients with advanced tumours, in which 13 patients with diagnosis of mCRC were included. One patient had a partial response, while another patient had a stable disease; the time to progression of these patients was 12 and 7 months, respectively (Young *et al*, 2006). These results suggest a potential anti-tumour effect of the metronomic approach in patients with mCRC.

One of the major concerns regarding the clinical application of metronomic chemotherapy relates to the dosing levels and the frequency of administration of the chemotherapeutic drugs. This important issue has generated confusion regarding the term 'metronomic' that has been sometimes associated with a simple, small reduction of a standard dose. Moreover, among the published trials, the dose for the metronomic chemotherapy is often arbitrarily chosen (e.g., the 'classic' fixed dose of 50 mg day^{-1} of CTX simply corresponds to a single tablet of the commercially available medicament) and no further efforts have been made to define the best dose. In our study, we evaluated three different dose levels of metronomic irinotecan that was infused continuously without breaks, starting from a reduction of 75% of the maximum tolerable dose of irinotecan when infused continuously more than 21 days every 28 days reported by Herben *et al* (1999). Moreover, we thoroughly investigated the changes in TSP-1 and VEGF expression/secretion in accessible compartments in metastatic patients such as peripheral mononuclear cells and plasma to confirm our preclinical data and find indexes of biological activity of the CPT-11 metronomic treatment.

Although our study was not comparative, the results show that the irinotecan metronomic chemotherapy produces clinical results such as those observed with other schedules of third/fourth line of treatment in patients with a diagnosis of metastatic colorectal carcinoma, both in terms of median PFS and median OS. Furthermore, our results were accompanied by a total absence of toxicity and in a heavily pretreated, irinotecan-resistant population with progressive disease. Moreover, G-CSF was not administered to patients receiving metronomic irinotecan chemotherapy and as such this could have an advantage, not only in terms of cost but also in terms of avoiding the possibility that the exogenous G-CSF might promote angiogenesis by mobilising circulating endothelial progenitor cells (CEPs) (Natori *et al*, 2002; Shaked and Kerbel, 2007). Our results suggest that metronomic irinotecan chemotherapy could work through a mechanism of action that is not related to the direct cytotoxic effect on tumour cells but rather through an antiangiogenic activity targeting proliferating endothelial cells as shown in the preclinical setting. Indeed, higher TSP-1 plasma levels were found during the metronomic irinotecan infusion when compared to the baseline values in single patients as well as the increased gene expression in the PBMC compartment, especially at the lower irinotecan doses. These findings clinically confirmed that low-dose CPT-11 inhibits angiogenesis, in part, by upregulating TSP-1 in tumour endothelial (Bocci *et al*, 2003), or tumour and tumour-associated stromal cells (Hamano *et al*, 2004), promoting endothelial cell apoptosis (de Castro Junior *et al*, 2006) and suppressing the mobilisation of circulating endothelial progenitors (Shaked *et al*, 2005). This linkage between PD parameters and dose levels could open a promising area of clinical investigation on surrogate markers for the activity of the metronomic chemotherapy approach. Indeed, a previous attempt to monitor putative surrogate markers such as VEGF, endostatin and TSP-1 plasma levels during metronomic CTX revealed a high degree of variability and no statistically significant relationships between these markers and disease progression or maintenance of stable disease in paediatric patients (Stempak *et al*, 2006). However, the heterogeneous patient population, the drugs that were involved and the

lack of a preclinical investigation of the doses that were used, and combination of drugs used all could have affected the outcome of the results. Colleoni *et al* (2002) described a reduction in serum VEGF of both responders and non-responders in breast cancer metastatic patients treated with metronomic CTX. In our study, the individual plasma VEGF concentrations of patients initially increased when compared to the baseline values in the first 3 weeks and then at lower doses constantly decreased. Despite the presence of a high variability, VEGF plasma levels may reflect both the initial elevated hypoxia of tumour tissue caused by the antiangiogenic therapy, as previously shown (Bocci *et al*, 2004; Motzer *et al*, 2006), and the low rate of VEGF secretion by tumour or tumour-associated stromal cells due to the long-term therapy (Colleoni *et al*, 2002).

This PK/PD study underlines the importance to conduct further comparative studies with BSC or bevacizumab to establish the feasibility of metronomic irinotecan approach. Moreover, this suggests further clinical steps that might be explored such as the administration of rubitecan, an oral camptothecin (Clark, 2006), or combination studies with other low-dose oral chemotherapeutic drugs already approved for colorectal cancer such as UFT (Munoz *et al*, 2006) or capecitabine. In addition, combination of a targeted antiangiogenic drug such as bevacizumab with metronomic irinotecan therapy might also be considered in patients not previously treated with this drug since such combinations show much greater antitumour efficacy compared to metronomic chemotherapy alone or the antiangiogenic drug alone (Klement *et al*, 2000; Kerbel and Kamen, 2004; Pietras and Hanahan, 2005).

ACKNOWLEDGEMENTS

We thank Dr Urban Emmenegger for the helpful discussion of this paper, Dr Laura Ciofi for the technical assistance and Mrs Cassandra Cheng for the excellent secretarial assistance. This work was entirely supported by a research grant from AIRC (Associazione Italiana per la Ricerca sul Cancro) to AF.

REFERENCES

Baruchel S, Diezi M, Hargrave D, Stempak D, Gammon J, Moghrabi A, Coppes MJ, Fernandez CV, Bouffet E (2006) Safety and pharmacokinetics of temozolomide using a dose-escalation, metronomic schedule in recurrent paediatric brain tumours. *Eur J Cancer* **42:** 2335–2342

Bocci G, Barbara C, Vannozzi F, Di Paolo A, Melosi A, Barsanti G, Allegrini G, Falcone A, Del Tacca M, Danesi R (2006) A pharmacokinetic-based test to prevent severe 5-fluorouracil toxicity. *Clin Pharmacol Ther* **80:** 384–395

Bocci G, Falcone A, Fioravanti A, Orlandi P, Di Paolo A, Fanelli G, Viacava P, Naccarato AG, Kerbel RS, Danesi R, Del Tacca M, Allegrini G (2007) *In vitro* and *in vivo* antiangiogenic and antitumour effects of metronomic irinotecan on colorectal cancer. *33rd Italian Society of Pharmacology Meeting: June 2007*, Cagliari, Italy

Bocci G, Fioravanti A, Orlandi P, Bernardini N, Collecchi P, Del Tacca M, Danesi R (2005a) Fluvastatin synergistically enhances the antiproliferative effect of gemcitabine in human pancreatic cancer MIAPaCa-2 cells. *Br J Cancer* **93:** 319–330

Bocci G, Francia G, Man S, Lawler J, Kerbel RS (2003) Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proc Natl Acad Sci USA* **100:** 12917–12922

Bocci G, Man S, Green SK, Francia G, Ebos JM, du Manoir JM, Weinerman A, Emmenegger U, Ma L, Thorpe P, Davidoff A, Huber J, Hicklin DJ, Kerbel RS (2004) Increased plasma vascular endothelial growth factor (VEGF) as a surrogate marker for optimal therapeutic dosing of VEGF receptor-2 monoclonal antibodies. *Cancer Res* **64:** 6616–6625

Bocci G, Tuccori M, Emmenegger U, Liguori V, Falcone A, Kerbel RS, Del Tacca M (2005b) Cyclophosphamide-methotrexate 'metronomic' chemotherapy for the palliative treatment of metastatic breast cancer. A comparative pharmacoeconomic evaluation. *Ann Oncol* **16:** 1243–1252

Chen HX, Mooney M, Boron M, Vena D, Mosby K, Grochow L, Jaffe C, Rubinstein L, Zwiebel J, Kaplan RS (2006) Phase II multicenter trial of bevacizumab plus fluorouracil and leucovorin in patients with advanced refractory colorectal cancer: an NCI Treatment Referral Center Trial TRC-0301. *J Clin Oncol* **24:** 3354–3360

Chong G, Dickson JL, Cunningham D, Norman AR, Rao S, Hill ME, Price TJ, Oates J, Tebbutt N (2005) Capecitabine and mitomycin C as third-line therapy for patients with metastatic colorectal cancer resistant to fluorouracil and irinotecan. *Br J Cancer* **93:** 510–514

Clark JW (2006) Rubitecan. *Expert Opin Investig Drugs* **15:** 71–79

Colleoni M, Orlando L, Sanna G, Rocca A, Maisonneuve P, Peruzzotti G, Ghisini R, Sandri MT, Zorzino L, Nole F, Viale G, Goldhirsch A (2006) Metronomic low-dose oral cyclophosphamide and methotrexate plus or minus thalidomide in metastatic breast cancer: antitumour activity and biological effects. *Ann Oncol* **17:** 232–238

Colleoni M, Rocca A, Sandri MT, Zorzino L, Masci G, Nole F, Peruzzotti G, Robertson C, Orlando L, Cinieri S, de BF, Viale G, Goldhirsch A (2002) Low-dose oral methotrexate and cyclophosphamide in metastatic breast cancer: antitumor activity and correlation with vascular endothelial growth factor levels. *Ann Oncol* **13:** 73–80

Coras B, Hafner C, Reichle A, Hohenleutner U, Szeimies RM, Landthaler M, Vogt T (2004) Antiangiogenic therapy with pioglitazone, rofecoxib, and trofosfamide in a patient with endemic Kaposi sarcoma. *Arch Dermatol* **140:** 1504–1507

de Castro Junior G, Puglisi F, de Azambuja E, El Saghir NS, Awada A (2006) Angiogenesis and cancer: a cross-talk between basic science and clinical trials (the 'do ut des' paradigm). *Crit Rev Oncol Hematol* **59:** 40–50

Di Paolo A, Bocci G, Danesi R, Del Tacca M (2006) Clinical pharmacokinetics of irinotecan-based chemotherapy in colorectal cancer patients. *Curr Clin Pharmacol* **1:** 311–323

Garcia AA, Hirte H, Fleming G, Yang D, Tsao-Wei DD, Roman L, Groshen S, Swenson S, Markland F, Gandara D, Scudder S, Morgan R, Chen H, Lenz HJ, Oza AM (2008) Phase II clinical trial of bevacizumab and low-dose metronomic oral cyclophosphamide in recurrent ovarian cancer: a trial of the California, Chicago, and Princess Margaret Hospital phase II consortia. *J Clin Oncol* **26:** 76–82

Glode LM, Barqawi A, Crighton F, Crawford ED, Kerbel R (2003) Metronomic therapy with cyclophosphamide and dexamethasone for prostate carcinoma. *Cancer* **98:** 1643–1648

Hamano Y, Sugimoto H, Soubasakos MA, Kieran M, Olsen BR, Lawler J, Sudhakar A, Kalluri R (2004) Thrombospondin-1 associated with tumor microenvironment contributes to low-dose cyclophosphamide-mediated endothelial cell apoptosis and tumor growth suppression. *Cancer Res* **64:** 1570–1574

Herben VM, Schellens JH, Swart M, Gruia G, Vernillet L, Beijnen JH, ten Bokkel Huinink WW (1999) Phase I and pharmacokinetic study of irinotecan administered as a low-dose, continuous intravenous infusion over 14 days in patients with malignant solid tumors. *J Clin Oncol* **17:** 1897–1905

Holen KD, Saltz LB (2001) New therapies, new directions: advances in the systemic treatment of metastatic colorectal cancer. *Lancet Oncol* **2:** 290–297

Hurwitz HI, Fehrenbacher L, Hainsworth JD, Heim W, Berlin J, Holmgren E, Hambleton J, Novotny WF, Kabbinavar F (2005) Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. *J Clin Oncol* **23:** 3502–3508

Jonker DJ, Karapetis CS, Moore M, Zalcberg JR, Tu D, Berry S, Koski S, Krahn M, Simes J, Tebbutt N, Van Hazel G, O'Callaghan CJ (2007) Randomized phase III trial of cetuximab monotherapy plus best supportive care (BSC) versus BSC alone in patients with pretreated metastatic epidermal growth factor receptor (EGFR)-positive colorectal carcinoma: a trial of the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) and the Australasian Gastro-Intestinal Trials Group (AGITG). *AACR Annual Meeting April 2007*, Vol 48, American Association for Cancer Research: Los Angeles, CA

Kerbel RS, Kamen BA (2004) The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* **4:** 423–436

Kieran MW, Turner CD, Rubin JB, Chi SN, Zimmerman MA, Chordas C, Klement G, Laforme A, Gordon A, Thomas A, Neuberg D, Browder T, Folkman J (2005) A feasibility trial of antiangiogenic (metronomic) chemotherapy in pediatric patients with recurrent or progressive cancer. *J Pediatr Hematol Oncol* **27:** 573–581

Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* **105:** R15–R24

Li WY, Koda RT (2002) Stability of irinotecan hydrochloride in aqueous solutions. *Am J Health Syst Pharm* **59:** 539–544

Masi G, Falcone A, Di Paolo A, Allegrini G, Danesi R, Barbara C, Cupini S, Del Tacca M (2004) A phase I and pharmacokinetic study of irinotecan given as a 7-day continuous infusion in metastatic colorectal cancer patients pretreated with 5-fluorouracil or raltitrexed. *Clin Cancer Res* **10:** 1657–1663

McCollum AD, Wu B, Clark JW, Kulke MH, Enzinger PC, Ryan DP, Earle CC, Michelini A, Fuchs CS (2006) The combination of capecitabine and thalidomide in previously treated, refractory metastatic colorectal cancer. *Am J Clin Oncol* **29:** 40–44

Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J Clin Oncol* **24:** 16–24

Munoz R, Man S, Shaked Y, Lee CR, Wong J, Francia G, Kerbel RS (2006) Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT–cyclophosphamide metronomic chemotherapy. *Cancer Res* **66:** 3386–3391

Natori T, Sata M, Washida M, Hirata Y, Nagai R, Makuuchi M (2002) G-CSF stimulates angiogenesis and promotes tumor growth: potential contribution of bone marrow-derived endothelial progenitor cells. *Biochem Biophys Res Commun* **297:** 1058–1061

Orlando L, Cardillo A, Ghisini R, Rocca A, Balduzzi A, Torrisi R, Peruzzotti G, Goldhirsch A, Pietri E, Colleoni M (2006a) Trastuzumab in combination with metronomic cyclophosphamide and methotrexate in patients with HER-2 positive metastatic breast cancer. *BMC Cancer* **6:** 225

Orlando L, Cardillo A, Rocca A, Balduzzi A, Ghisini R, Peruzzotti G, Goldhirsch A, D'Alessandro C, Cinieri S, Preda L, Colleoni M (2006b) Prolonged clinical benefit with metronomic chemotherapy in patients with metastatic breast cancer. *Anticancer Drugs* **17:** 961–967

Piantadosi S (2005) Translational clinical trials: an entropy-based approach to sample size. *Clinical Trials* **2:** 182–192

Pietras K, Hanahan D (2005) A multitargeted, metronomic, and maximum-tolerated dose 'chemo-switch' regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. *J Clin Oncol* **23:** 939–952

Scartozzi M, Falcone A, Pucci F, Braconi C, Pierantoni C, Cavanna L, Franciosi V, Berardi R, Beretta G, Masi G, Allegrini G, Zaniboni A, Labianca R, Cascinu S (2006) Capecitabine and mitomycin C may be an effective treatment option for third-line chemotherapy in advanced colorectal cancer. *Tumori* **92:** 384–388

Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben-David Y, Lawler J, Henkin J, Huber J, Hicklin DJ, D'Amato RJ, Kerbel RS (2005) Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. *Cancer Cell* **7:** 101–111

Shaked Y, Kerbel RS (2007) Antiangiogenic strategies on defense: on the possibility of blocking rebounds by the tumor vasculature after chemotherapy. *Cancer Res* **67:** 7055–7058

Stempak D, Gammon J, Halton J, Moghrabi A, Koren G, Baruchel S (2006) A pilot pharmacokinetic and antiangiogenic biomarker study of celecoxib and low-dose metronomic vinblastine or cyclophosphamide in pediatric recurrent solid tumors. *J Pediatr Hematol Oncol* **28:** 726–728

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* **92:** 205–216

Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, Canon JL, Van Laethem JL, Maurel J, Richardson G, Wolf M, Amado RG (2007) Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* **25:** 1658–1664

Vogt T, Hafner C, Bross K, Bataille F, Jauch KW, Berand A, Landthaler M, Andreesen R, Reichle A (2003) Antiangiogenetic therapy with pioglitazone, rofecoxib, and metronomic trofosfamide in patients with advanced malignant vascular tumors. *Cancer* **98:** 2251–2256

Young SD, Whisseli M, Noble JC, Cano PO, Lopez PG, Germond CJ (2006) Phase II clinical trial results involving treatment with low-dose daily oral cyclophosphamide, weekly vinblastine, and rofecoxib in patients with advanced solid tumors. *Clin Cancer Res* **12:** 3092–3098

einstein
Official Publication of the Instituto Israelita de Ensino e Pesquisa Albert Einstein

ISSN: 1679-4508 | e-ISSN: 2317-6385

REVIEW

Standardization of the infusion sequence of antineoplastic drugs used in the treatment of breast and colorectal cancers

Padronização da ordem de infusão de medicamentos antineoplásicos utilizados no tratamento dos cânceres de mama e colorretal

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DOI: 10.1590/S1679-45082018RW4074

ABSTRACT

The definition of antineoplastic administration sequences can help planning of therapeutic regimens in a more rational way, and thus optimize chemotherapy effects on patients, increasing efficacy and reducing toxic effects. In this way, this study aimed to evaluate the infusion order of antineoplastic agents of the main therapeutic protocols used in the treatment of colorectal and breast cancer which are used in a tertiary hospital, identifying possible interactions dependent on the infusion sequence. For the definition of protocols adopted in the hospital, medical prescriptions were used in the period of January to March 2016 and a literature review was conducted to search for studies assessing the sequence of administering the selected regimens. The databases used were SciELO, LILACS and MEDLINE, in addition to Micromedex Solutions® and UpToDate®. A total of 19 protocols were identified for antineoplastic therapy, 11 for colorectal cancer and 8 for breast cancer. The selected articles provided evidence for administration order of 19 protocols, and three protocols did no report relevance of infusion sequence. Sequence-dependent interactions were mainly related to toxicity, pharmacokinetics and efficacy of the drug combination. The definition of the infusion sequence has a great impact on the optimization of therapy, increasing efficacy and safety of the protocols containing combined antineoplastic therapies.

Keywords: Administration, intravenous; Antineoplastic agents/administration & dosage; Breast neoplasms; Colorectal neoplasms

RESUMO

A definição de sequências de administração de antineoplásicos pode proporcionar o planejamento dos esquemas terapêuticos de forma mais racional e, assim, otimizar o efeito da quimioterapia nos pacientes, aumentando a eficácia e reduzindo o aparecimento de efeitos tóxicos. Desta forma, o objetivo deste estudo foi avaliar a ordem de infusão dos antineoplásicos constituintes dos principais protocolos terapêuticos para o tratamento dos cânceres de mama e colorretal utilizados em um hospital terciário, identificando possíveis interações dependentes da sequência de infusão. Para definição dos protocolos adotados na instituição, foram utilizadas as prescrições no período de janeiro a março de 2016, sendo então realizada uma revisão de literatura, para buscar estudos que avaliaram a sequência de administração dos esquemas selecionados. Para tanto, as seguintes

How to cite this article:
Silva AA, Carlotto J, Rotta I. Standardization of the infusion sequence of antineoplastic drugs used in the treatment of breast and colorectal cancers. einstein (São Paulo). 2018;16(2):eRW4074.

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Received on:
Mar 30, 2017

Accepted on:
Nov 6, 2017

Conflict of interest:
none

bases de dados foram utilizadas: SciELO, LILACS e MEDLINE, além das plataformas Micromedex Solutions® e UpToDate®. Foram identificados 19 protocolos para terapia antineoplásica, sendo 11 para câncer colorretal e 8 para câncer de mama. Os artigos selecionados forneceram evidências para ordem de administração de 19 protocolos, e em 3 protocolos, não foi evidenciada a relevância da sequência infusional. As interações dependentes de sequência foram principalmente relacionadas à toxicidade, farmacocinética e eficácia da combinação de fármacos. A definição da sequência infusional possui grande impacto na otimização da terapia, aumentando a eficácia e a segurança dos protocolos, contendo terapias combinadas de antineoplásicos.

Descritores: Administração intravenosa; Antineoplásicos/administração & dosagem; Neoplasias da mama; Neoplasias colorretais

INTRODUCTION

Cancer is a worldwide public health issue. In 2012, there were 14.1 million cases of cancer around the world, and it is estimated this number will reach 24 million, in 2025.[1] According to the *Instituto Nacional de Câncer "José Alencar Gomes da Silva"* (INCA), in 2016/2017, Brazil had approximately 600 thousand new cases of cancer; in that, breast (28.1%) and colorectal cancer (8.6%) were the most prevalent among women, and prostate (28.6%), tracheal, bronchial and lung (8.1%), and colorectal (7.8%), in males. Non-melanoma skin cancer was not included in this estimate and is the most prevalent in both sexes.[2] Colorectal cancer is among the five most frequent neoplasms, and its incidence is not homogeneous throughout Brazil; it is more prevalent in the South and Southeast regions of the country.[3]

The combination of several drugs with different mechanisms of action is an effective strategy in cancer treatment and provides many benefits. First, the association of two or more drugs with different mechanisms of actions can delay cell mutations and the process of adjustment to cancer. Second, the synergistic effect of the drugs, that is, the combined action of medications leading to potentiated biological effect.[4] The pharmacological mechanisms involved in the process of interactions among intravenous solutions are basically pharmacokinetic interactions (involving factors that accelerate or delay absorption, distribution, metabolization and elimination of the drugs used) and pharmacodynamic interactions (factors leading to dysfunction in the pharmacological receptor binding).[5] Several antineoplastic agents (*e.g.* doxorubicin, docetaxel, paclitaxel, etc.) are metabolized via the cytochrome P450 pathway (CYP), and other chemotherapeutic drugs (*e.g.* taxanes and platinum agents) present high levels of protein binding. Moreover, many chemotherapeutic drugs have specific mechanisms of action during the cell cycle, and can increase cytotoxicity or antagonize the mechanism of the second agent.[6]

Recognizing these pharmacokinetic interactions between drugs is important to optimize doses of cytotoxic agents in combined chemotherapy. Many drugs called "cell cycle specific drugs" (CCS) are effective against cancer and act on cells that are in the cell cycle. A second group of agents called "cell cycle nonspecific drugs" (CCNS) are able to kill tumor cells, regardless of their being in the cell cycle or at rest.[7] Regarding the infusion order, if the specific antineoplastic agents are administered before nonspecific cycles, maximized effect in cells with high cell division rates, such as neoplastic cells, is theoretically expected. The reason is when the cell cycle is interrupted at the time of division, nonspecific agents can more easily act in the DNA.[8] Furthermore, it is argued that it is preferable to first administer the vesicant antineoplastic drug, considering vascular integrity decreases with time. It is therefore advantageous to infuse the vesicant antineoplastic agent when the vein is more stable and less irritated.[9]

As a focus of this study, we have chosen to work with protocols used for treatment of breast and colorectal cancers, for being the most prevalent in women (breast and colorectal) and are among the most prevalent in men (colorectal), especially in the South and Southeast regions of Brazil.

Even though many reports have been published on this topic, there are no studies containing the evaluation of infusion sequence for all protocols used in antineoplastic therapy. Therefore, to define the infusion sequence, we must often rely on pharmacokinetic and pharmacodynamic evaluations and on drug characteristics. In the literature, there is not yet one single source compiling the ideal administration sequences for the main protocols used in antineoplastic therapy.

OBJECTIVE

To identify interactions that depend on the infusion sequence to establish recommendations for the administration of antineoplastic agents in protocols used in breast and colorectal cancer treatments.

METHODS

This is a literature review study about the evaluation of infusion sequence of antineoplastic drugs in protocols used in colorectal and breast cancer treatments, employed in a tertiary hospital in the city of Curitiba (State of Paraná).

To define therapeutic protocols containing antineoplastic drug combinations used at the organization, we analysed medical prescriptions of adult patients seen at the oncology outpatient clinic. We selected prescriptions given between January and March 2016 that included the combinations of antineoplastic drugs used in breast and colorectal cancer treatment. We excluded prescriptions containing regimens in which the antineoplastic drugs had not been administered on the same day, or those that included only one antineoplastic agent, since the objective of the study was to determine the medication infusion sequence.

Based on the therapeutic protocols selected from medical prescriptions, we conducted a literature review to search for studies evaluating the administration sequence of the selected regimens. To that end, we used the databases Scientific Electronic Library Online (SciELO), Latin American and Caribbean Health Sciences Literature (LILACS) and MEDLINE (PubMed). The keywords to identify studies related to the theme included any combination of the protocol name and/or antineoplastic drug, with the English words "administration", "sequencing" or "interactions". Additionally, we used the software Micromedex Solutions® and UpToDate® to collect information on the drugs and on possible drug interactions. We included all articles about protocols of interest, giving preference to those conducted in humans and excluding the studies containing protocols of drugs not administered on the same day of treatment.

For situations without a clearly established infusion sequence or with disagreement among authors, we evaluated pharmacokinetics, pharmacodynamics and characteristics of each antineoplastic drug to define the most appropriate sequence. This study was approved by the Research Ethics Committee, protocol 1.776.532, CAAE: 59998016.0.0000.0096.

RESULTS

A total of 408 prescriptions containing treatment protocols for colorectal and breast cancer were evaluated. Most patients were female (65.8%) and mean age was 53.56 years.

Table 1 shows therapeutic protocols used at the organization for breast and colorectal cancer with the respective number of patients for whom they were prescribed.

Table 1. Therapeutic protocols

Protocols	n (%)
AC*	49 (27.53)
FLOX†	19 (10.67)
B-FOL†	18 (10.11)
Cisplatin + gemcitabine	17 (9.55)
MAYO†	15 (8.43)
TC‡	10 (5.62)
Cisplatin + irinotecan	9 (5.06)
Fluorouracil + folinic acid	7 (3.93)
Gemcitabine + docetaxel	7 (3.93)
FOLFIRI§	6 (3.37)
FOLFOX†	5 (2.81)
IFL§	2 (1.12)
MC DONALD†	2 (1.12)
Paclitaxel + pamidronate	2 (1.12)
M-FOLFOX	2 (1.12)
Paclitaxel + zoledronic acid	2 (1.12)
Docetaxel + pamidronate	1 (0.56)
Trastuzumab + pertuzumab	1 (0.56)
CAF*	1 (0.56)
Cisplatin + paclitaxel	1 (0.56)
FOLFOXIRI**	1 (0.56)
Trastuzumab + paclitaxel	1 (0.56)

* Protocol AC was composed of doxorubicin and cyclophosphamide; † protocols FLOX and B-FOL were composed of oxaliplatin, folinic acid and fluorouracil, in different combination regimens; protocols MAYO and MC DONALD included folinic acid and fluorouracil; ‡ protocol TC was composed of docetaxel and cyclophosphamide; § protocols FOLFIRI and IFL comprised irinotecan, folinic acid and fluorouracil in different combination regimens; ‖ protocol FOLFOX was composed of oxaliplatin, folinic acid and fluorouracil; ¶ protocol CAF comprised doxorubicin, fluorouracil and cyclophosphamide; ** protocol FOLFOXIRI included irinotecan, oxaliplatin, folinic acid and fluorouracil.

In total, 178 patients used 22 different protocols containing a combination of antineoplastic drugs.

The bibliographic search yielded 36 articles that were the basis to evaluate these protocols. The selected articles brought some evidence towards the administration sequence of 19 protocols (Table 2), while for 3 protocols there was no evidence of relevance in the infusion sequence (Table 3). Interactions dependent on the sequence were mainly related to toxicity, pharmacokinetics and efficacy of drug combination.

Table 2. Suggested sequence for the therapeutic protocol and reasons for infusion order

Protocol	Suggested sequence Agent				Reasons for suggestion
	First	Second	Third	Fourth	
AC	Doxorubicin CCNS[7] Vesicant[8]	Cyclophosphamide CCNS[7]	-	-	Compatible in Y[1,9] Cyclophosphamide is a prodrug catalyzed directly by cytochrome P450, mainly through CYP2B6.[10,11] Doxorubicin is a (moderate) inhibitor of the same enzyme.[12] To avoid delayed plasma clearance of doxorubicin due to cyclophosphamide metabolism, it is recommended to infuse doxorubicin before cyclophosphamide.[5]
CAF	Doxorubicin CCNS[7] Vesicant[8]	Fluorouracil CCS[7]	Cyclophosphamide CCNS[7]	-	Compatible in Y[1,9] Fluorouracil acts in specific cell cycle phases, while doxorubicin and cyclophosphamide are CCNS.[7] However, doxorubicin has a high tissue vesicant potential, which reinforces the importance of it being administered first.[8] Cyclophosphamide is a prodrug catalyzed directly by cytochrome P450, especially through CYP2B6.[10,11] Doxorubicin is a (moderate) inhibitor of the same enzyme.[12] To avoid delayed plasma clearance of doxorubicin due to cyclophosphamide metabolism, it is recommended to infuse doxorubicin before cyclophosphamide.[5] Regarding fluorouracil and cyclophosphamide, it has been observed that fluorouracil sensitizes the DNA so it can be attacked by alkylating agents.[13]
Cisplatin + Gemcitabine	Gemcitabine CCS[7]	Cisplatin CCNS[7]	-	-	Compatible in Y[1,9] Gemcitabine is a CCS drug while cisplatin is CCNS, which justifies this infusion sequence. Also, gemcitabine administration (4 or 24 hours) before cisplatin administration proved less toxic, causing less leukopenia.[14]
Cisplatin + irinotecan	Cisplatin CCNS[7]	Irinotecan CCS[7]	-	-	Compatible in Y[1,9] Irinotecan is a CCS drug, while cisplatin is CCNS. However, with previous administration of cisplatin, there is an increase in the synergistic effect, with the presence of toxicity regardless of the administration sequence.[15,16]
Cisplatin + paclitaxel	Paclitaxel CCS[7]	Cisplatin CCNS[7]	-	-	Compatible in Y[1,9] Paclitaxel is a CCS drug, whereas cisplatin is CCNS, which justifies this infusion sequence.[7] When cisplatin is administered first, paclitaxel clearance is reduced, and myelosuppression is more severe. It has been suggested that this decrease in paclitaxel clearance, after cisplatin, may be due to cytochrome P450 inhibition, which is responsible for paclitaxel metabolism.[17]
Docetaxel + pamidronate	Docetaxel CCS[7]	Pamidronate	-	-	Compatible in Y[1,9] No studies were found on administration order. The recommendation is to administer docetaxel first, considering pamidronate may cause nephrotoxicity, which manifests as nephritic syndrome, kidney function deterioration and renal failure, which could alter docetaxel excretion.[18]
FOLFIRI	Irinotecan CCS[7] + Folinic acid (60 minutes prior)	Fluorouracil CCS[7] (bolus)	Fluorouracil CCS[7] (continuous infusion – 46 hours)	-	Incompatible in Y (irinotecan – fluorouracil)[19] Folinic acid stabilizes thymidylate synthase when administered before fluorouracil, increasing the efficacy and cytotoxicity of the latter.[20,21] Moreover, for better action of folinic acid, a minimum 60-minute period is required for drug distribution and intracellular metabolism.[5] We observed a synergistic effect when there was previous exposure to irinotecan, intensifying fluorouracil-induced DNA damage.[22] According to a study by Falcone et al., toxicity was affected by the administration sequence of irinotecan and fluorouracil, with acceptable toxicity when irinotecan was followed by fluorouracil.[23] To provide faster patient care, the concomitant initial infusion of irinotecan and folinic acid was proposed, followed by fluorouracil in bolus and fluorouracil in continuous infusion.[5] It is worth mentioning the importance of clearing the Y system between the infusions of irinotecan and fluorouracil, due to incompatibility.

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Table 2. Suggested sequence for the therapeutic protocol and reasons for infusion order

Protocol	Suggested sequence				Reasons for suggestion
	Agent				
	First	Second	Third	Fourth	
FOLFOXIRI	Irinotecan CCS[27]	Oxaliplatin CCNS[27] + Folinic acid (60 minutes prior)	Fluorouracil CCS[27] (continuous infusion – 46 hours)	-	Incompatible in Y (irinotecan – fluorouracil)[28] Not tested in Y (fluorouracil – oxaliplatin)[28] Irinotecan is a prodrug and thus requires hepatic microsomal activation. Therefore, whenever possible, this type of drug should be prioritized in multiple infusions.[28] Also, some studies demonstrated there may be an increase in cholinergic side effects of irinotecan when administered after oxaliplatin.[29] Dodds et al., observed the cholinergic effects of irinotecan are manifested by inhibiting acetylcholinesterase.[29] Similarly to other alkylating agents, oxaliplatin can also inhibit this enzyme, which can potentiate the cholinergic effects of irinotecan. Regarding the combination between oxaliplatin and fluorouracil, Qin et al. observed *in vitro* synergistic effect, *i.e.*, apoptosis was more prominent when cells were treated with oxaliplatin first and then with fluorouracil.[28] Furthermore, some studies demonstrated oxaliplatin can inhibit the main enzyme of fluorouracil metabolism (dihydropyrimidine dehydrogenase).[28] A synergistic effect was observed when there was previous exposure to irinotecan, intensifying DNA damage induced by fluorouracil.[27] According to a study by Falcone et al., toxicity was affected by the administering sequence of irinotecan and fluorouracil, with acceptable toxicity when irinotecan was followed by fluorouracil.[28] Folinic acid stabilizes thymidylate synthase when administered before fluorouracil, increasing efficacy and cytotoxicity of the latter.[15,28] Moreover, for better action of folinic acid, a minimum 60-minute period is required for the distribution of the drug and intracellular metabolism.[28] It is worth mentioning the importance of cleaning the Y system between the infusions of irinotecan and fluorouracil, due to incompatibility.
FLOX/5-FOL	Oxaliplatin CCNS[27]	Folinic acid (60 minutes prior)	Fluorouracil CCS[27]	-	Not tested in Y (fluorouracil – oxaliplatin).[18] Folinic acid stabilizes thymidylate synthase when administered before fluorouracil, increasing efficacy and cytotoxicity of the latter.[19,20] Moreover, for better action of folinic acid, a minimum 60-minute period is required for distribution of the drug and intracellular metabolism.[5] Regarding the combination between oxaliplatin and fluorouracil, Qin et al. observed *in vitro* synergistic effect, *i.e.*, apoptosis was more prominent when cells were treated with oxaliplatin first and then with fluorouracil.[28] Furthermore, some studies demonstrated that oxaliplatin can inhibit the main enzyme of fluorouracil metabolism (dihydropyrimidine dehydrogenase).[28]
FOLFOX	Oxaliplatin CCNS[27] + Folinic acid (60 minutes prior)	Fluorouracil CCS[27] (bolus)	Fluorouracil CCS[27] (continuous infusion – 46 hours)		Not tested in Y (fluorouracil – oxaliplatin).[18] Folinic acid stabilizes thymidylate synthase when administered before 5-FU, increasing the efficacy and cytotoxicity of the latter.[19,20] Moreover, for better action of the folinic acid, a minimum 60-minute period is required for distribution of the drug and intracellular metabolism.[5] Regarding the combination between oxaliplatin and 5-FU, Qin et al., observed *in vitro* synergistic effect; apoptosis was more prominent when cells were treated with oxaliplatin first and then with 5-FU.[28] Furthermore, some studies demonstrated oxaliplatin can inhibit the main enzyme of 5-FU metabolism (dihydropyrimidine dehydrogenase).[28] To provide more efficient patient care, concomitant initial infusion of oxaliplatin and folinic acid was proposed, followed by fluorouracil in bolus and fluorouracil in continuous infusion.[30]

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Table 2. Suggested sequence for the therapeutic protocol and reasons for infusion order

Protocol	Suggested sequence Agent				Reasons for suggestion
	First	Second	Third	Fourth	
IFL	Irinotecan CCS[7]	Folinic acid (60 minutes prior)	Fluorouracil CCS[7]	-	Incompatible in Y (irinotecan – fluorouracil).[15] Folinic acid stabilizes thymidylate synthase when administered before fluorouracil, increasing the efficacy and cytotoxicity of the latter.[16,20] Moreover, for better action of the folinic acid, a minimum 60-minute period is required for distribution of the drug and intracellular metabolism.[5] A synergistic effect was observed when there was previous exposure to irinotecan, intensifying DNA damage induced by fluorouracil.[16] According to a study by Falcone et al., toxicity was affected by the administering sequence of irinotecan and fluorouracil, with acceptable toxicity when irinotecan was followed by fluorouracil.[20] It is worth mentioning the importance of cleaning the Y system between the infusions of irinotecan and fluorouracil, due to incompatibility.
MAYO/ McDonald/ fluorouracil + folinic acid	Folinic acid (60 minutes prior)	Fluorouracil CCS[7]	-	-	Compatible in Y[15] Folinic acid stabilizes thymidylate synthase when administered before fluorouracil, increasing the efficacy and cytotoxicity of the latter.[16,20] Moreover, for better action of the folinic acid, a minimum 60-minute period is required for distribution of the drug and intracellular metabolism.[5]
Paclitaxel + zoledronic acid	Paclitaxel CCS[7]	Zoledronic acid	-	-	Compatible in Y[15] Increase of synergistic effects due to increased apoptosis.[28]
Paclitaxel + pamidronate	Paclitaxel CCS[7]	Pamidronate	-	-	Compatible in Y[15] No studies were found describing an administration sequence. The recommendation is to administer paclitaxel first, considering pamidronate can cause nephrotoxicity, which manifests as nephritic syndrome, kidney function deterioration and renal failure, which could alter paclitaxel excretion.[12]
TC	Docetaxel CCS[7]	Cyclophosphamide CCNS[7]	-	-	Compatible in Y[15] Docetaxel is a CCS drug, while cyclophosphamide is a CCNS drug, which justifies this infusion sequence. Cyclophosphamide is a prodrug, catalyzed directly through cytochrome P450.[29] Docetaxel is also oxidated by cytochrome P450 enzymes, especially by CYP3A4 in the liver.[30] We found a few controversial studies that relate ifosfamide and docetaxel because ifosfamide and cyclophosphamide are similar.[30] Schijvers et al. observed the AUC of ifosfomide and its metabolites were smaller when docetaxel was administered first.[31] A study by Ando et al., suggests that docetaxel can competitively inhibit the biotransformation of the prodrug ifosfamide through the isoenzyme CYP3A4 of cytochrome P450.[32] However, the studies we found present no clear evidence for an administration sequence of these drugs.
Trastuzumab + paclitaxel	Trastuzumab	Paclitaxel CCS[7]	-	-	Compatible in Y[15] Lee et al., observed pre-treatment with trastuzumab resulted in better sensitization of breast cancer cells, i.e., trastuzumab followed by paclitaxel increased the activation and induction of programmed cell death or cell apoptosis.[33] Moreover, due to the possible infusion reaction of the monoclonal antibody, it is recommended that trastuzumab be infused first.[34,35]

CCNS: cell-cycle nonspecific; CCS: cell-cycle specific; AUC: area under the curve.

Table 3. Combination of chemotherapeutic agents in which sequence has no effect on efficacy or toxicity

Carboplatin + paclitaxel[36,37]
Gemcitabine + docetaxel[38,39]
Trastuzumab + pertuzumab[40-42]

DISCUSSION

There are few studies in the literature evaluating the infusion sequence of antineoplastic drugs in chemotherapy protocols. The definition of more adequate administration sequences, considering the

constituent drugs, can help planning of therapeutic regimens in a more rational way, and thus optimize chemotherapy effects on patients, increasing efficacy and reducing toxic effects. The main criteria to be considered when defining the ideal order are: pharmacokinetics/pharmacodynamics (including the cell cycle phase in which the antineoplastic drugs act) and drug characteristics (vesicant/irritant and incompatibilities). Moreover, considering that the main target is cell division, the drugs affect all normal tissues in quick division, and they will probably produce some toxicity, either at high or low levels.[43]

Based on information related to each drug's pharmacokinetics and pharmacodynamics, it is possible to evaluate antagonism or synergism, in relation to efficacy and safety. The synergistic effect is one of the main reasons for using combined chemotherapy. Synergy is defined as an expected additive effect when individual drugs are combined. Antagonism is when the combination of two drugs reduces or nullifies the effects of one or both. The combination of folinic acid (LV) and fluorouracil (5FU) is an example of two drugs that synergistically act on the same target, yielding antitumoral results.[44] Folinic acid increases the effect of 5FU by stabilizing the binding of its converted form (fluorodeoxyuridine acid) to thymidylate synthase, contributing to inhibition of this important enzyme in DNA repair and replication.[19,20]

Among the effects related to the pharmacokinetics ofthe drugs studied, the most frequent were related to metabolism/biotransformation and antineoplastic medication elimination/excretion. Although some drugs are metabolized in their place of absorption, the primary metabolism site is the liver, especially in the P450 cytochrome enzyme complex. Drugs, food and some herbs can induce or inhibit enzymes involved in drug metabolism.[45] Therefore, it is important to understand the role of enzymes and transporters in the metabolism of antineoplastic agents and the mechanisms through which they modulate their expression and activity.

Enzyme inhibition usually leads to an increase in the metabolic rate and in the serum concentration of the drug, which can result in increased therapeutic response or toxicity. Enzyme inhibition effects are relatively fast, with their initial effects appearing in 24 hours. Therefore, patients must be frequently monitored.[46] A well-known example of drug interaction due to enzyme inhibition is the one between cyclophosphamide and doxorubicin. Cyclophosphamide is a substrate of the enzyme CYP2B6, which means it is a prodrug and requires biotransformation to produce its pharmacologically active cytotoxic compounds. Doxorubicin is a (moderate) inhibitor of this same enzyme. Therefore, CYP inhibitors can reduce the metabolism of substrates of this pathway, leading to decreased serum concentration of cyclophosphamide.[12]

Most antineoplastic drugs affect mainly cell division. In many cases, the antiproliferative action of antineoplastic drugs is directly in the DNA, resulting in permanent damage and initiating cell apoptosis. A better result of these antineoplastic drugs can be obtained through the action during the S phase of the cell cycle, when the cell is synthetizing a new DNA.[44] Thus, regarding infusion order, if we administer a CCS antineoplastic drug before a CCNS agent, we can theoretically expect a maximization of the effects on cells with a high cell division rate, such as the neoplastic cells. Thus, with an interrupted cell cycle, antineoplastic agents can more easily act in the DNA.

In addition, the higher the exposure to antineoplastic drugs, the less stable and more fragile veins become. As a consequence, drugs administered last have a higher chance of leakage, regardless of the technique employed. It is better to administer the vesicant antineoplastic drug first, when the vein is more stable and less irritated. Another possibility is using the "sandwich technique": a non-vesicant antineoplastic drug first, then the vesicant drug, and lastly another non-vesicant. However, vein integrity decline due to successive cytotoxic cycles suggests vesicant drugs are safer when administered first.[47] Therefore, administering vesicant drugs first proves more advantageous and safer for patients.

The limitations of this study include the fact that, in some cases, the infusion sequence was not clearly described in the literature, such as: a) the combination of pamidronate and paclitaxel or docetaxel – the infusion order was based on the fact that pamidronate can cause nephrotoxicity, which could alter the excretion of the other drugs; and b) the combination of cyclophosphamide and docetaxel, whose data were extrapolated from studies with ifosfomide, which belongs to the same drug class of cyclophosphamide.

Nevertheless, this review showed that many infusion sequences have already been well-defined in the literature, regarding safety, efficacy, prevention of excessive toxicity or reduced efficacy. Therefore, understanding the potential drug interactions, medical teams can minimize risks by prescribing adequate drugs with an appropriate infusion order and monitoring signs of interactions.

CONCLUSION

Defining an infusion sequence greatly optimizes treatment. Such sequences must be based on studies

(preferably carried out in humans, about specific sequence evaluations), and pharmacokinetics, pharmacodynamics and drug properties (vesicant drugs or incompatibilities). By means of this research, we propose increasing efficacy and safety of protocols that include combined antineoplastic treatments, and standardizing the administration sequences.

ACKNOWLEDGMENTS

Thanks to the Hospital Pharmacy at the *Complexo Hospital de Clínicas da Universidade Federal do Paraná*, and to the Multiprofessional Residency Program, for supporting the development of this service.

REFERENCES

1. World Cancer Research Fund International [Internet]. London: World Cancer Research Fund International [cited 2017 Jan 25]. Available from: http://www.wcrf.org
2. Brasil. Ministério da Saúde. Instituto Nacional de Câncer José de Alencar Gomes da Silva (INCA). Estimativa 2016: incidência de câncer no Brasil [Internet]. Rio de Janeiro (RJ): INCA; 2015 [citado 2017 Jul 28]. Disponível em: http://santacasadermatoazulay.com.br/wp-content/uploads/2017/06/estimativa-2016-v11.pdf
3. Habr Gama A. [Colorectal cancer: the importance of its prevention]. Arq Gastroenterol. 2005;42(1):2-3. Portuguese.
4. He Z, Huang J, Xu Y, Zhang X, Tang Y, Huang C, et al. Co-delivery of cisplatin and paclitaxel by folic acid conjugated amphiphilic PEG-PLGA copolymer nanoparticles for the treatment of non-small lung cancer. Oncotarget. 2015;6(39):42150-68.
5. Bonassa EM, Gato MI. Terapêutica Oncológica para Enfermeiros e Farmacêuticos. 4a ed. São Paulo: Atheneu; 2012.
6. Mancini R, Modlin J. Chemotherapy Administration Sequence: a review of the literature and Creation of a Sequencing Chart. J Hematol Oncol Pharm. 2011;1(1):17-25. Review.
7. Almeida VL, Leitão A, Reina LC, Montanari CA, Donnici CL, Lopes MT. [Cancer and cell cicle-specific and cell cicle nonspecific anticancer DNA-interactive agents: an introduction]. Quim Nova. 2005;28(1):118-29. Portuguese.
8. Rodrigues R. Ordem de Infusão de Medicamentos Antineoplásicos - Sistematização de informações para auxiliar a discussão e criação de protocolos assistenciais. São Paulo: Atheneu; 2015.
9. How C, Brown J. Extravasation of cytotoxic chemotherapy from peripheral veins. Eur J Oncol Nurs. 1998;2(1):51-8.
10. Micromedex 2.0 Solutions. Intravenous (IV) Compatibility Search [Internet]. Ann Arbor (MI): Truven Health Analytics Inc. 2013 [cited 2017 Jan 20]. Available from: http://www-micromedexsolutions-com.ez22.periodicos.capes.gov.br/micromedex2/librarian/CS/359299/ND_PR/evidencexpert/ND_P/evidencexpert/DUPLICATIONGHIELDSYNC/282BE0/ND_PG/evidencexpert/ND_B/evidencexpert/ND_AppProduct/evidencexpert/ND_T/evidencexpert/PFActionId/evidencexpert.FindIVCompatibility?navItem=topIVSis.locPage=true
11. Chang TK, Weber GF, Crespi CL, Waxman DJ. Differential Activation of Cyclophosphamide and Ifosphamide by cytochromes p-450 2b and 3a in human liver microsomes. Cancer Res. 1993;53(23):5629-37.
12. Bachmann KA, Lewis JD, Fuller MA, Bonfiglio MF. Interações medicamentosas: o novo padrão de interações medicamentosas e fitoterápicas. Um guia completo dos substratos, indutores e inibidores de enzimas do citocromo P450. 2a ed. Barueri (SP): Manole; 2006.
13. Santelli G, Valeriote F, Vietti T, Coulter D. Cytotoxic effect of 5-fluorouracil plus cyclophosphamide against transplantable leukemias. Eur J Cancer. 1981; 17(5):629-34.
14. Kroep JR, Peters GJ, van Moorsel CJ, Catik A, Vermorken JB, Pinedo HM, et al. Gemcitabine-cisplatin: a schedule finding study. Ann Oncol. 1999; 10(12):1503-10.
15. de Jonge MJ, Verweij J, Planting AS, van Der Burg ME, Stoter G, de Boer-Dennert MM, et al. Drug administration sequence does not change pharmacodynamics and kinetics of irinotecan and cisplatin. Clin Cancer Res. 1998;5(8):2012-7.
16. Han JY, Lim HS, Lee DH, Ju SY, Lee SY, Kim HY, et al. Randomized Phase II study of two opposite administration sequences of irinotecan and cisplatin in patients with advanced nonsmall cell lung carcinoma. Cancer. 2006; 106(4):873-80.
17. Kennedy MJ, Zahurak ML, Donehower RC, Noe DA, Sartorius S, Chen TL, et al. Phase I and pharmacologic study of sequences of paclitaxel and cyclophosphamide supported by granulocyte colony-stimulating factor in women with previously treated metastatic breast cancer. J Clin Oncol. 1996;14(3):783-91.
18. Micromedex 2.0Solumtions. Pamidronate Disodium, Medication Safety, Precautions [Internet]. Ann Arbor (MI): Truven Health Analytics Inc. 2013 [cited 2017 Jan 20]. Available from: http://www-micromedexsolutions-com.ez22.periodicos.capes.gov.br/micromedex2/librarian/PFDefaultActionId/evidencexpert.DoIntegratedSearch#
19. Jolivet J. Role of leucovorin dosing and administration schedule. Eur J Cancer. 1995;31A(7-8):1311-5. Review.
20. Rustum YM, Cao S, Zhang Z. Rationale for treatment design: biochemical modulation of 5-fluorouracil by leucovorin. Cancer J Sci Am. 1998;4(1):12-8. Review.
21. Grivicich I. A importância da sequência de administração do irinotecan e 5-fluorouracil na inibição da proliferação do carcinoma de cólon humano in vitro [tese]. Porto Alegre: Universidade Federal do Rio Grande do Sul; 1999.
22. Falcone A, Di Paolo A, Masi G, Allegrini G, Danesi R, Lencioni M, et al. Sequence effect of irinotecan and fluorouracil treatment on pharmacokinetics and toxicity in chemotherapy-naive metastatic colorectal cancer patients. J Clin Oncol. 2001;19(15):3458-62.
23. Valencak J, Raderer M, Kornek GV, Henja MH, Scheithauer W. Irinotecan-related cholinergic syndrome induced by coadministration of oxaliplatin. J Natl Cancer Inst. 1998;90(2):160.
24. Dodds HM, Bishop JF, Rivory LP. More about: irinotecan-related cholinergic syndrome induced by coadministration of oxaliplatin. J Natl Cancer Inst. 1998;91(1):91-2.
25. Qin B, Tanaka R, Shibata Y, Arita S, Ariyama H, Kusaba H, et al. In-vitro schedule-dependent interaction between oxaliplatin and 5-fluorouracil in human gastric cancer cell lines. Anticancer Drugs. 2006;17(4):445-53.
26. Fischel JL, Formento P, Ciccolini J, Rostagno P, Etienne MC, Catalin J, et al. Impact of the oxaliplatin-5 fluorouracil-folinic acid combination on respective intracellular determinants of drug activity. Br J Cancer. 2002;86(7):1162-8.
27. Buzaid AC, Hoff PM, Maluf FC. Mini-MOC - poket do manual de oncologia clínica do Hospital Sírio-Libanês. São Paulo: Dendrix; 2009.
28. Neville-Webbe HL, Evans CA, Coleman RE, Holen I. Mechanisms of the synergistic interaction between the bisphosphonate zoledronic acid and the chemotherapy agent paclitaxel in breast cancer cells in vitro. Tumor Biol. 2006;27(2):92-103.
29. Miyoshi Y, Ando A, Takamura Y, Taguchi T, Tamaki Y, Noguchi S. Prediction of response to docetaxel by CYP3A4 mRNA expression in breast cancer tissues. Int J Cancer. 2002;97(1):129-32.
30. Bramwell VH, Mouridsen HT, Santoro A, Blackledge G, Somers R, Verwey J, et al. Cyclophosphamide versus ifosfamide: final report of a randomized phase II trial in adult soft tissue sarcomas. Eur J Cancer Clin Oncol. 1987;23(3):311-21.

31. Schrijvers D, Pronk L, Highley M, Bruno R, Locci-Tonelli D, De Bruijn E, et al. Pharmacokinetics of ifosfamide are changed by combination with docetaxel: results of a phase I pharmacologic study. Am J Clin Oncol. 2000;23(4):358-63.

32. Ando Y. Possible metabolic interaction between docetaxel and ifosfamide. Br J Cancer. 2000;82(2):497.

33. Lee S, Yang W, Lan KH, Sellappan S, Kios K, Hortobagyi G, et al. Enhanced sensitization to taxol-induced apoptosis by herceptin pretreatment in ErbB2-overexpressing breast cancer cells. Cancer Res. 2002;62(20):5703-10.

34. Chung CH. Managing premedications and the risk for reactions to infusional monoclonal antibody therapy. Oncologist. 2008;13(6):725-32. Review.

35. Hannawa IS, Bestul DJ. Rituximab tolerability when given before or after CHOP. J Oncol Pharm Pract. 2011;17(4):381-6.

36. Markman M, Elson P, Kulp B, Peterson G, Zanotti K, Webster K, et al. Carboplatin plus paclitaxel combination chemotherapy : impact of sequence of drug administration on treatment-induced neutropenia. Gynecol Oncol. 2003;91(1):118-22.

37. Huizing MT, Giaccone G, van Warmerdam LJ, Rosing H, Bakker PJ, Vermorken JB, et al. Pharmacokinetics of paclitaxel and carboplatin in a dose-escalating and dose-sequencing study in patients with non-small-cell lung cancer. The European Cancer Centre. J Clin Oncol. 1997;15(1):317-29.

38. Harita S, Watanabe Y, Kiura K, Tabata M, Takigawa N, Kuyama S, et al. Influence of altering administration sequence of docetaxel, gemcitabine and cisplatin in patients with advanced non-small cell lung cancer. Anticancer Res. 2006;26(2B):1637-41.

39. Rizvi NA, Spiridonidis CH, Davis TH, Bhargava P, Marshall JL, Dahut W, et al. Docetaxel and gemcitabine combinations in non-small cell lung cancer. Semin Oncol. 1999;26(5 Suppl 16):27-31; discussion 41-2. Review.

40. Nahta R, Hung M, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res. 2004;64(7):2343-6.

41. Richard S, Selle F, Lotz JP, Khalil A, Gligorov J, Soares DG. Pertuzumab and trastuzumab: the rationale way to synergy. An Acad Bras Cienc. 2016;88 Suppl 1:565-77. Review.

42. Cortés J, Fumoleau P, Bianchi GV, Petrella TM, Gelmon K, Pivot X, et al. Pertuzumab monotherapy after trastuzumab-based treatment and subsequent reintroduction of trastuzumab: activity and tolerability in patients with advanced human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol. 2012;30(14):1594-600.

43. Rang HP, Dale MM, Ritter JM, Flower RJ, Henderson G. Rang & Dale Farmacologia. 7a ed. Rio de Janeiro: Elsevier; 2012.

44. Shah MA, Schwartz GK. The relevance of drug sequence in combination chemotherapy. Drug Resist Updat. 2000;3(6):335-56.

45. Scripture CD, Figg WD. Drug interactions in cancer therapy. Nat Rev Cancer. 2006;6(7):546-58. Review. Erratum in: Nat Rev Cancer. 2006;6(9):741.

46. Yap KY, Chui WK, Chan A. Drug interactions between chemotherapeutic regimens and antiepileptics. Clin Ther. 2008;30(3):1385-407. Review.

47. How C, Brown J. Extravasation of cytotoxic chemotherapy from peripheral veins. Eur J Oncol Nurs. 1998;2(1):51-8.

Phase 1 expansion study of irinotecan liposome injection (nal-IRI) in patients with metastatic breast cancer (mBC): findings from the cohort with active brain metastasis (BM)

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TRLS-06

Presented at the Society for Neuro-Oncology (SNO) Inaugural Conference on Brain Metastases | New York, NY, USA | August 16–17, 2019

Disclosures

Author disclosures

- COI for Dr Anders: research funding PUMA, Lilly, Merck, Seattle Genetics, Nektar, Tesaro, G1-Therapeutics
- Compensated consultant role: UMA (9/2018–9/2019), Genentech (1/2019–), Eisai (1/2019–present), Ipsen (2/2019–present)
- Royalties: UpToDate, Jones and Bartlett

Funding

- This study was sponsored by Ipsen

Liposomal Irinotecan

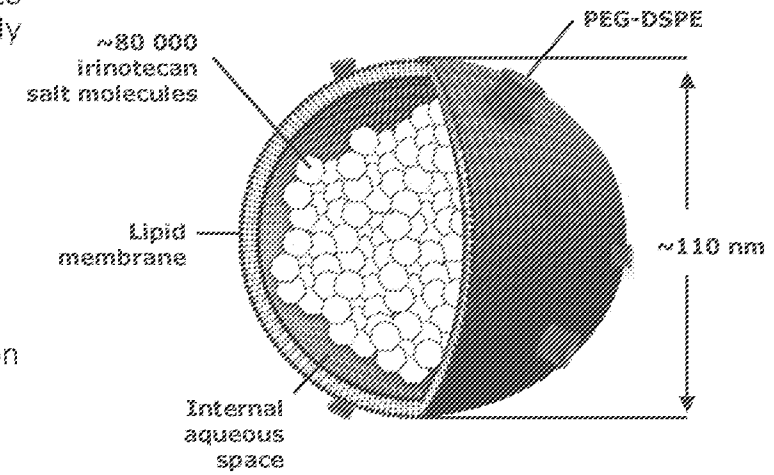
Liposomal irinotecan (nal-IRI) is a long-acting, liposomal encapsulation of irinotecan

- The encapsulated nal-IRI delivery system (nal-IRI) was developed to maximize efficacy of irinotecan and to reduce toxicities commonly associated with the non-liposomal formulation[1–3]

nal-IRI pharmacokinetics

- The half-life of total irinotecan following administration of nal-IRI is 25.8 hours
- 95% of irinotecan remains contained within the liposome during circulation
- Approximately fivefold higher levels of drug are found in tumors compared with plasma at 72 hours, suggesting local metabolic activation of irinotecan

- nal-IRI has been shown to accumulate in brain metastases (BM) and to prolong survival in animal models of breast cancer (BC) BM[4]
- Approved for treatment of metastatic adenocarcinoma of the pancreas after progression on gemcitabine-based therapy (70 mg/m^2 FBE) in combination with 5-FU and LV[5]



Nanoliposomal irinotecan

1. Maeda H *et al. J Control Release* 2000;65:271–84; 2. Bertrand N *et al. Adv Drug Deliv Rev* 2014;66:2–25; 3. Roy AC *et al. Ann Oncol* 2013;24:1567–73; 4. Mohammad AS *et al. Pharm Res* 2018;35:31; 5. Onivyde US IP. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/207793lbl.pdf (Accessed August 2019). FBE, free-base equivalent. 5_FU, 5-fluorouracil; LV, leucovorin.

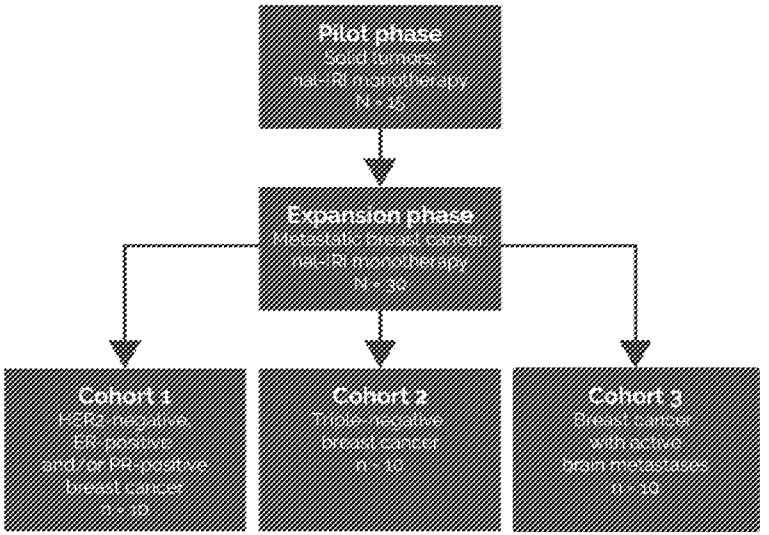
Study Objectives and Treatment

Study objective

- The expansion part of the phase 1 trial CITS (NCT01770353) assessed the efficacy and safety of nal-IRI monotherapy in patients with mBC
- This study evaluated patients with BC and active BM (cohort 3)

Treatment

- Pilot phase: 70 mg/m^2 (FBE) every 2 weeks
- At the start of the expansion phase, the dosing regimen was 70 mg/m^2 every 2 weeks
- Amendment during expansion phase: 50 mg/m^2 (FBE) every 2 weeks, then escalated to 70 mg/m^2 (FBE) every 2 weeks if tolerated



CITS study diagram

BC, breast cancer; BM, brain metastases; CITS, Cross Indication Translational Study; ER, estrogen receptor; FBE, free-base equivalent; HER2, human epidermal growth factor receptor 2; mBC, metastatic breast cancer; nal-IRI, liposomal formulation of irinotecan; PR, progesterone receptor

Study Methods

Study design

- Open-label, non-randomized, expansion, phase 1 trial

Key inclusion criteria

- Any sub-type of mBC with active BM
- Radiographic evidence of new or progressive CNS metastases after radiation therapy, with ≥ 1 lesion ≥ 1 cm in the longest dimension on gadolinium-enhanced MRI

Other key inclusion criteria

- ≥ 1 to ≤ 5 prior lines of cytotoxic therapy in a metastatic setting
- Neurologically stable
- No evidence of diffuse leptomeningeal disease on brain MRI/CSF
- Imaging not consistent with pseudo-progression following prior radiotherapy

Efficacy assessments

- Tumor assessments by CT or MRI every 8 weeks
- Change in non-CNS tumor burden was assessed using RECIST v1.1[1]
- Change in CNS tumor burden was assessed using modified RECIST criteria[2]

Safety assessments

- AEs were tabulated using NCI CTCAE (protocol version 4.02)

1. Eisenhauer EA *et al. Eur J Cancer* 2009;45:228–47; 2. Anders C *et al. Breast Cancer Res Treat* 2014;146:557–66. AE, adverse event; BM, brain metastases; CNS, central nervous system; CSF, cerebrospinal fluid; CT, computed tomography; mBC, metastatic breast cancer; MRI, magnetic resonance imaging; NCI CTCAE, National Cancer Institute Common Terminology Criteria for Adverse Events; RECIST, Response Evaluation Criteria in Solid Tumours

Baseline Demographics and Characteristics

Demographics	Cohort 1 (N = 10)	Cohort 2 (N = 10)	Cohort 3[a] (N = 10)	Total population (N = 30)
Sex, female, n (%)	10 (100)	10 (100)	10 (100)	30 (100)
Age, years				
Median (range)	56 (49–68)	52.5 (37–70)	45.5 (29–63)	53 (29–70)
Race, n (%)				
White	8 (80)	8 (80)	7 (70)	23 (77)
Black/African	0	1 (10)	1 (10)	2 (7)
Other	2 (20)	1 (10)	2 (20)	5 (16)
Months since metastatic diagnosis				
Median (range)	63.7 (16–87)	20.7 (0–34)	32.4 (8–55)	24.0 (0–87)
Number of prior cytotoxic anti-cancer regimens				
Median (range)	3.0 (1–6)	3.0 (0–5)	3.0 (1–6)[b]	3.0 (0–6)

[a]Triple-negative, n = 4; ER-positive or PR-positive and HER2-positive, n = 3; ER-positive or PR-positive and HER2-negative, n = 2; ER-negative or PR-negative and HER2-positive, n = 1. [b]Anthracycline, n = 6; taxane, n = 10; capecitabine, n = 7. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor

Safety

	Cohort 3 N = 10
Exposure	
Median (range), weeks	13.9 (10–105)
Adverse events, n (%)	
Any TEAE	10 (100)
Grade 3	6 (60)
Grade 4	2 (20)
Grade 5	0
TEAE related to nal-IRI	10 (100)
Grade ≥ 3	3 (30)
TEAE related to nal-IRI leading to dose adjustment	4 (40)
SAEs	7 (70)
SAE related to nal-IRI	1 (10)

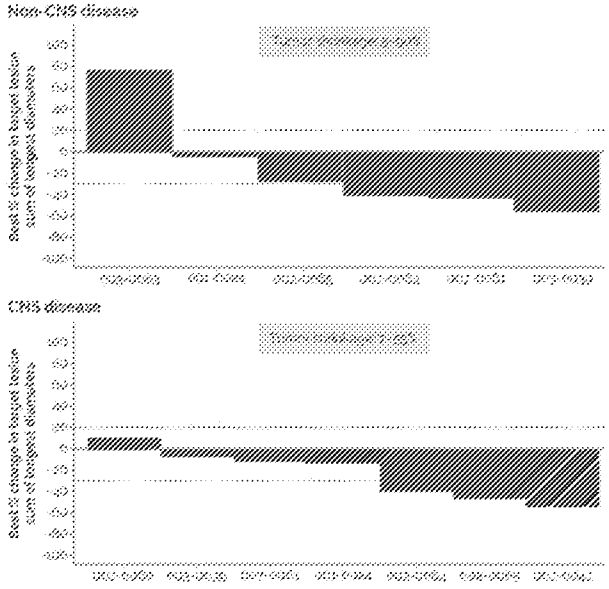
TEAEs related to nal-IRI and of grade ≥ 3	
Diarrhea	2
Hypophosphatemia	1
Hypokalemia	1
Anemia	1

If a patient experienced > 1 event in a category, the patient was counted only once in that category

nal-IRI, liposomal formulation of irinotecan; SAE, serious adverse event; TEAE, treatment-emergent adverse event

Clinical Response

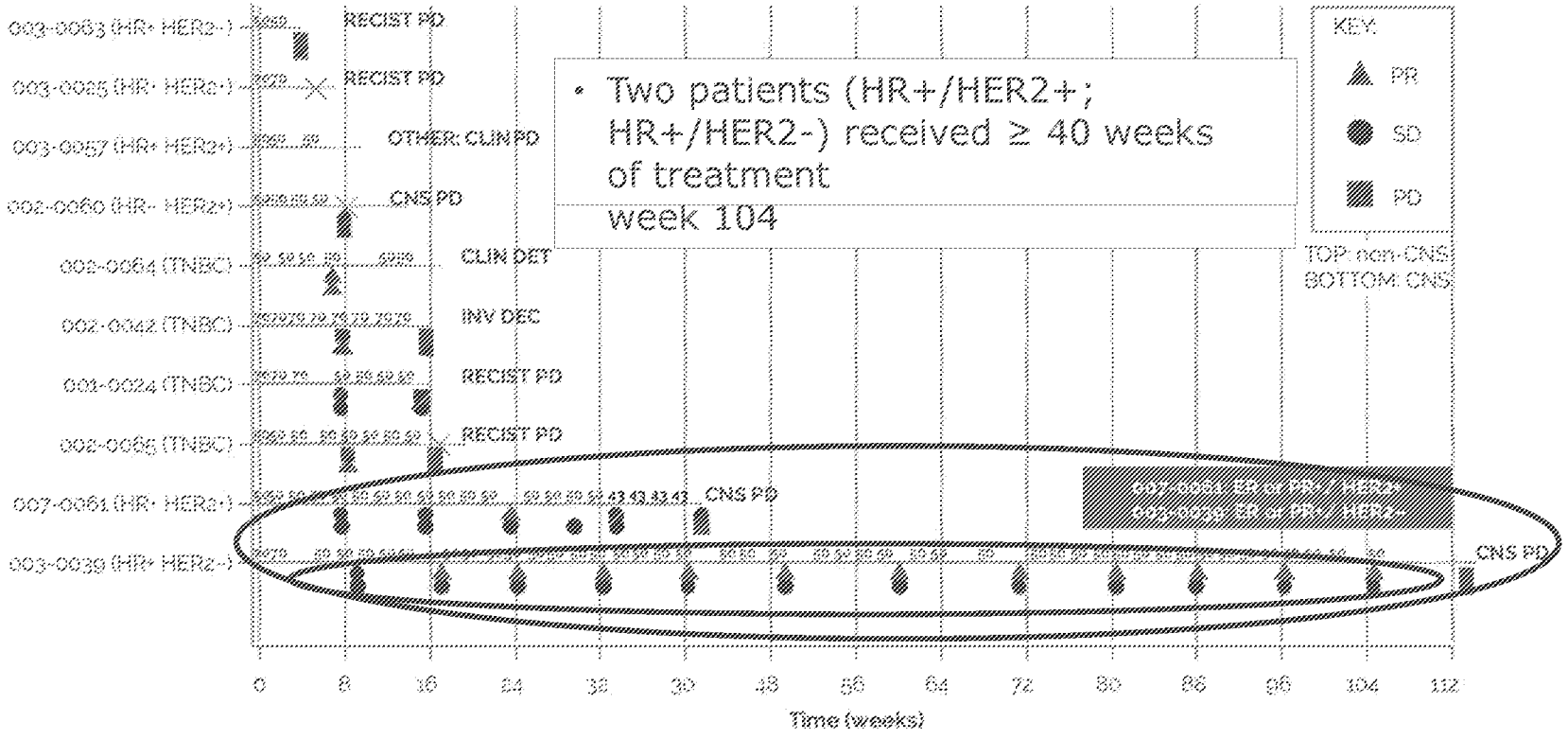
	Cohort 3 (N = 10)	
Best overall response, n (%)	**Non-CNS disease**	**CNS disease**
Complete response	0	0
Partial response	3 (30)	3 (30)
Stable disease	2 (20)	3 (30)
Progressive disease	3 (30)	2 (20)
Not evaluable	2 (20)	2 (20)
Objective response rate		
Complete response + partial response, n (%) [95% CI]	3 (30) [6.7–65.3]	3 (30) [6.7–65.3]
Duration of objective response		
Months, median (range)	4.1 (0.0–22.2)	1.8 (0.0–1.9)



- Six patients achieved CNS disease control (3 partial response; 3 stable disease)
- Among 7 patients with serial evaluation of CNS metastases post-treatment, 6 patients achieved a reduction in target CNS lesions compared with baseline

CI, confidence interval; CNS, central nervous system

Duration of Response



CLIN, clinical; CNS, central nervous system; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; INV DEC, investigator decision; PD, progressive disease; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumours; SD, stable disease; TNBC, triple-negative breast cancer

Conclusions

- Among heavily pretreated patients with mBC and BM, treatment with nal-IRI achieved > 30% ORR for systemic and CNS disease
  - Six patients (including TNBC, HR+/HER2-, HR+/HER2+) achieved CNS disease control (3 PR; 3 SD)
  - One patient had durable CNS SD and non-CNS PR, which were maintained for 2 years
- GI toxicities, in particular diarrhea, were the most common nal-IRI-related TEAEs
- SAEs related to nal-IRI were uncommon
- Further exploration of nal-IRI in patients with mBC and active BM is warranted

BM, brain metastases; CNS, central nervous system; HR, hormone receptor; HER2, human epidermal growth factor receptor 2; GI, gastrointestinal; mBC, metastatic breast cancer; nal-IRI, liposomal formulation of irinotecan; ORR, objective response rate; PR, partial response; SAE, serious adverse event; SD, stable disease; TEAE, treatment-emergent adverse event; TNBC, triple-negative breast cancer

Acknowledgments

Medical writing support

- The authors thank Isabelle Kaufmann of Oxford PharmaGenesis, Oxford, UK, for providing medical writing support, which was sponsored by Ipsen in accordance with Good Publication Practice guidelines

Acknowledgments

- The authors thank all patients involved in the study, as well as their caregivers, care team, investigators and research staff in participating institutions

BREAST CANCER—LOCAL/REGIONAL/ADJUVANT

Pharmacokinetic (PK) characterization of irinotecan liposome injection in patients (pts) with metastatic breast cancer (mBC).

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Duke Cancer Institute, Durham, NC; Honor Health Research Institute, Scottsdale, AZ; University of California San Francisco, San Francisco, CA; Ipsen, Cambridge, MA; Ipsen Innovation, Les Ulis Cedex, France; Mayo Clinic, Phoenix, AZ; H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL; Washington University School of Medicine in St. Louis, St. Louis, MO

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Abstract Disclosures

Abstract

e12003

Background: Irinotecan liposome injection (nal-IRI) uses intraliposomal stabilisation technology to enable high drug load and in-vivo stability. This analysis characterizes the PK profile of nal-IRI in pts with mBC. **Methods:** The expansion of NCT01770353 enrolled 30 pts with mBC over three cohorts (Cohort 1: ER+ and/or PR+ BC [C-1]; Cohort 2: Triple Negative BC [C-2]; and Cohort 3: BC with active Brain Metastasis [C-3]). Key inclusion criteria: ECOG ≤1; adequate organ function; and >1 and ≤5 prior lines of cytotoxic therapy in metastatic setting. Most pts received nal-IRI 60 mg/m^2 salt-based equivalent (50 mg/m^2 free base equivalent [FBE]) or 80 mg/m^2(70 mg/m^2 FBE) q2w by iv infusion, based on tolerance; 2 pts were treated at 40 mg/m^2 (35 mg/m^2 FBE). Plasma samples were collected over 360h across cycles 1, 2 &3, and analysed using LC-MS/MS for total irinotecan. Data were analysed by a non-compartmental approach using Phoenix Winnonlin, and were compared with values from studies in other tumor types. **Results:** 21 patients were evaluable for PK analysis (C-1, n=6; C-2, n=7; and C-3, n=8). No trend for accumulation was observed in cycle 2 or 3, when comparing total irinotecan C_{max} & exposure (area under the curve at 168 h [AUC_{168}]) versus cycle 1. At 40-80 mg/m^2(35-70 mg/m^2 FBE), C_{max} of irinotecan tended to increase proportionally with total dose, and was comparable to studies in other tumour types (see Table). Among 29 pts who received nal-IRI, partial response (per RECIST) was observed in

10 pts. The most related TEAEs (≥ 25%) were diarrhea, nausea, vomiting, hypokalaemia, decreased appetite and fatigue. Stable disease was observed in 5 pts. **Conclusions:** PK parameters in patients with mBC were comparable to historical studies in patients with other tumour types. The safety profile of nal-IRI monotherapy appeared consistent with gastrointestinal and blood disorders.

Study	Dose (Salt base equivalent) mg/m²	Total irinotecan C_{max} (µg/ml) after nal-IRI administration		
		N	Mean	SD
PEP0203 NCT02884128	60	3	28.9	15.7
	80	6	29.2	5.2
PIST-CRC_01 NCT00940758	80	6	41.2	4.1
CITS Pilot NCT01770353	80	13	39.0	7.6
mBC Expansion NCT01770353 Cycle 1 only	60	12	28.1	5.8
	80	5	44.4	7.9

*NA=not applicable

at week 1 (HR 1.06, 95% CI 1.01–1.11, p=0.02), lower SRS dose (HR 0.43, 95% CI 0.20–0.91, p=0.03), and larger tumor volume (HR 1.52, 95% CI 1.05–2.20, p=0.03) were significantly associated with LR, but not histology, rCBV at baseline, change of rCBV at week 1 from baseline, or any rCBF parameters. Higher rCBV at week 1 (above the median) was associated with significantly higher risk of LR than lower rCBV (44% vs 0% at 1 year, respectively, p=0.02). CONCLUSIONS: DSC-PMR and specifically rCBV at week 1 may be a promising imaging biomarker to predict treatment response of brain metastasis after SRS and warrant further investigation.

TRLS-03. PHASE II TRIAL OF GDC-0084 IN COMBINATION WITH TRASTUZUMAB FOR PATIENTS WITH HER2-POSITIVE BREAST CANCER BRAIN METASTASES (BCBM)

Jose Pablo Leone, Lorenzo Trippa, Lindsey Millikin, Chelsea Andrews, Jennifer Ligibel, Heather Parsons, Wenya Bi, Jean Zhao, Eric Winer, and Nancy Lin

BACKGROUND: The PI3K/Akt/mTOR is an important pathway in BCBM. Mutations in PIK3CA or PTEN loss are associated with trastuzumab resistance. Inhibition of PI3K and mTOR led to durable responses in 3 of 5 patient-derived xenografts (PDX) models of BCBM. GDC-0084 is a potent, brain-penetrant inhibitor of class I PI3K and mTOR. METHODS: This is a single-center, phase II study to evaluate the efficacy of the combination of GDC-0084 with trastuzumab for the treatment of central nervous system (CNS) metastases in patients with HER2-positive breast cancer. Patients will receive GDC-0084 (45 mg daily) and trastuzumab (8 mg/kg loading dose, then 6 mg/kg every 3 weeks). Two cohorts will be enrolled: Cohort A: a single-arm, two-stage, phase II cohort; and Cohort B: a pre-surgical window cohort. Inclusion criteria include unequivocal evidence of new and/or progressive HER2-positive CNS metastases, at least one measurable (≥10 mm) CNS metastasis (Cohort A), clinical indication for CNS metastasis resection (Cohort B). Primary endpoint for Cohort A is objective response rate (ORR) in the CNS per Response Assessment in Neuro-Oncology Brain Metastases (RANO-BM) criteria. For Cohort B, the primary endpoint is the correlation between p4EBP1 levels in the resected CNS tumor tissue from patients and intracranial response to GDC-0084/trastuzumab in the PDX model generated from the same patient. Secondary endpoints include overall survival, safety and patient-reported outcomes. Mandatory blood and cerebrospinal fluid with optional tumor biopsy will be collected at baseline, on-treatment and at progression. In Cohort A, we will enroll 37 patients in a Simon two-stage design. If ≥4 responses are seen, the regimen will be considered successful. This design has 90% power with alpha < 10%. Cohort B will enroll 10 patients. The trial opened in February, 2019. NCT03765983.

TRLS-04. NEAR INFRARED FLUORESCENT DYE LOCALIZES BRAIN METASTASIS PRIOR TO DURAL OPENING AND IS MORE SENSITIVE THAN WHITE LIGHT IN BRAIN METASTASIS SURGERY

Love Buch, Steve Cho, Ryan Salinas, Jasmin Hussain, and John Lee

INTRODUCTION: To improve surgical resection of brain tumors, our lab has pioneered a novel fluorescent dye technique, Second-Window Indocyanine-Green (SWIG), that relies on passive delivery and accumulation of indocyanine-green (ICG) in neoplastic tissue via the enhanced permeability and retention effect. We hypothesize that SWIG can provide early localization of brain metastasis prior to dural opening and can improve identification of surgical margins. METHODS: Subjects were prospectively enrolled in clinical trial after informed consent. Approximately 24 hours prior surgery, subjects were infused intravenously with 2.5mg/kg or 5mg/kg of ICG. Intraoperatively, a dedicated near-infrared (NIR) camera was used to detect ICG signal. After bone flap removal, the NIR imaging system was positioned above the presumed location of tumor. Additional NIR images were obtained after dural opening, corticectomy, and after conventional white-light surgical resection. RESULTS: We enrolled 50 patients with 51 total intraparenchymal brain metastases (23 lung, 7 breast, 8 GU/GI, 4 melanoma, and 7 others). Prior to dural opening, NIR signal was identified in 35 patients at an average depth of 4.3mm with SBR = 5.3 ± 3.7. In the seven patients where NIR signal could not be identified prior to dural opening, tumor depth was an average of 8.4mm from cortical surface. Upon dural opening and tumor identification, all 51 tumors demonstrated strong NIR signal with SBR = 6.2 ± 2.8. With white light alone, sensitivity/specificity/PPV/NPV for tumor detection was 83%, 94%, 98%, 57%. With NIR, sensitivity/specificity/PPV/NPV for tumor detection was 100%, 29%, 85%, 100%. DISCUSSION: NIR fluorophores are superior to visible light fluorophores in their depth of penetration. All contrast-enhancing brain metastasis accumulate ICG using our SWIG technique, and NIR fluorescence could be used to localize brain metastasis prior to dural opening. NIR fluorophores are likely to represent the next phase in tumor visualization given the rapid growth of fluorophores targeted to systemic cancers.

TRLS-05. EARLY RESULTS FROM A PROSPECTIVE PHASE I/II DOSE ESCALATION STUDY OF NEOADJUVANT RADIOSURGERY FOR BRAIN METASTASES

Erin Murphy, Kailin Yang, John Suh, Jennifer Yu, Cathy Schiltero, Alireza Mohammadi, Glen Stevens, Lilyana Angelov, Michael Vogelbaum, Gene Barnett, Manmeet Ahluwalia, Gennady Neyman, and Samuel Chao

OBJECTIVES: Single-session stereotactic radiosurgery (SRS) alone for brain metastases larger than 2cm in maximal dimension results in local control of only 50%. Surgical resection followed by SRS to the resection cavity can result in leptomeningeal failure (LMD). This Phase I/II study aims to determine the safety and local control of neoadjuvant SRS at escalating doses followed by surgical resection of brain metastases greater than 2 cm. METHODS: Radiosurgery dose was escalated at 3 Gy increments from currently accepted RTOG standard. If no dose-limiting toxicities (DLT) were observed, the dose was escalated. Patients underwent surgical resection of brain metastases within 2 weeks and were followed with brain MRIs and neurologic evaluations every 3 months. RESULTS: 27 patients were enrolled. For tumor size >2.0–3.0 cm, 2 patients completed treatment at 18 Gy and 3 patients at 21Gy. For tumor size >3.0–4.0 cm, 4 patients were treated at 15 Gy and 9 patients were treated at 18 Gy and 1 patient at 21 Gy. For tumor size >4.0–5.0 cm, 1 patient was treated at 12 Gy and 7 patients at 15 Gy. No DLT have occurred. With a mean follow-up of 13.1 months, the 6 and 12 month local control was 93.8% and 72.3%, respectively. Six and 12 month distant brain control was 38.6% and 25.8%. Overall survival at 12 months was 53.5%. One patient developed LMD 5 months following SRS. 4 patients (15%) had acute grade 1/2 toxicity, and no grade 3/4 toxicity was observed. CONCLUSIONS: Neoadjuvant SRS with dose escalation followed by surgical resection for brain metastases greater than 2 cm results in local control comparable to postoperative SRS or WBRT, and demonstrates acceptable acute toxicity. A low rate of LMD failure was found. The Phase II portion of the trial will be conducted at the maximum tolerated SRS doses.

TRLS-06. PHASE 1 EXPANSION STUDY OF IRINOTECAN LIPOSOME INJECTION (NAL-IRI) IN PATIENTS WITH METASTATIC BREAST CANCER (MBC): FINDINGS FROM THE COHORT WITH ACTIVE BRAIN METASTASIS (BM)

Carey Anders, Pamela Munster, Donald Northfelt, Hyo Sook Han, Cynthia Ma, Fiona Maxwell, Tiffany Wang, Bruce Belanger, Bin Zhang, Yan Moore, and Jasgit C Sachdev

BACKGROUND: nal-IRI is a liposomal formulation of irinotecan (topoisomerase-1 inhibitor). Preclinical data show that nal-IRI accumulates in BMs and prolongs survival in animal models of BM. Findings from a phase 1 expansion study (NCT01770353), evaluating patients with mBC and active BM, are reported. METHODS: This phase 1 expansion study enrolled patients with mBC who received multiple prior lines of cytotoxic therapy in the metastatic setting, including one cohort with mBC and active BM, defined as radiographic evidence of new or progressive central nervous system (CNS) metastases after radiation therapy with ≥1 lesion of ≥1 cm in the longest dimension on gadolinium-enhanced magnetic resonance imaging. Patients received nal-IRI 50 mg/m^2 (free-base equivalent; FBE) every two weeks (q2w) as an intravenous infusion over 90 minutes, escalating to 70 mg/m^2 FBE q2w, if tolerated. RECIST v1.1 and modified RECIST criteria were used to asses non-CNS and CNS disease, respectively. RESULTS: In total, 30 patients were enrolled (10 with active BM). Median age was 53 years (range 29–70 years) and median number of prior cytotoxic anti-cancer regimens was 3 (range 0–6); 29 patients received ≥1 dose of nal-IRI 50 mg/m^2 FBE. Overall, nal-IRI monotherapy appeared to be well tolerated, and achieved ≥30% objective response rates for both CNS and non-CNS disease. Among the 10 patients with active BM, 6 achieved CNS disease control (3 partial responses [PRs] and 3 stable disease [SD]), including one patient with durable CNS SD and non-CNS PR for 2 years. Among 7 patients with serial evaluation of CNS metastases posttreatment, 6 patients achieved a reduction in target CNS lesions compared with baseline. CONCLUSION: Treatment with nal-IRI resulted in CNS disease control among 6 of 10 heavily pretreated patients with mBC and active BM. Further exploration of nal-IRI in patients with mBC and active BM is warranted.

TRLS-07. BRAINSTORM: OUTCOMES FROM A MULTI-INSTITUTIONAL PHASE I/II STUDY OF RRX-001 IN COMBINATION WITH WHOLE BRAIN RADIATION THERAPY FOR PATIENTS WITH BRAIN METASTASES

Michelle Kim, Hemant Parmar, Matthew Schipper, Theresa Devasia, Madhava Aryal, Santosh Kesari, Steven O'Day, Aki Morikawa, Daniel Spratt, Larry Junck, Aaron Mammoser, James Hayman, Theodore Lawrence, Christina Tsien, Robert Aiken, Sharad Goyal, Nacer Abrouk, Malcolm Trimble, Yue Cao, and Christopher Lao

INTRODUCTION: To determine the recommended Phase II dose of RRx-001, a radiosensitizer with vascular normalizing properties, when used with

VOLUME 25 · NUMBER 24 · AUGUST 20 2007

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Phase III Study Comparing a Semimonthly With a Monthly Regimen of Fluorouracil and Leucovorin As Adjuvant Treatment for Stage II and III Colon Cancer Patients: Final Results of GERCOR C96.1

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Submitted April 19, 2007; accepted May 30, 2007.

Supported by grants from the Association Pour la Recherche en Cancérologie (APREC) and from Wyeth (Lederle and Puteaux, France) and Baxter (Maurepas, France).

Presented in part at the 41st Annual Meeting of the American Society of Clinical Oncology, Orlando, FL, May 13-17, 2005.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/07/2524-3732/$20.00

DOI: 10.1200/JCO.2007.12.2234

ABSTRACT

Purpose

This randomized, 2 × 2 factorial study compared a semimonthly regimen (fluorouracil [FU] and leucovorin [LV] semi-monthly is LV5FU2) with a monthly regimen of FU and LV (mFU/LV) as well as 24 weeks versus 36 weeks of each regimen as adjuvant treatment of stage II and III colon cancer.

Patients and Methods

LV5FU2 was administered semimonthly for 2 days as racemate (dl) or levogyre (l-; 200 or 100 mg/m^2) as a 2-hour infusion, followed by 400 mg/m^2 FU bolus and a 600-mg/m^2 FU 22-hour continuous infusion. FU and LV were administered monthly (mFU/LV) for 5 days as dl- or l-LV 15-minute infusion, followed by a 400 mg/m^2 FU 15-minute infusion. The primary end point was disease-free survival (DFS).

Results

Between September 1996 and November 1999, 905 patients with stage II (43%) and III (57%) colon cancer were enrolled. The median follow-up was 6 years. There was no statistically significant difference between mFU/LV and LV5FU2 in terms of DFS (150 v 148 events; hazard ratio [HR],1.01; 95% CI, 0.806 to 1.269, $P = .94$) and overall survival (OS; 104 v 103 events; HR,1.02; 95% CI, 0.77 to 1.34; $P = .91$). No statistical difference was observed between 24 or 36 weeks of chemotherapy. Median survival from metastatic relapse was 24 months. The survival of patients with metastatic relapse (n = 243) was significantly longer for patients with a longer time from random assignment to relapse (< 1, 1 to 2, ≥ 2 years; log-rank test for trend P, .0497).

Conclusion

DFS and OS were not statistically different between treatment groups and treatment durations. These data confirm the value of LV5FU2 as control arm in the Multicenter International Study of Oxaliplatin/5FU-LV in the Adjuvant Treatment of Colon Cancer and Pan-European Trials in Adjuvant Colon Cancer studies.

J Clin Oncol 25:3732-3738. © 2007 by American Society of Clinical Oncology

INTRODUCTION

Colorectal carcinoma is the most common gastrointestinal malignancy, and each year worldwide 500,000 people die of the disease and nearly 1 million patients are newly diagnosed. As many as 40% to 50% of patients who undergo potentially curative surgery alone will ultimately relapse and die of metastatic disease.[1] Over the past years, considerable progresses have been made in the treatment of patients with colon cancer. The use of optimized, leucovorin (LV)-modulated fluorouracil (FU) regimens have increased the median survival of patients with metastatic (stage IV) colon cancer from barely 6 months without treatment to more than 12 months.[2] The use of continuous infusion allowed the administration of high doses of LV and FU, leading to enhanced efficacy with acceptable toxicity.[3] The addition of new chemotherapeutic agents (eg, oxaliplatin, irinotecan) to these regimens has further extended median survival to approximately 20 months.[4-6]

The purpose of adjuvant chemotherapy is to reduce the risk of recurrence of the cancer either locally or with distant metastases, which could be due to remaining nondetectable tumor cells after

surgery. The Intergroup trial (INT-0035) was the first large-scale study to demonstrate a significant effect of adjuvant treatment in stage III colon cancer patients with FU plus levamisole given for 1 year.[7] The efficacy of FU bolus and LV became the standard chemotherapy for stage III colon cancer in 1996.[8,9] Available clinical trial data do not support the routine use of adjuvant chemotherapy for all stage II patients, but suggest that it should be considered, in case of high-risk patients.[10]

Concerning the duration of adjuvant chemotherapy, the INT-0089 study showed that 6 months of FU plus LV (Roswell Park schedule or North Central Cancer Treatment Group and Mayo Clinic regimen) were equivalent in term of OS to 12 months of FU plus levamisole.[9] The North Central Cancer Treatment Group and National Cancer Institute of Canada Clinical Trials Group study showed that 6 months of combined FU and LV were equivalent to 12 months of combined FU, LV, and levamisole.[11] Based on these results, a semimonthly regimen of FU and LV (LV5FU2) was selected as the control arm of the Multicenter International Study of Oxaliplatin/5FU-LV in the Adjuvant Treatment of Colon Cancer (MOSAIC) study, which showed a significant advantage in terms of disease-free survival (DFS) at 3 years for oxaliplatin, FU, and LV (FOLFOX4).[12] In 2004, adjuvant chemotherapy with FU modulated by LV combined with oxaliplatin (FOLFOX4) became an accepted standard of care for patients with stage III colon cancer (MOSAIC study).[12]

This study was undertaken to compare the therapeutic ratio, efficacy, and toxicity of a monthly schedule of a 15-minute infusion of FU plus high-dose LV for 5 consecutive days with LV5FU2, given for different durations (24 *v* 36 weeks) in the adjuvant setting, after resection of stage II and III colon or high rectum cancer.

In 2003, we reported the early results of this study (C96-1), which failed to show, after a median follow-up of 41 months, a statistically significant difference in DFS or overall survival (OS) between LV5FU2 and monthly mFU/LV or between treatment durations.[13] These early results also showed LV5FU2 to be less toxic than mFU/LV.[13,14] Herein, we report the final DFS and OS results of the C96-1 study with a median follow-up of 6 years. We also carry out prognostic factor analyses and further analyses of patients with metastatic relapses.

PATIENTS AND METHODS

Our methods have been described previously.[14,15] Patients with stage II or III colon or high rectum (Gastrointestinal Tumor Study Group 1975) cancer were randomly assigned to two adjuvant chemotherapy regimens (LV5FU2 or mFU/LV) and two treatment durations (24 or 36 weeks) using a 2 × 2 factorial design. A dynamic minimization procedure was used to stratify patients according to institution, cancer stage (II or III), number of affected nodes for stage III cancer (≤ 4 or > 4), adjacent organs invasion, and time between surgery and random assignment (< 20, 20 to 35, or 36 to 49 days).

Chemotherapy Administration Schedules

Patients assigned to the LV5FU2 group received racemate (*dl*-)LV 200 mg/m^2 (or levogyre [*l*-]LV 100 mg/m^2, according to drug availability in each institution) as a 2-hour infusion, followed by bolus FU 400 mg/m^2 and a 22-hour infusion of FU 600 mg/m^2 for 2 consecutive days every 14 days. The use of implantable ports and disposable or electronic pumps allowed chemotherapy to be administered on an outpatient basis. Patients in this group received 12 or 18 cycles of treatment depending on whether they were randomly assigned to the 24- or the 36-week treatment group. Patients could be treated in the 7 days after random assignment. Patients in the mFU/LV group received an infusion of *dl*-LV 200 mg/m^2 (or *l*-LV 100 mg/m^2) for 15 minutes, followed by a 15-minute bolus FU 400 mg/m^2 for 5 consecutive days every 28 days. In this study, we chose mFU/LV as the reference arm. mFU/LV differs from the monthly Mayo Clinic regimen by a higher dose of LV (200 *v* 20 mg/m^2), a different period of administration (15 minutes *v* bolus), and a slightly lower dose per day of FU (400 *v* 425 mg/m^2). In the case of grade 2 toxicity, FU was administered over 60 minutes; for toxicities greater than 2, FU was given over 60 minutes and the dose decreased to 300 mg/m^2. Appropriate adjustment in dosage was made according to toxicity.

Follow-Up Procedures

Before random assignment, every 6 months for 5 years, and then annually, patients were assessed for: medical history, physical examination, renal and liver function tests, complete blood cell count, carcinoembryonic antigen (CEA), chest x-ray, abdominal ultrasound or computerized tomography scan. Colonoscopy was performed within 6 months of the end of chemotherapy, and repeated every 3 years if the previous colonoscopy was normal, or every year if the previous colonoscopy showed polyps with polypectomy. The protocol was reviewed and approved by the ethics review committee of Saint-Antoine Hospital (Paris, France). All patients provided written informed consent before inclusion in the trial.

Statistical Methodology

DFS was assessed by recording colorectal cancer relapse, second colorectal cancer, or death. Two-sided statistical tests were performed on the DFS (primary efficacy criterion), OS, and toxicities. Survival curves were estimated by the Kaplan-Meier method. Patients lost to follow-up were censored at the last documented visit. The differences in DFS and in OS were evaluated using a log-rank test, or an unadjusted proportional hazards regression model to assess hazard ratios and their 95% CIs. Proportional hazards regression models of DFS and OS were used to identify significant prognostic covariates. Treatment effect was estimated using a proportional hazard regression model after adjustment for all significant prognostic factors.

RESULTS

A total of 905 patients with stage II (43%) and III (57%) cancer were randomly assigned in this study between July 1996 and November 1999. Four patients (0.4%) were ineligible but were nevertheless included in the analyses performed as intent to treat (two patients with metastatic disease at inclusion in the LV5FU2 arm, one patient with metastatic disease at inclusion in the mFU/LV arm, and one patient with low rectum cancer in the mFU/LV arm). All patients entered onto the study could potentially be followed for more than 5 years after random assignment and, at the time of the final analysis, the median follow-up time was 6.06 years in the mFU/LV group and 6.03 years in the LV5FU2 group. As described in our earlier report,[14,15] the clinical and pathologic characteristics of our patients were well-balanced among the study arm.

Cancer Recurrence and DFS

Table 1 summarizes patient status at 6 years median follow-up. For the whole population, 246 patients relapsed with distant metastasis, 10 presented local relapse alone, five had second colon or rectal cancer alone, and 37 died without relapse, for a total of 298 events, evenly distributed between the LV5FU2 and mFU/LV arms (148 and 150 events, respectively). Figure A1 (online only) shows the DFS curves for both regimens. There was no significant difference between the LV5FU2 and mFU/LV regimens (DFS at 6 years, 66% and 65%, respectively; hazard ratio [HR], 1.01; 95% CI, 0.81 to 1.27; $P = .74$). Similar results were obtained when data were analyzed for treatment duration (Fig A2, online only): 151 events were observed in the 24-week group versus 147 events in the 36-week group (HR, 0.97; 95% CI, 0.77 to 1.22; $P = .63$).

Table 1. Patient Status After a Median Follow-Up of 6 Years

Variable	mFU/LV		LVFU2		24 Weeks		36 Weeks	
	No.	%	No.	%	No.	%	No.	%
No. of patients	453		452		454		451	
Median follow up, years	6.06		6.03		6.14		6.00	
Deaths, all causes	104	22.9	103	22.8	100	22.0	107	23.7
Total No. of patients with events*	150	33.1	148	32.7	151	33.3	147	32.6
Metastatic relapse	129	28.5	117	25.9	126	27.8	120	26.6
Local relapse alone	3	0.7	7	1.5	5	1.1	5	1.1
Death without relapse	16	3.5	21	4.6	18	4.0	19	4.2
Second colon or rectal cancer	2	0.4	3	0.7	1	0.2	2	0.4

Abbreviations: mFU/LV, fluorouracil and leucovorin monthly; LVFU2, fluorouracil and leucovorin semimonthly.
*Events were metastatic or local relapses, second colon or rectal cancer, or death.

OS

At the time of this analysis, 103 and 104 patients died in the LV5FU2 and mFU/LV arms, respectively (Fig 1). The OS at 6 years was 76% for LV5FU2 and 78% for mFU/LV (HR, 1.02; 95% CI, 0.77 to 1.34; $P = .91$). According to treatment duration, 100 and 107 patients died in the 24- and 36-week groups, respectively (Fig A3, online only). The OS at 6 years was 78% and 75% for 24 weeks versus 36 weeks, respectively (HR, 1.11; 95% CI, 0.84 to 1.45; $P = .47$).

Survival of Patients With Metastatic Relapse

The three patients with metastatic disease at inclusion were excluded from this analysis of survival of patients with metastatic relapse. The 243 patients who presented a metastatic relapse during the trial were distributed as follows: 128 and 115 for mFU/LV and LV5FU2 groups, respectively. The 167 patients who died during the trial were distributed as follows: 88 and 81 patients for mFU/LV and LV5FU2 groups, respectively. The time from metastatic relapse to death was not significantly different between the two groups (HR, 1.09; 95% CI, 0.81 to 1.48; P, .56; Fig 2). Median survival from metastatic relapse was 24 months. Data on the treatment of the metastatic relapse were available for 183 patients (75%). Most of the patients with metastatic disease who relapsed were treated with oxaliplatin- and/or CPT11-based chemotherapy (Table 2). Thirty-eight percent of patients with metastatic disease who relapsed had surgery with a curative intent.

The survival of patients with metastatic relapse was significantly different according to time from random assignment to relapse. The patients were classified according to the time to metastatic relapse into one of three categories: less than 1 year, 1 to 2 years, or ≥ 2 years. These categories were chosen to divide the population into roughly equal groups. The Kaplan-Meier curves showed that the time from metastatic relapse to death was longer for patients with a longer time to metastatic relapse (Fig 5; P, .0497 by the log-rank test for trend).

Subgroup Analysis

Although the trial was not powered to look at stage II or stage III patients separately, descriptive treatment comparisons were carried out on the stage II and III subpopulations.

Among patients with stage II, in term of DFS, 74 events were recorded, equally distributed between the LV5FU2 and mFU/LV groups with 37 events and a DFS at 6 years of 80% in both arms (HR, 1.02; 95% CI, 0.64 to 1.60). In term of OS, 21 and 24 deaths occurred in LV5FU2 and mFU/LV with an OS at 6 years of 88% and 89%, respectively (HR, 0.89; 95% CI, 0.50 to 1.60).

Among patients with stage III, in term of DFS, 224 events were recorded, 111 and 113 in the LV5FU2 and mFU/LV arms and a DFS at 6 years of 54% and 55%, respectively (HR, 1.01; 95% CI,

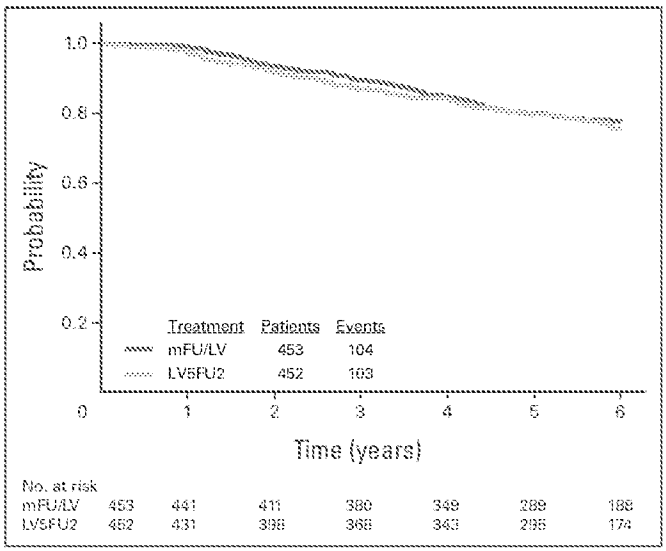

Fig 1. Overall survival according to treatment. mFU/LV, monthly fluorouracil and leucovorin; LV5FU2, semimonthly fluorouracil and leucovorin.

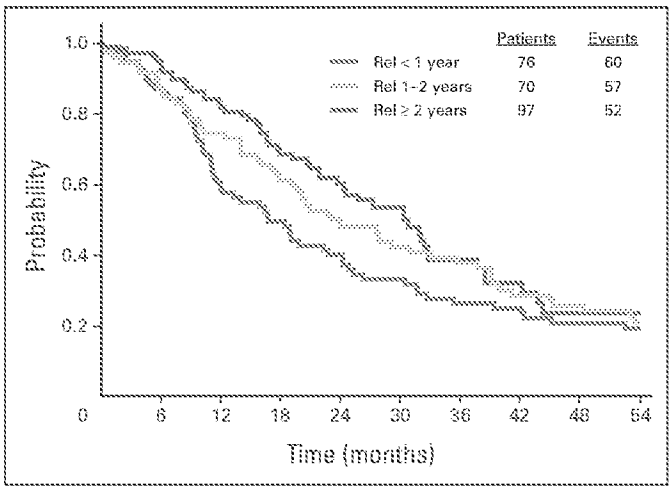

Fig 2. Survival after metastatic relapse according to the time to relapse (Rel).

Table 2. Available Data Concerning Patients With Metastatic Relapse (n = 243)

Variable	Patients* (n = 183) No.	%
Patient with surgery (curative intent) of metastatic disease	70	38
Surgery alone	9	5
Surgery and chemotherapy	61	33
Patients without surgery or chemotherapy for metastatic disease	16	9
Adjuvant chemotherapy after surgery of metastatic disease	41	22
Type of chemotherapy (either adjuvant after metastasis surgery or palliative)	158	86
Fluoropyrimidine ± LV alone	11	6
Fluoropyrimidine ± LV + oxaliplatin or irinotecan	78	42
Fluoropyrimidine ± LV + oxaliplatin and irinotecan	69	38

Abbreviation: LV, leucovorin.
*For the 243 patients with metastatic relapse, data concerning treatment of metastatic relapses were not available for 60 patients. Analysis of therapy of metastatic relapse was not programmed by the protocol and collect of these data was done retrospectively.

0.78 to 1.31). In term of OS, 78 and 79 deaths occurred with an OS at 6 years of 67% and 69%, respectively (HR, 1.02; 95% CI, 0.75 to 1.40).

Prognostic Variables

Table 3 presents the prognostic impact of patient and tumor-related factors on recurrence and death. In univariate analyses, stage, adhesion, or invasion to adjacent organ, regional peritoneum involvement (a tumor nodule resected in the pericolorectal adiposite tissue of a primary carcinoma without histological evidence of residual lymph node), obstruction, histologic differentiation, and type of stage II (high *v* low risk) were all significant prognostic factors for both DFS and OS. Other factors such as age, sex, and time interval between surgery and treatment, localization, and perforation were not found to be statistically significant either for DFS or for OS. These univariate analyses were confirmed in a multivariate proportional hazards regression model in which stage, adhesion, or invasion to adjacent organ, regional peritoneum involvement, obstruction, and poor histologic differentiation all remained statistically significant (Table 4). After adjustment for these factors, there was still no significant difference between LV5FU2 and mFU/LV in terms of either DFS (HR, 1.05; 95% CI, 0.84 to 1.32; $P = .66$) or OS (HR, 1.06; 95% CI, 0.81 to 1.39; $P = .69$).

Concerning stage, we considered stage II patients to be high risk if at least one of the following factors was present: adhesion or invasion to adjacent organ (T4), obstruction, perforation, number of examined nodes fewer than 10, and poor histologic differentiation. Of 391 stage II patients (197 treated by mFU/LV and 194 treated by LV5FU2), 243 were considered high-risk stage II (132 treated by mFU/LV and 111 treated by LV5FU2; Table A1, online only). High risk was a significant prognostic factor for both DFS and OS ($P = .0004$) and OS ($P = .0002$; Table 3). Multivariate proportional hazards regression model were fitted for stage II and stage III patients separately (Table 4). In stage II patients, high risk was a significant prognostic factor for both DFS and OS. In stage III patients, number of affected nodes, adhesion or invasion to adjacent organ, and regional peritoneum involvement were significant prognostic factors for both DFS and OS.

DISCUSSION

With a median follow-up of 6 years, this study did not demonstrate any efficacy difference between LV5FU2 and mFU/LV. Even though the study was not designed as an equivalence study, the point estimate of the DFS and OS hazard ratios are close to 1, and the limits of the 95% DFS CI (0.81 to 1.27) suggest that the HRs of the two regimens do not differ by more than 20% to 25%, and can therefore be considered approximately equivalent in terms of efficacy, despite major differences in toxicity.[13,14] Three other studies[15-17] comparing an infusional FU schedule with or without LV to the Mayo Clinic regimen did not demonstrate any significant difference in terms of DFS or OS. Two of these studies compared FU delivered by protracted infusion (PVI 5FU).[15,16] The third one, the Pan-European Trials in Adjuvant Colon Cancer (PETACC-2) study randomly assigned 1,601 patients with stage III colon cancer to 6 months of LV5FU2 or two other FU continuous infusion schedule (Spanish Cooperative Group for Gastrointestinal Tumor Therapy = FU 3 g/m^2 weekly or Arbeitsgemeinschaft fur Internistische Onkologie = LV 500 mg/m^2 and FU 2.6 g/m^2 weekly for 6 weeks with 2 weeks rest) and the Mayo Clinic regimen.[17] Similar to our own previously published safety results,[13,14] infusional FU schedules with or without LV were less toxic as compared with the Mayo regimen (Table A2). In PETACC-2, the LV5FU2 regimen appeared to be the best tolerated infusional regimen.[17] The major drawback of continuous infusion with FU is the need for an implantable port with its associated adverse effects.

This study evaluated also duration of chemotherapy (24 *v* 36 weeks), and failed to demonstrate a significant improvement in patient DFS or OS when chemotherapy was given for 36 weeks as compared with 24 weeks. In the literature, 6 months of adjuvant chemotherapy with FU and LV were also shown to be equivalent to 12 months of the same chemotherapy[11] and 6 months of FU plus LV with or without levamisole was shown to be equivalent to 1 year of FU plus levamisole.[8,9] All these results indicate that an increase in the duration of chemotherapy with FU and LV beyond 6 months (24 weeks) is not associated with better efficacy.

Updated analyses of this trial produced some interesting results. The time between surgery and randomization was not a prognostic factor in this study where this parameter was a stratification criterion. In univariate and multivariate prognostic analyses (Tables 3 and 4), high risk stage II disease, as defined in the MOSAIC study[10,18,19] (invasion and/or adhesion to adjacent organ, obstruction, perforation, poor histological differentiation, number of examined nodes fewer than 10) was shown to be the most potent prognostic factor for relapse or death. Obstruction, in and of itself, was also an independent prognostic factor for the whole population.

Table 3. Impact of Prognostic Variables on DFS and OS for All Patients

Variable	No. of Patients	DFS at 6 Years (%)	*P*	OS at 6 Years (%)	*P*
Age, years					
≤ 60	395	67	.48	0.79	.058
> 60	508	64		0.74	
Sex					
Male	485	66	.95	0.76	.60
Female	416	66		0.78	
Stage					
II	391	80	< .0001	0.88	< .001
III	514	55		0.68	
III, 1–4 N+	405	59		0.72	
III, > 4 N+	109	40	< .0001	0.53	.001
Delay surgery inclusion, days					
< 20	52	74	.62	0.77	.99
≥ 20 and < 35	547	65		0.77	
≥ 35	302	66		0.76	
Primary tumor localization					
Proximal*	357	67		0.77	
Distal†	516	65	.50	0.77	.51
Multiple sites	10	50		0.70	
Adhesion or invasion to adjacent organ					
No	794	67	.0002	0.78	.004
Yes	104	53		0.65	
Regional peritoneum involvement					
No	813	68	< .001	0.79	.0025
Yes	88	46		0.66	
Obstruction					
No	744	68	< .0011	0.79	.0001
Yes	155	53		0.65	
Perforation					
No	828	67	.0798	0.77	.58
Yes	70	55		0.71	
Histological differentiation					
Well and moderately well	819	66	.019	0.77	.026
Poor	26	52		0.64	
Stage II high risk‡	243	77	.0004	86	.0002
Stage II low risk§	147	90		97	

Abbreviations: DFS, disease-free survival; OS, overall survival; N+, node positive.
*Proximal: Caecum, right colon, flexures, and transverse colon.
†Distal: Left colon and sigmoid, rectosigmoid, and high rectum.
‡Stage II high risk of relapse if minimum one of these factors are present: invasion or adhesion to adjacent organ, obstruction, perforation, poor histologic differentiation, number of examined nodes fewer than 10.
§Stage II low risk if 0 these factors are present.

We analyzed the OS of patients with metastatic relapses. The median time between metastatic relapse and death was 24 months, which is much longer than the reported 14.2 months observed in the Adjuvant Colon Cancer End Points (ACCENT) meta-analysis.[20] One possible explanation is that most patients with metastatic relapse in our study had surgery of their metastasis with a curative intent and/or were treated with a combination of oxaliplatin or CPT11 with FU and LV (Table 2), which has been shown to contribute to increase the median OS for metastatic disease.[4-6] In ACCENT, a majority of adjuvant studies had been carried out before the approval of oxaliplatin and irinotecan.[20] This study (C96-1) was the most recent one to be included in ACCENT.[20] A median OS of 24 months was close to the OS observed for patients with an initial diagnosis of metastatic disease, which reflects the progress in the management of metastatic colorectal cancer based a multidisciplinary approach (surgery of metastasis, use of oxaliplatin and irinotecan). The high proportion of patients with surgery for metastatic relapses (38%) underscores the importance of an intense follow-up for patients at risk of a relapse.[21] The time from metastatic relapse to death was longer for patients with a late metastatic relapse. This observation suggests that for future studies in first-line metastatic colorectal cancer, patients should be stratified according to time from initial diagnosis to metastasis.

One of the decisive advantages of LV5FU2 is that this regimen can be safely combined with other cytotoxic agents in colorectal

Table 4. Multivariate Proportional Hazards Regression Models for DFS and OS

Patients	DFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
All						
Stage II v III 1-4 N+	0.40	0.31 to 0.53	< .0001	0.39	0.28 to 0.55	< .0001
Stage III > 4 N+ v III 1-4 N+	1.80	1.35 to 2.41	< .0001	1.84	1.31 to 2.58	.0004
Adhesion or invasion to adjacent organ	1.57	1.15 to 2.16	.0048	1.66	1.16 to 2.39	.0058
Regional peritoneum involvement	1.85	1.34 to 2.56	.0002	2.19	1.53 to 3.15	< .0001
Obstruction	1.42	1.08 to 1.86	.014	1.69	1.24 to 2.31	.0009
Stage II						
High risk*	2.63	1.49 to 4.64	.0008	4.34	1.84 to 10.21	.0008
Stage III						
Stage III 1-4 N+ v III > 4 N+	1.79	1.33 to 2.39	.0001	1.83	1.30 to 2.57	.0005
Adhesion or invasion to adjacent organ	1.66	1.15 to 2.38	.0065	1.88	1.24 to 2.87	.0033
Regional peritoneum involvement	1.80	1.22 to 2.62	.0027	2.16	1.44 to 3.31	.0002

Abbreviations: DFS, disease-free survival; OS, overall survival; HR, hazard ratio; N+, node positive
*Stage II high risk of relapse if minimum one of these factors: invasion or adhesion to adjacent organ, obstruction, perforation, poor histologic differentiation, number of examined nodes fewer than 10.

cancer, such as oxaliplatin or CPT11. Combination of LV5FU2 with oxaliplatin or CPT11 in metastatic disease had a more favorable safety profile while extending both progression-free survival[3,6,22] and OS.[6,22] These results validate the choice of LV5FU2 as the control group of trials testing the addition of oxaliplatin (MOSAIC)[12] or irinotecan (PETACC-3)[23] to adjuvant chemotherapy for patients with stage II and III colon cancer.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. 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.

Employment or Leadership Position: Pascal Piedbois, Astra Zeneca (U) **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** Thierry André, Baxter; Aimery de Gramont, Baxter **Research Funding:** None **Expert Testimony:** None **Other Remuneration:** None

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REFERENCES

1. Obrand DI, Gordon PH: Incidence and patterns of recurrence following curative resection for colorectal carcinoma. Dis Colon Rectum 40:15-24, 1997

2. Advanced Colorectal Cancer Meta-Analysis Project: Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: Evidence in terms of response rate. J Clin Oncol 10:896-903, 1992

3. de Gramont A, Bosset JF, Milan C, et al: Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: A French Intergroup study. J Clin Oncol 15:808-815, 1997

4. de Gramont A, Figer A, Seymour M, et al: Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 18:2938-2947, 2000

5. Tournigand C, André T, Louvet C, et al: FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: A randomized GERCOR study. J Clin Oncol 22:229-237, 2004

6. Goldberg RM, Sargent DJ, Morton RF, et al: A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 22:23-30, 2004

7. Moertel CG, Fleming TR, Macdonald JS, et al: Levamisole and fluorouracil as adjuvant therapy of resected colon carcinoma. N Engl J Med 322:352-358, 1990

8. Wolmark N, Rockette H, Mamounas E, et al: Clinical trial to assess the relative efficacy of fluorouracil and leucovorin, fluorouracil and levamisole, and fluorouracil, leucovorin, and levamisole in patients with Dukes' B and C carcinoma of the colon: Results from National Surgical Adjuvant Breast and Bowel Project C-04. J Clin Oncol 17:3553-3559, 1999

9. Haller DG, Catalano PJ, Macdonald JS, et al: Phase III study of fluorouracil, leucovorin, and levamisole in high-risk stage II and III colon cancer: final report of Intergroup 0089. J Clin Oncol 23:8671-8678, 2005

10. André T, Sargent D, Tabernero J, et al: For the GERCOR (French Oncology Research Group): Current issues in adjuvant treatment of stage II colon cancer. Ann Surg Oncol 13:887-898, 2006

11. O'Connell MJ, Laurie JA, Kahn M, et al: Prospectively randomized trial of postoperative adjuvant chemotherapy in patients with high-risk colon cancer. J Clin Oncol 16:295-300, 1998

12. André T, Boni C, Mounedji-Boudiaf L, et al: Oxaliplatin, 5-fluorouracil and leucovorin as adjuvant treatment of colon cancer: Results of the international randomized MOSAIC trial. N Engl J Med 23:2343-2351, 2004

13. André T, Colin P, Louvet C, et al: For the GERCOR Group, Paris France: Bimonthly regimen of 5-fluorouracil and leucovorin (LV5FU2) as adjuvant therapy in stage II, and III colon cancer: 4-year results of a randomized trial. J Clin Oncol 21:2896-2903, 2003

14. André T, Colin P, Louvet C, et al: Randomized adjuvant study comparing two schemes of 5-fluorouracil and leucovorin in stage B2 and C colon adenocarcinoma: Study design and preliminary safety results. Semin Oncol 28:35-40, 2001 (supp 1)

15. Saini A, Norman AR, Cunningham D, et al: Twelve weeks of protracted venous infusion of fluorouracil is as effective as 6 months of bolus 5FU and folinic acid as adjuvant treatment in colorectal cancer. Br J Cancer 16:1859-1865, 2003

16. Poplin EA, Benedetti JK, Estes NC, et al: Phase III Southwest Oncology Group 9415/Intergroup 0153 randomized trial of fluorouracil, leucovorin, and levamisole vs. fluorouracil continuous infusion and levamisole for adjuvant treatment of stage III and high-risk stage II colon cancer. J Clin Oncol 23:1819-1825, 2005

17. Cerrato A, Köhne C, Bedenne L, et al: For AIO/CAO, FFCD, TTD, EORTC: Folinic acid modulated bolus 5-FU or infusional 5-FU or infusional 5-FU adjuvant treatment of patients of UICC stage III colon cancer: Preliminary analysis of the PETACC-2-study. J Clin Oncol 24:161s, 2006 (abstr 3563)

18. Hickish T, Boni C, Navarro M, et al: Stage II patients in the "MOSAIC" trial evaluating oxaliplatin/5FU/LV as adjuvant treatment of colon cancer: A subpopulation analysis. Ann Oncol 15:iii76, 2004 (suppl 3; abstr 284P)

19. Andre T, Tournigand C, Achille E, et al: GERCOR (French Oncology Research Group): Adjuvant treatment of colon cancer Mosaic study's main results. Bull Cancer 93:S5-S9, 2006 (suppl 1)

20. O'Connel MJ for the Adjuvant Colon Cancer Endpoints (ACCENT) Group: Survival following recurrence in patients with adjuvant colon cancer: Findings from the 20,800 patient ACCENT Dataset: NSAPB Foundation, Pittsburgh, PA. American Society of Clinical Oncology Gastrointestinal Symposium 223, January 19-21, 2007 (abstr 277), Orlando, FL

21. Renehan AG, Egger M, Saunders MP, et al: Impact on survival of intensive follow up after curative resection for colorectal cancer: Systematic review and meta-analysis of randomised trials. BMJ 324:813, 2002

22. Douillard JY, Cunningham D, Roth A, et al: Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: A multicentre randomised trial. Lancet 355:1041-1047, 2000

23. Van Cutsem E, Labianca R, Hossfeld D, et al: Randomized phase III trial comparing infused-irinotecan/5-fluorouracil (5FU)/folinic acid (AF) vs. 5FU/FA (AF) in stage III colon cancer patients (PETACC-3; V307). J Clin Oncol 23:3s, 2005 (abstr 8)

Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Annals of Oncology 20: 251–257, 2009
doi:10.1093/annonc/mdn557
Published online 20 August 2008

Randomized study of weekly irinotecan plus high-dose 5-fluorouracil (FUIRI) versus biweekly irinotecan plus 5-fluorouracil/leucovorin (FOLFIRI) as first-line chemotherapy for patients with metastatic colorectal cancer: a Spanish Cooperative Group for the Treatment of Digestive Tumors Study

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Received 7 May 2008; revised 11 July 2008; accepted 14 July 2008

Background: Irinotecan plus infusional 5-fluorouracil/leucovorin (FOLFIRI) is accepted as a reference treatment for the first-line treatment of patients with metastatic colorectal cancer (MCRC). The aim of this study was to demonstrate that a regimen without leucovorin (LV) (FUIRI) is not inferior to the standard FOLFIRI (response rate).
Patients and methods: Chemotherapy-naive patients with MCRC were randomized to receive either irinotecan (180 mg/m^2 on day 1) + 5-fluorouracil (5-FU) (400 mg/m^2 bolus and 600 mg/m^2 22-h infusion) + LV (200 mg/m^2 on days 1–2) (FOLFIRI) every 2 weeks or irinotecan (80 mg/m^2) + 5-FU (2.250 mg/m^2 48-h infusion) (FUIRI) weekly.
Results: In all, 346 patients were included, 173 in each arm. In the intention-to-treat analysis, the response rates for FOLFIRI and FUIRI were 57% [95% confidence interval (CI) 49% to 64%] and 51% (95% CI 43% to 59%), respectively ($P = 0.2809$). No statistically significant differences were observed between FOLFIRI and FUIRI regarding median progression-free survival (8.3 versus 8.4 months; $P = 0.4339$) nor median overall survival (21.6 versus 19.2 months; log-rank test $P = 0.2941$). Grade 3/4 neutropenia was significantly more frequent on FOLFIRI arm (27% versus 9%), while the proportion of diarrhea was higher on FUIRI arm (21% versus 42%).
Conclusion: FUIRI represents a valid alternative without LV to the FOLFIRI regimen as MCRC first-line treatment.
Key words: colorectal cancer, 5-fluorouracil, irinotecan

introduction

Until recently, the standard treatment for metastatic colorectal cancer (MCRC) has been 5-fluorouracil (5-FU)-based chemotherapy [1]. Furthermore, the efficacy of continuous infusion 5-FU relative to that of bolus 5-FU was a subject of ongoing debate. Most studies comparing continuous infusion with bolus administration are in favor of continuous infusion in relation to response rate and, probably, time to progression, although overall survival (OS) is not clearly better. The meta-analysis of six randomized trials in a total of 1219 patients with CRC showed higher response rates and slightly longer survival with continuous infusion 5-FU than with bolus 5-FU [2, 3]. In addition, the incidence of grades 3 and 4 hematotoxicity was lower with continuous infusion 5-FU. There is, however, no consensus on the optimal schedule of continuous infusion 5-FU. The most popular continuous infusion schedules in Europe are the de Gramont regimen (France) [4], AIO (Germany) [5] and Treatment of Digestive Tumors (TTD) (Spain) [6]. However, the response rates and median survival times achieved with infusional 5-FU alone are not optimal and more efficacious therapies are needed.

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Currently, after the publication of several phase III studies of combination of 5-FU, leucovorin (LV), and irinotecan or oxaliplatin, these regimens are considered as a reference first-line treatment for MCRC [7, 8]. Irinotecan in combination with infusional 5-FU/LV, in particular, is accepted as a standard regimen for the first-line treatment of patients with advanced or MCRC [9–11]. In addition to having improved therapeutic efficacy, infusional 5-FU/LV in combination with irinotecan has the advantage of a favorable toxicity profile compared with bolus 5-FU/LV combination therapy [12, 13].

In a previous phase I/II clinical trial conducted by our group in patients with advanced colorectal cancer, the combination of irinotecan 80 mg/m^2 plus TTD regimen (5-FU 2.25 g/m^2 given as 48-h i.v. infusion) in a weekly schedule was shown as an active and safe regimen for the first-line treatment of advanced colorectal cancer [14]. Therefore, the TTD regimen may constitute an alternative to other 5-FU/LV-modulated regimens for combining with irinotecan as first-line colorectal cancer treatment.

To date, no randomized clinical trials have compared the different 5-FU/irinotecan schedules. In order to confirm the value of the TTD/irinotecan (FUIRI) schedule in the treatment of previously untreated MCRC, a randomized study was designed to demonstrate that the efficacy of our regimen without LV is not inferior to the standard 5FULV2/CPT-11 (FOLFIRI).

patients and methods

This multicenter, prospective study was carried out by the Cooperative Group for the TTD group.

Local ethics committee approval was obtained before enrollment of any patient into the study, which was carried out in accordance with the Declaration of Helsinki and its subsequent amendments as well as Good Clinical Practice guidelines. Signed informed consent was obtained from all patients before study entry.

eligibility criteria

Patients with histological confirmed MCRC with at least one measurable lesion, chemotherapy naive or having finished prior adjuvant chemotherapy ≥6 months before were included. An interval of 4 weeks must have elapsed since the end of surgery and/or radiotherapy. Other eligibility criteria included the following: age 18–75 years; World Health Organization performance status of two or less and life expectancy of >3 months; adequate hematological parameters (hemoglobin ≥10 g/dl, absolute neutrophil count ≥2 × 10^9/l, and platelets ≥150 × 10^9/l); total bilirubin ≤1.25 × the upper limits of normal (ULN) and serum transaminase levels ≤3 × ULN (total bilirubin ≤1.5 × ULN, serum transaminase levels ≤ 5 × ULN, in presence of liver metastases); creatinine ≤1.25 ULN and; absence of a second primary tumor other than nonmelanoma skin cancer or *in situ* cervical carcinoma. Patients with central nervous system metastasis, unresolved bowel obstruction or subobstruction, Crohn's disease, ulcerative colitis, history of chronic diarrhea or known contraindications to fluorouracil (severe cardiac disease, including myocardial infarction within the 12 months before entry onto the trial, uncontrolled hypertension or angina pectoris) were excluded.

treatment schedule

Patients on the FOLFIRI arm received irinotecan 180 mg/m^2 as a 90-min i.v. infusion on day 1 with 5-FU 400 mg/m^2 bolus and 600 mg/m^2 by 22-h infusion, plus LV 200 mg/m^2 on days 1 and 2, every 2 weeks. FUIRI consisted of weekly irinotecan 80 mg/m^2 as a 30-min i.v. infusion followed immediately by 5-FU 2250 mg/m^2 given as a 48-h i.v. infusion; treatment was administered weekly without any rest period between cycles. For both arms, a treatment cycle lasted 6 weeks, and therapy was continued until disease progression, severe toxicity, treatment refusal (unrelated to toxicity), investigator decision or death or until patient was lost to follow-up.

Irinotecan was administered according to the guidelines used for irinotecan monotherapy, including recommendations for the use of concurrent antiemetics, atropine, and loperamide. Patients were assessed for toxicity before each infusion using the National Cancer Institute—Common Toxicity Criteria, version 2.0, April 1999 [15]. For any patient with severe toxicity, therapy had to be delayed until complete normalization, and the dose of 5-FU and irinotecan had to be reduced to 80% of the previous dose for all further administrations. In case of grade 4 neutropenia or febrile neutropenia concomitant with diarrhea, preventive oral antibiotic therapy was indicated. At the end of the treatment period, patients were followed up every 3 months to document death, progression, or further cancer treatment.

evaluations during the study

Physical examination and laboratory studies, including complete blood counts with differential, serum liver function tests, and actual or estimated creatinine clearance, were carried out within 7 days of enrollment; electrocardiography and carcinoembryonic antigen were determined within 21 days before treatment start; and chest radiograph or computed tomography (CT) scan and abdominal CT scan were completed within 28 days of enrollment. Women of childbearing potential had a negative serum pregnancy testing within 7 days of registration. During treatment, safety assessments and blood counts were carried out before each infusion (weekly in arm A and every 2 weeks in arm B). Serum chemical values were assessed at the beginning of each treatment cycle (every 6 weeks).

Tumor response studies were carried out using the response evaluation criteria in solid tumors criteria [16] every 12 weeks or sooner if clinically indicated, until the disease progressed or the patient died. The duration of response was measured from the first documentation of response to disease progression. Disease-free survival was defined as the time interval from randomization until progression or death. The time to treatment failure was calculated from randomization to the date of treatment failure, regardless of cause of it (disease progression, relapse, death, or any other cause of discontinuing the study treatment). Without contradictory data, patients who died or were lost to follow-up were assumed to have progressed at the time they were last documented to be progression free. OS was measured from the randomization to date of death.

statistical considerations

Randomization was centralized without patients' stratification. We planned to include 328 patients (164 per arm) to show a significant difference in response rate (overall response rate, complete, and partial responses) between treatment groups, assuming response rates of 38% in the FOLFIRI group and 38% ± 15% in the FUIRI group, by use of two-tailed chi-square tests ($\alpha = 0.05$, $\beta = 0.2$).

Chi-square tests were used to compare categorical variables between treatment groups. For continuous variables, we used Student's *t*-tests. The Kaplan–Meier method was used to estimate survival curves, and the log-rank test was used to compare the curves. Cox proportional hazards modeling was used to calculate hazard ratios and 95% confidence intervals (CIs). All randomized patients were included in an intention-to-treat analysis; patients who canceled before the initiation of therapy were excluded from the toxicity analyses (Figure 1).

results

From October 2001 to October 2005, a total of 346 patients from 18 Spanish centers were enrolled onto the study and

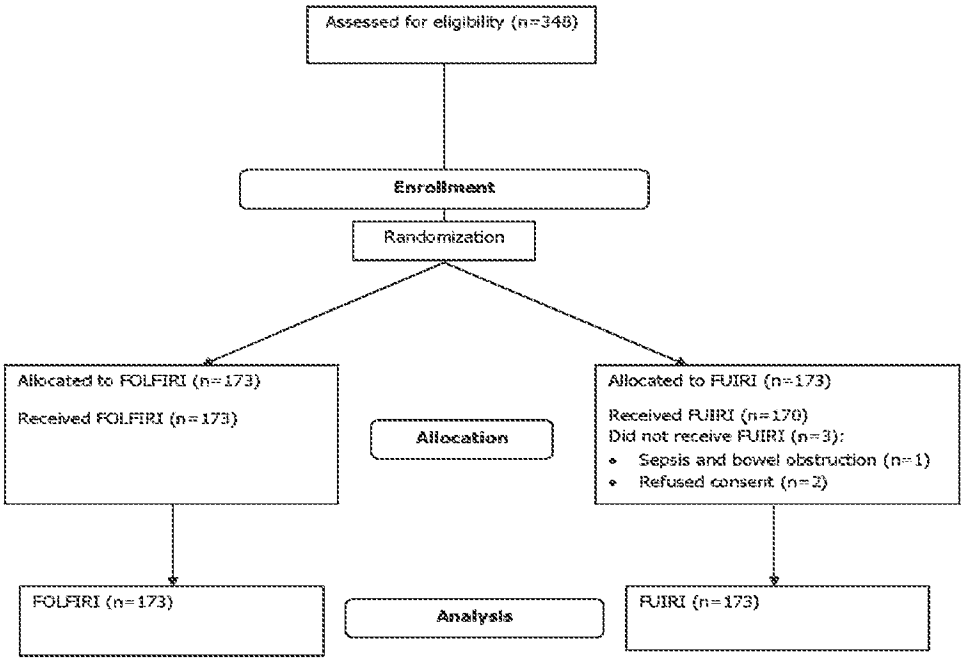

Figure 1. Consort flowchart.

randomly assigned to FOLFIRI ($n = 173$) or FUIRI ($n = 173$). Table 1 presents the characteristics of the patients, which were balanced among the treatment groups: nevertheless, the rectum was the primary tumor site in a higher proportion of patients in the FUIRI group (31%) than in the FOLFIRI group (24%). Fifty per cent of the patients had an Eastern Cooperative Oncology Group performance status of zero at baseline, and more than two-thirds of patients in each group had at least two organs involved, with the liver being the most common site of metastatic disease.

treatment compliance

Three patients did not receive any treatment infusion, all of them in the FUIRI group, two because consent was withdrawn and another due to toxicity (sepsis and bowel obstruction) before starting treatment. As presented in Table 2, the median number of administered cycles was 4 in both arms (range 1–24). Sixty-two per cent of patients in the FOLFIRI group and 55% in the FUIRI group received more than three complete cycles (18 weeks) of chemotherapy.

The median relative intensity of the doses of irinotecan and 5-FU (calculated as the actual dose delivered divided by the intended dose) were significantly higher in the FOLFIRI group than in the FUIRI group (89% versus 83%, $P = 0.002$).

toxicity

Grades 3 and 4 toxic effects occurring at a frequency of 3% or more are shown in Table 3. Diarrhea was the most frequent grade 3 or 4 non-hematological toxic effect in each of the treatment groups, being significantly more frequent in the FUIRI group ($P < 0.00001$): most of the cases were grade 3 (19 of 21 in the FOLFIRI arm and 36 of 42 in the FUIRI arm). Grade 3 or 4 hematological toxicity was higher in the FOLFIRI arm, reaching statistically significance for neutropenia

Table 1. Patient characteristics

Characteristic	FOLFIRI (173)		FUIRI (173)	
	No. of patients	%	No. of patients	%
Sex				
Male	110	64	110	64
Female	63	36	63	36
Age, years				
Median	63		63	
Range	29–75		28–75	
Eastern Cooperative Oncology Group				
0	86	50	88	51
1	79	45	74	43
2	8	5	11	6
Primary site				
Colon	130	75	113	65
Rectum	41	24	54	31
Both	–	–	5	3
Nonspecified	2	1	1	1
Metastatic site				
Liver	121	70	133	77
Lung	53	31	55	32
Lymph nodes	39	23	24	14
Abdomen	35	20	21	12
Other	18	10	11	6
No. of metastatic sites				
1	103	60	116	67
>2	70	40	57	33
Prior therapy				
Radiotherapy	14	8	23	13
Surgery	153	88	153	88
Adjuvant chemotherapy	38	22	37	21

FOLFIRI, 5-fluorouracil/leucovorin.

($P < 0.00001$); for leucopenia the difference was close to significance ($P = 0.0672$).

During the first 60 days, five patients died in the FOLFIRI arm and four in the FUIRI arm. There were two therapy-related deaths in the FOLFIRI arm (sepsis and febrile neutropenia: both in the first 60 days) and four in the FUIRI arm (two septic shock, one sudden death, and one diarrhea: two in the first 60 days, one septic shock, and one diarrhea).

response to treatment

Thirty-five patients (14%) who did not complete the first cycle of treatment were included in the efficacy analysis as nonevaluable, 10 on FOLFIRI arm and 25 on FUIRI arm, mainly due to toxicity, consent withdrawn or not cancer-related death. In the intent-to-treat analysis (Table 4), where all randomized patients were included (346 patients), the response rates for the FOLFIRI and the FUIRI arms were 57% (95% CI 49% to 64%) and 51% (95% CI 43 to 59%), respectively: no statistically significant differences were detected ($P = 0.2809$) (Table 4). The median duration of response was 7.2 (95% CI 5.9–8.5) months in the FOLFIRI group and 6.1 (95% CI 5.6–7.5) months in the FUIRI group ($P = 0.3137$). Secondary resection of liver metastases was possible in 10 patients in each arm of treatment.

survival analysis

After a median follow-up of 17.2 months (95% CI 15.7% to 18.5%), 160 patients in the FOLFIRI arm and 158 in the FUIRI arm had progressed or died. The median progression-free survival in the FOLFIRI group was 8.3 months (95% CI 7.3–8.9 months) versus 8.4 months (95% CI 7.1–9.1 months) in the FUIRI group ($P = 0.4339$; Figure 2). The median OS was 21.6 months (95% CI 19.9–25.3 months) in patients treated with FOLFIRI compared with 19.2 months (95% CI 17.4–23.8 months) in the FUIRI group ($P = 0.2941$; Figure 3).

Seventy-five per cent of patients in the FOLFIRI group and 68% in the FUIRI group ($P = 0.122$) received additional second-line treatment (Table 5). No significant differences were founded in the proportion of patients receiving further treatment with oxaliplatin between the two groups (72% FOLFIRI versus 62% FUIRI).

Table 2. Number of cycles and relative dose intensities

	Arm A (FOLFIRI) (No. of patients = 173)	Arm B (FUIRI) (No. of patients = 173)
No. of cycles		
Total	847	810
Median per patient	5	4
Range	1–24	1–21
Relative dose intensity (%)		
Irinotecan	89	83
Fluorouracil	89	83

FOLFIRI, 5-fluorouracil/leucovorin.

Table 3. Maximum toxicity (>3%) per patient (%) according to NCI grade

	Arm A (FOLFIRI) (No. = 173)		Arm B (FUIRI) (No. = 173)		P (grade 3–4)
	3	4	3	4	
Hematological					
Leucopenia	24	4	25	1	NS
Neutropenia	22	27	31	9	<0.00001
Non-hematological					
Diarrhea	54	21	47	42	<0.00001
Nausea	50	3	59	4	NS
Vomiting	40	4	42	8	NS
Abdominal pain	33	6	41	8	NS
Astenia/fatigue	54	10	59	18	NS
Anorexia	26	3	39	8	NS
Stomatitis	42	4	38	3	NS
Infection	28	3	30	6	NS
(no neutropenia)					
Febrile neutropenia	–	3	–	2	NS

NCI, National Cancer Institute; FOLFIRI, 5-fluorouracil/leucovorin; NS, not significant.

Table 4. Antitumoral efficacy (intention-to-treat analysis: 346 patients)

Parameter	Arm A (FOLFIRI)		Arm B (FUIRI)	
	No. of patients	%	No. of patients	%
Complete response	15	9	15	9
Partial response	83	48	73	42
Stable disease	44	25	49	28
Progressive disease	21	12	11	6
Nonevaluable	10	6	25	15
Total	173	100	173	100

FOLFIRI, 5-fluorouracil/leucovorin.

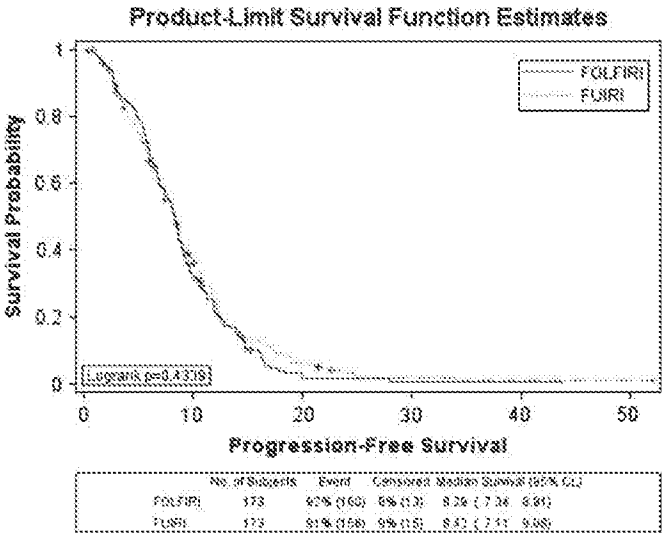

Figure 2. Progression-free survival.

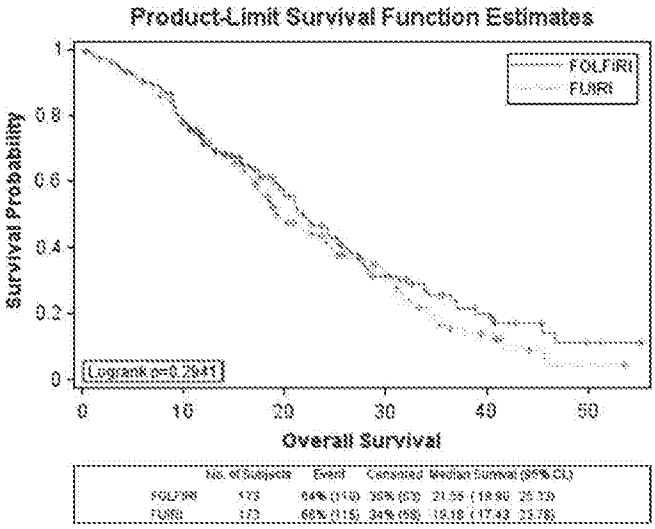

Figure 3. Overall survival.

Table 5. First second-line treatment administered to patients in the two treatment groups

Regimen	Arm A (FOLFIRI)[a]		Arm B (FUIRI)[b]	
	No. of patients	%	No. of patients	%
Oxaliplatin based	93	71	72	62
Irinotecan based	17	13	27	23
Fluoropyrimidines	9	7	9	8
Cetuximab	6	5	5	4
Others	5	4	4	3
Total	130	100	117	100

[a]One hundred and thirty (75%) of 173 patients received second-line treatment.

[b]One hundred and seventeen (68%) of 173 patients received second-line treatment.

FOLFIRI, 5-fluorouracil/leucovorin.

discussion

This trial is the first randomized study comparing two European regimens of 5-FU ± LV in combination with irinotecan in the treatment of advanced colorectal cancer. This study provides convincing evidence that FUIRI is an active regimen for the treatment of patients with previously untreated advanced colorectal cancer, without statistically significant differences in efficacy with the FOLFIRI regimen and with a different toxicity profile. The control regimen of 5-FU/LV/irinotecan selected has been one of the most commonly and active used (and tested in phase III studies) treatments for MCRC in Europe, having showed increased response rates, time to progression, and survival in comparison with the same combination without irinotecan [9].

In the present trial, we used the schedule we had previously developed in a phase I/II study [14] in which 5-FU is not modulated by LV, in order to increase the dose intensity of continuous infusion 5-FU. As we have previously demonstrated, the addition of LV to high doses of FU does not increase activity but does increases toxicity and treatment cost [17, 18].

Although comparisons over time and studies may be problematic, the efficacy results showed in the present study for both arms are among the highest reported to date in randomized trials in patients with advanced colorectal cancer treated with an irinotecan/5-FU combination [9–11, 19–24]: Table 6 summarizes the results of the randomized studies of irinotecan (CPT-11) in combination with 5-FU continuous infusion as first-line treatment of colorectal cancer. Indeed, it is noteworthy that the median survival times achieved by patients treated in both arms are similar to the best reported in randomized multicenter trials including either irinotecan- or oxaliplatin-based combinations. This may be a consequence of the high proportion of patients who received a second-line therapy, mainly based in oxaliplatin, what strongly suggests that all patients should receive all available active drugs during the

Table 6. Randomized studies of CPT-11 + 5-FU continuous infusion as first-line treatment of colorectal cancer

Schedule	N	RR %	Progression free survival, median (months)	Overall survival, median (months)	Neutropenia[a]	Diarrhea[b]
FOLFIRI/AIOIRI (9)	198 (101/97)	34.8	6.7	17.4	46/29	13/44
AIOIRI (11)	214	62.2[c]	8.5	20.1	7	29[d]
FOLFIRI (19)	172	31	7.0	14	10	10
FOLFOXIRI (20)	137	43.0	–	21.5	35	28
FOLFIRI (20)	146	33.6	–	19.5	28	11
FOLFOXIRI (21)	122	66	9.8	22.6	50	20
FOLFIRI (21)	122	41	6.9	16.7	28	12
Simplified FOLFIRI (22)	109	56	8.5	21.5	24	14
Simplified FOLFIRI (24)	144	47.2	7.6	23.1	43	14
FOLFIRI (present study)	173	57	8.3	21.6	27	21
FUIRI (present study)	173	51	8.4	19.2	9	42

[a]Percentage of patients with NCI grades 3–4 neutropenia.

[b]Percentage of patients with NCI grades 3–4 diarrhea.

[c]RR in patients with measurable disease.

[d]Toxicity in the dose reduced cohort (5-FU 2.0 g/m^2).

5-FU, 5-fluorouracil; RR, response rate; FOLFIRI, 5-fluorouracil/leucovorin; NCI, National Cancer Institute.

course of their disease, as have been argued previously by other authors [10, 11, 22, 23].

The safety profile of both regimens was consistent with that reported in previous randomized studies using the same or similar schedules [9–11, 19–24]: the FOLFIRI schedule was associated with higher frequency of grade 3 or 4 hematological toxicity while non-hematological toxic effects (mainly diarrhea) were more frequent with FUIRI. In particular, the safety profile of the FUIRI regimen was comparable to that reported by Köhne et al. [11] using a similar regimen (AIO) based on 5-FU administered as high-dose continuous infusion (plus LV). Indeed, in that study, the initial 5-FU dose of 2300 mg/m^2 was reduced to 2000 mg/m^2 due to an elevated percentage of grades 3–4 diarrhea (36% of patients) and three toxic deaths in the irinotecan + AIO arm. Nevertheless, the proportion of grade 4 diarrhea during treatment with FUIRI was much lower than in our prior phase I/II study [14]: this fact underlines the importance of the strict adherence to guidelines for patient surveillance and dose adjustment followed by the investigators participating in this study. The death rates within the first 60 days of treatment and the proportion of toxic deaths were comparable between both arms (no statistically significant differences were observed) in the expected range with these regimens.

In conclusion, FUIRI represents a valid alternative to the standard FOLFIRI regimen as a first-line option of care for patients with MCRC. Our regimen seems suitable to be combined with new targeted agents, such as cetuximab or bevacizumab: in fact, bevacizumab has been approved in Europe and the United States for combination with 5-FU/LV and irinotecan on the basis of the increases in OS, progression-free survival, and response rate over the same regimen without bevacizumab and the addition of bevacizumab cause an increase of neutropenia that could be less pronounced in case of combination of FUIRI with bevacizumab [25].

funding

Pfizer.

acknowledgements

The physicians listed below cared for the patients in this study. The authors thank them for their cooperation and support: Dr Valladares Ayerbes, Complejo Hospitalario Juan Canalejo Marítimo de Oza, La Coruña, Dr Martinez Villacampa, Instituto Catalán de Oncología, Hospital Duran i Reinals, Barcelona, Dr Benavides Orgaz, Hospital Regional Universitario Carlos Haya, Málaga, Dr Aranda Aguilar, Hospital Universitario Reina Sofía, Córdoba, Dr Massutti Sureda, Hospital General Universitario de Alicante, Alicante, Dr Marcuello Gaspar, Hospital Santa Creu i Sant Pau, Barcelona, Dr Constenla Figueiras, Complejo Hospitalario de Pontevedra, Pontevedra, Dr Camara Vicario, Fundación Hospital Alcorcón, Madrid, Dr Carrato Mena, Hospital General Universitario de Elche, Alicante, Dr Sánchez Rovira, Hospital Ciudad de Jaén, Jaén, Dr Almenar Cubells, Hospital Universitario, Dr Peset, Valencia, Dr Llanos Muñoz, Hospital Universitario de Canarias, Tenerife, Dr Sastre Valera, Hospital Universitario Clínico San Carlos, Madrid, Dr Galán Brotons, Hospital de Sagunto, Valencia, Dr Etxeberria Larrea, Instituto Oncológico, Guipúzcoa, Dr García López, Hospital Ramón y Cajal, Madrid, Dr Checa Ruiz, Instituto de Oncología Corachán, Barcelona, Dr Aranda Bellido, Hospital de Mérida, Badajoz. Monitoring, Statistics and Data Management: Pivotal. TTD (Spanish Cooperative Group for the Treatment of Digestive Tumors): Ruiz de Mena, I. Author's disclosures of potential conflicts of interest: the authors indicated no potential conflicts of interest.

references

1. Advanced Colorectal Cancer Meta-Analysis Project. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer. Evidence in terms of response rate. J Clin Oncol 1992; 10: 896–903.
2. Meta-Analysis Group in Cancer. Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. J Clin Oncol 1998; 16: 301–308.
3. Meta-Analysis Group in Cancer. Toxicity of fluorouracil in patients with advanced colorectal cancer: effect of administration schedule and prognostic factors. J Clin Oncol 1998; 16: 3537–3541.
4. de Gramont A, Bosset JF, Milan C et al. Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: a French intergroup study. J Clin Oncol 1997; 15: 808–815.
5. Köhne C-H, Schöffski P, Wilke H et al. Effective biomodulation by leucovorin of high-dose infusion fluorouracil given as a weekly 24-hour infusion: results of a randomized trial in patients with advanced colorectal cancer. J Clin Oncol 1998; 16: 418–426.
6. Aranda E, Díaz-Rubio E, Cervantes A et al. Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with weekly high-dose 48-hour continuous-infusion fluorouracil for advanced colorectal cancer: a Spanish Cooperative Group for Gastrointestinal Tumor Therapy (TTD) study. Ann Oncol 1998; 9: 727–731.
7. Venook A. Critical evaluation of current treatments in metastatic colorectal cancer. Oncologist 2005; 10: 250–261.
8. O'Neil BH, Goldberg RM. Chemotherapy for advanced colorectal cancer: let's not forget how we got here (until we really can). Semin Oncol 2005; 32: 35–42.
9. Douillard JY, Cunningham D, Roth AD et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000; 355: 1041–1047.
10. Saltz LB, Cox JV, Blanke C et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. N Engl J Med 2000; 343: 905–914.
11. Köhne C-H, van Cutsem E, Wils J et al. Phase III study of weekly high-dose infusional fluorouracil plus folinic acid with or without irinotecan in patients with metastatic colorectal cancer: European Organisation for Research and Treatment of Cancer Gastrointestinal Group Study 40986. J Clin Oncol 2005; 23: 4856–4865.
12. Sargent DJ, Niedzwiecki D, O'Connell MJ et al. Recommendation for caution with irinotecan, fluorouracil, and leucovorin for colorectal cancer. N Engl J Med 2001; 345: 144–145.
13. van Cutsem E, Douillard JY, Köhne C-H. Toxicity of irinotecan in patients with colorectal cancer. N Engl J Med 2001; 345: 1351–1352.
14. Aranda E, Carrato A, Cervantes A et al. On behalf of Grupo Español de Tratamiento de Tumores Digestivos (TTD). Phase I/II trial of irinotecan plus high-dose 5-fluorouracil (TTD regimen) as first-line chemotherapy in advanced colorectal cancer. Ann Oncol 2004; 15: 559–567.
15. Cancer Therapy Evaluation Program. Common Toxicity Criteria, Version 2.0. 1998; http://ctep.cancer.gov/reporting/ctc.html.
16. Therasse P, Arbuck SG, Eisenhauer EA et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000; 92(3): 205–216.

17. Díaz-Rubio E, Aranda E, Camps C et al. A phase II study of weekly 48-hour infusions with high-dose fluorouracil in advanced colorectal cancer: an alternative to biochemical modulation. J Infus Chemother 1994; 4: 58–61.
18. Aranda E, Cervantes A, Dorta J et al. A phase II trial of weekly high-dose continuous infusion 5-fluorouracil plus oral leucovorin in patients with advanced colorectal cancer. Cancer 1995; 76: 559–563.
19. Colucci G, Gebbia V, Paoletti G et al. Phase III randomized trial of FOLFIRI versus FOLFOX4 in the treatment of advanced colorectal cancer: a multicenter study of the Gruppo Oncologico Dell'Italia Meridionale. J Clin Oncol 2005; 23: 4866–4875.
20. Souglakos J, Androulakis N, Syrigos K et al. FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) vs FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) as first-line treatment in metastatic colorectal cancer (MCC): a multicentre randomised phase III trial from the Hellenic Oncology Research Group (HORG). Br J Cancer 2006; 94: 798–805.
21. Falcone A, Ricci S, Brunetti I et al. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. J Clin Oncol 2007; 25: 1670–1676.
22. Tournigand C, André T, Achille E et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. J Clin Oncol 2004; 22: 229–237.
23. Goldberg RM, Sargent DJ, Morton RF et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004; 22: 23–30.
24. Fuchs CS, Marshall J, Mitchell E et al. Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: results from the BICC-C Study. J Clin Oncol 2007; 25(30): 4779–4786.
25. Hurwitz H, Fehrenbacher L, Novotny W et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350: 2335–2342.

Antitumor efficacy of a liposomal formulation of irinotecan in preclinical gastric cancer models: Augmenting its response by antiangiogenic agents

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Background

- *Gastric Adenocarcinoma (GAC):*
  - The 3rd most common cause of cancer-related deaths worldwide
  - Five-year survival rate 31%
  - Standard treatment: FLOT [5-FU, Leucovorin, Oxaliplatin, Docetaxel]
  - Median progression-free survival (PFS) and overall survival (OS): 5.2 and 11.1 months
  - Dismal prognosis factors: late-stage diagnosis; early and aggressive invasion with metastatic disease; resistance to conventional chemotherapy; post-operative recurrence common
  - Second-line therapy: Taxanes and Irinotecan (cytotoxic agents), Trastuzumab, Ramucirumab (molecular targeted agents)
- *Angiogenesis:*
  - Essential process for tumor growth and metastasis
  - Potential target for cancer therapy
  - Angiogenesis play an important role in pathogenesis of GAC
- *Liposomal irinotecan (Onivyde, nal-IRI):*
  - Superior efficacy and safety over water-soluble, non-liposomal irinotecan
  - Enhanced circulation- 70X higher irinotecan in blood than conventional irinotecan
  - Enhanced drug availability- 6X higher SN38 (active metabolite) in tumor compared to plasma in human
  - Approved second-line therapy for metastatic pancreatic ductal adenocarcinoma
- *DC101 (DC):*
  - Murine version of Ramucirumab, an approved therapy for NSCLC and gastric cancer
  - Monoclonal antibody targeting VEGFR2
- *Cabozantinib (Cab):*
  - Potent small molecule inhibitor that targets c-Met, VEGFR2, Axl, Ret, and Kit signaling
  - Approved treatment for medullary thyroid cancer and renal cell carcinoma
- *Nintedanib (Nin):*
  - Potent triple angiokinase inhibitor of VEGFR1/2/3, FGFR1/2/3, PDGFR-α/β
  - Approved therapy for NSCLC (EU)

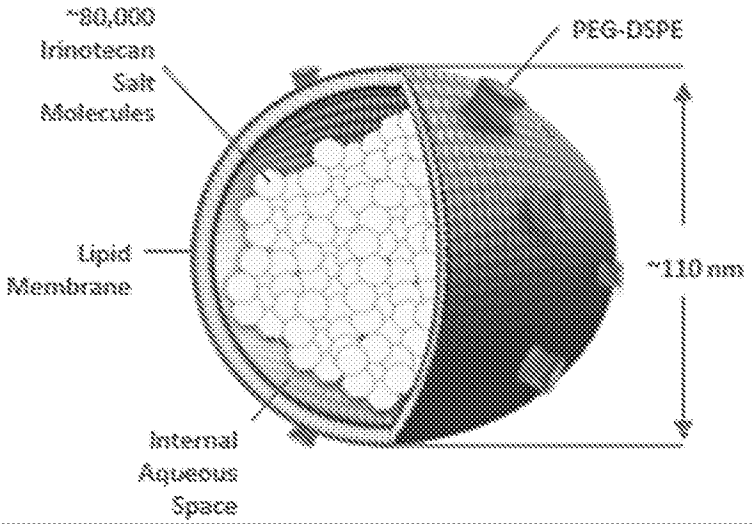
Study Objectives

Based on improved pharmacokinetics and drug biodistribution of nal-IRI compared with free irinotecan and a crucial role of angiogenesis in the progression and metastasis of GAC, we aimed to evaluate the antitumor response of nal-IRI, in combination with mechanistically diverse antiangiogenic agents, in preclinical models of GAC.

Methods

- Animal survival experiments were performed in the peritoneal dissemination xenograft model in NOD-SCID mice using $10x10^6$ human GAC MKN-45 or KATO-III cells (n=5-7).
- Tumor growth and pharmacokinetic studies were performed in subcutaneous xenografts in NOD-SCID mice using $7.5x10^6$ human GAC MKN-45 cells (n=5-7).
- *In vivo* drug doses: nal-IRI (10 mg/kg, 1x/wk), IRI (50 mg/kg, 1x/wk), DC101 (40 mg/kg, 2x/wk), Cab (30 mg/kg, 5x/wk) and Nin (25 mg/kg, 5x/wk) were delivered intraperitoneally.
- *In vitro* cell proliferation was evaluated in three GAC cell lines (MKN-45, KATO-III and SNU-5).
- Mechanistic evaluation: immunoblot and Immunohistochemistry analysis in tumor samples.
- Animal studies were performed following an approved IACUC protocol of Indiana University.

Liposomal irinotecan

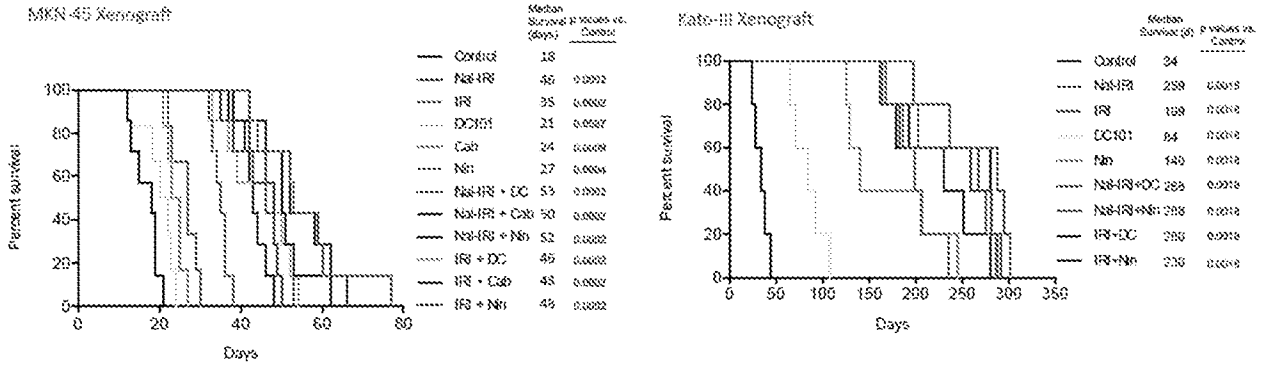


Gastric cancer cell lines used

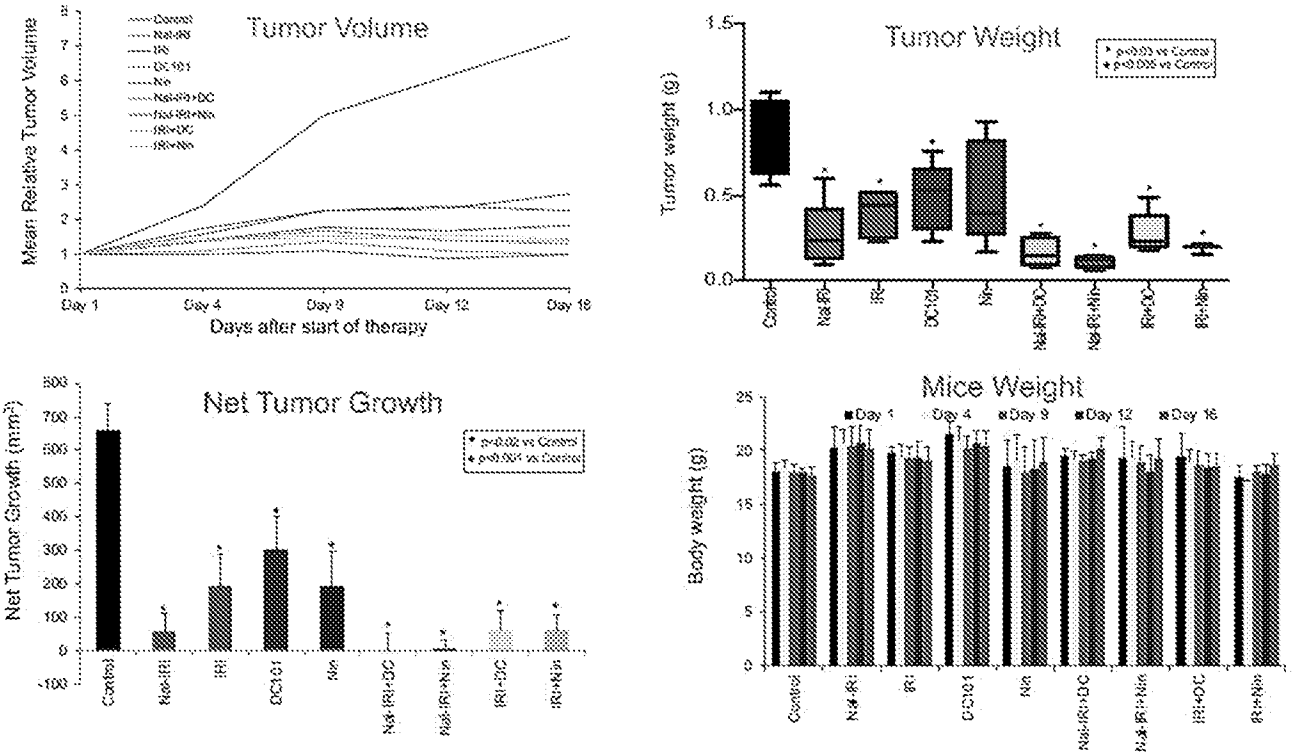
Cell line	Origin	Histology	Oncogene Expression
MKN-45	Liver metastasis	Poorly differentiated adenocarcinoma	c-met+, Ecad+
KATO-III	Pleural effusion	Signet ring cell carcinoma	c-met+, FGFR2/K-sam+
SNU-5	Malignant ascites	Poorly differentiated adenocarcinoma	myc+, erb B2+

Results

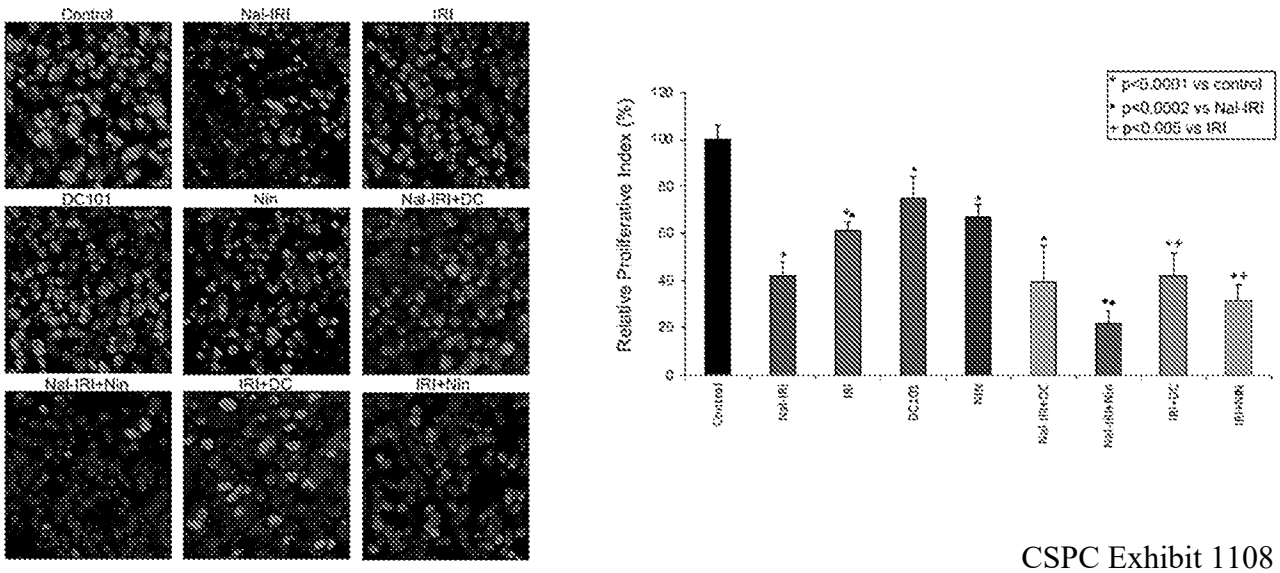
Nal-IRI, IRI and Antiangiogenic Agents Therapy: Animal survival in peritoneal dissemination xenografts



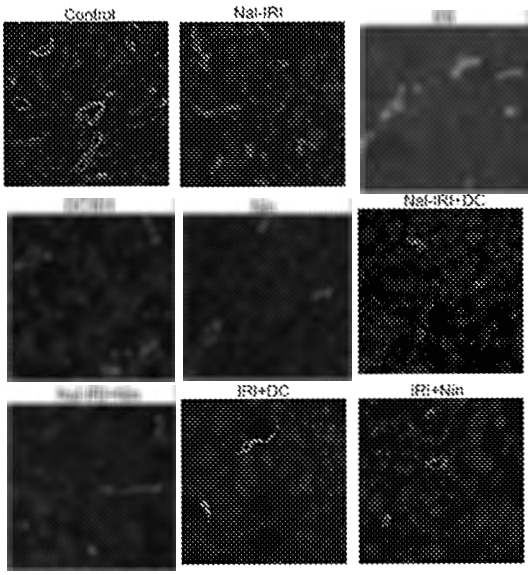
Nal-IRI, IRI and Antiangiogenic Agents Therapy: Tumor growth in subcutaneous xenografts

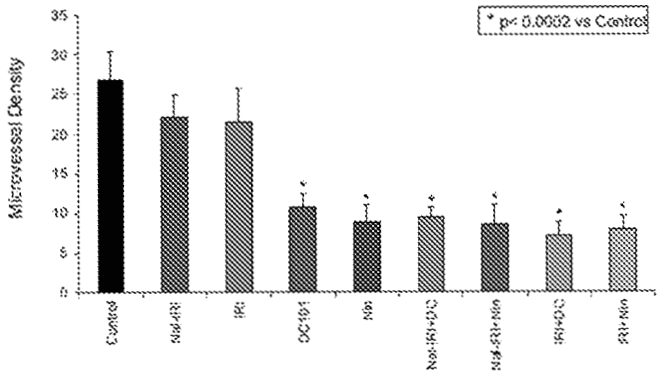


Nal-IRI, IRI and Antiangiogenic Agents Therapy: Tumor cell proliferation analysis (Ki67 staining)

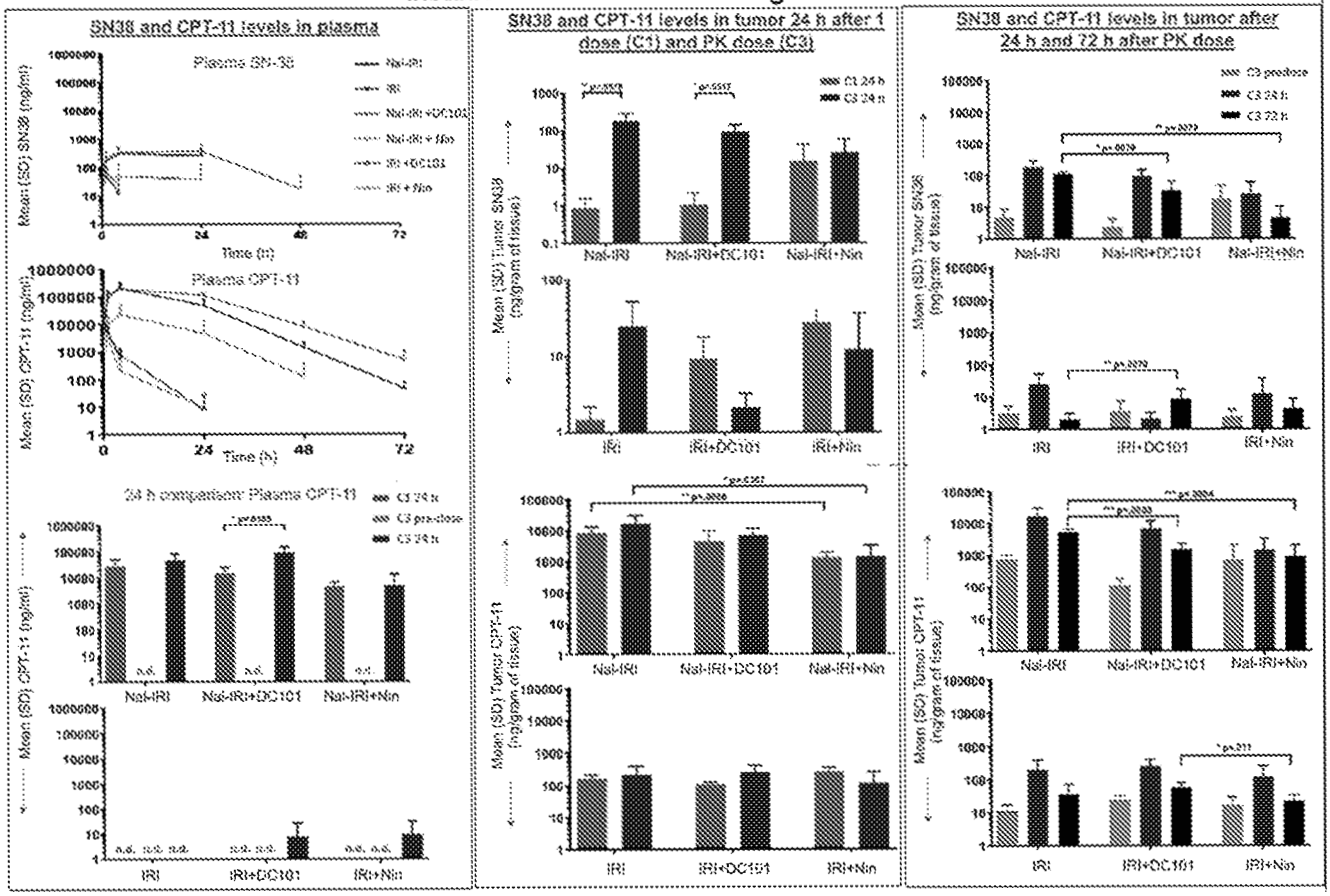


Nal-IRI, IRI and Antiangiogenic Agents Therapy: Tumor vasculature analysis (endomucin staining)

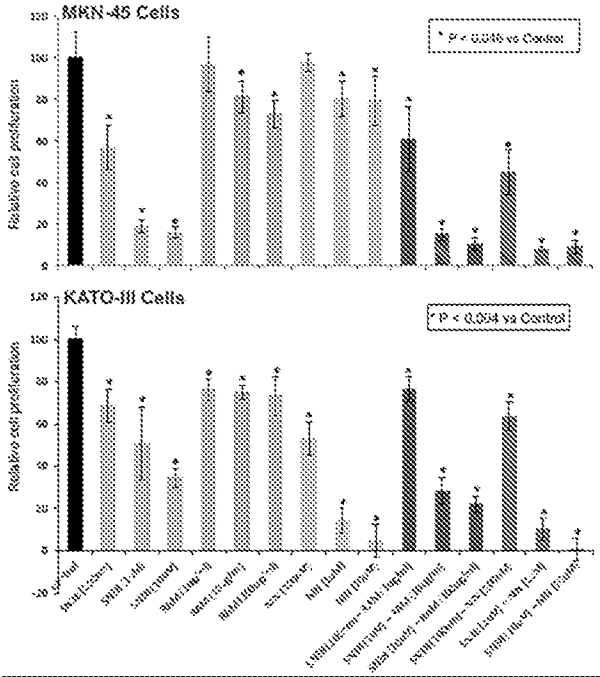


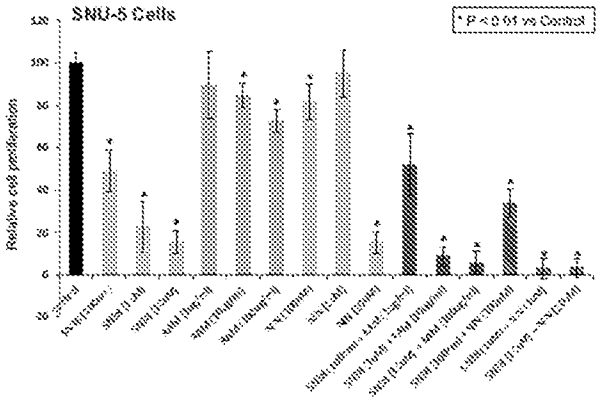


Nal-IRI, IRI and Antiangiogenic Agents Therapy: Pharmacokinetic analysis in MKN-45 tumors- Metabolite levels after IP drug deliveries



Nal-IRI, IRI and Antiangiogenic Agents Treatment: *In vitro* GAC cell proliferation





Summary

- Nal-IRI increased animal survival (>156%), this extension in survival was >30% compared with IRI
- Addition of nintedanib and DC101 further improved (range 3-40%) animal survival caused by nal-IRI and IRI
- Nal-IRI exhibited greater tumor growth inhibition (92%) than IRI (71%) in GAC subcutaneous xenografts
- The combinations of nal-IRI and IRI with antiangiogenic agents demonstrated additive response on tumor growth inhibition
- Nal-IRI was most effective in reducing tumor cell proliferation (by 58%), followed by IRI (39%), nintedanib (33%), DC101 (25%)
- Combinations of nal-IRI or IRI with antiangiogenic agents demonstrated an additive effect in reducing tumor cell proliferation
- Tumor vasculature was reduced by nintedanib (65%) and DC101 (58%), while nal-IRI and IRI showed no effect
- Pharmacokinetic studies: compared with IRI, nal-IRI increased the retention, circulation time and tumor levels of CPT-11 (IRI) and its active metabolite SN38
- Addition of nintedanib or DC101 did not cause any significant increase in plasma or tumor levels of SN38 or CPT-11 (IRI)
- In the nal-IRI group, addition of nintedanib exhibited increased clearance of SN38 and CPT-11 in plasma and tumor
- *In vitro* cell viability assay demonstrated a dose-dependent growth inhibitory effects of SN-38 on GAC cells
- Combinations of SN38 with antiangiogenic agents demonstrated additive effects on cell proliferation inhibition

Conclusion

Nal-IRI has greater antitumor efficacy than IRI, and its antitumor effects can be enhanced by antiangiogenic agents suggesting that this combination has potential for improving clinical GAC therapy

Acknowledgements

This work has been financially supported by Merrimack Pharmaceuticals, Ipsen Biopharmaceuticals and Indiana University School of Medicine funds.

Antitumor efficacy of a liposomal formulation of irinotecan in preclinical gastric cancer models: Augmenting its response by antiangiogenic agents
HARPER
Background
Results
Summary
Conclusion
Acknowledgments
Gastric cancer cell lines used

CANCER RESEARCH

Experimental and Molecular Therapeutics

Abstract 553: Antitumor efficacy of a liposomal formulation of irinotecan in preclinical gastric cancer models: Augmenting its response by antiangiogenic agents

Niranjan Awasthi, Margaret A. Schwarz, Changhua Zhang, Stephan Klinz, Florence Meyer-Losic, Benjamin Beaufils, Arunthathi Thiagalingam, and Roderich E. Schwarz

DOI: 10.1158/1538-7445.AM2020-553 Published August 2020

Proceedings: AACR Annual Meeting 2020; April 27-28, 2020 and June 22-24, 2020; Philadelphia, PA

Abstract

BACKGROUND: Gastric adenocarcinoma (GAC) remains the 3rd most common cause of cancer-related deaths worldwide. Systemic chemotherapy is commonly a fundamental treatment for metastatic GAC that usually leads to a modest patient benefit, resulting in a 5-year survival rate of 31%. A liposomal formulation of irinotecan (nal-IRI) has shown improved pharmacokinetic and drug biodistribution compared with free irinotecan (IRI) in preclinical studies. Angiogenesis plays a crucial role in the progression and metastasis of GAC. We evaluated the therapeutic efficacy of nal-IRI in combination with nintedanib, a tyrosine kinase inhibitor targeting FGFR, PDGFR and VEGFR, and DC101, a monoclonal antibody targeting VEGFR2 in preclinical models of GAC.

METHODS: *In vitro* cell proliferation was evaluated in three GAC cell lines (MKN-45, KATO-III and SNU-5) by WST-1 assay. Protein expression was measured by Western blot analysis in MKN-45 cell lysates. Animal survival studies were performed using two cell lines MKN-45 and KATO-III cells in peritoneal dissemination models in NOD/SCID mice (n=5-7). Tumor growth and pharmacokinetic studies were performed in GAC cell-derived xenografts. Mechanistic evaluation included IHC and Immunoblot analysis in tumor tissues.

RESULTS: Animal survival was increased by nal-IRI (>156%) and IRI (>94%) therapy compared with PBS-treated controls. Importantly, nal-IRI led to a >30% extension of animal survival compared with IRI. The addition of

antiangiogenic agents nintedanib or DC101 led to a further improvement (range 3-40%) in animal survival caused by nal-IRI and IRI. In GAC cell-derived subcutaneous xenografts, compared to controls, nal-IRI demonstrated greater tumor growth inhibition (92%) than IRI (71%). Here, the combinations of nal-IRI or IRI with antiangiogenic agents exhibited an additive response. Ki67 staining of tumor sections revealed that nal-IRI was most effective in reducing intratumoral proliferation (by 58%), followed by IRI (39%), nintedanib (33%), and DC101 (25%). Combinations of nal-IRI or IRI with antiangiogenic agents demonstrated an additive effect in reducing tumor cell proliferation. As expected, tumor vasculature (assessed by endomucin staining) was reduced by nintedanib (65%) and DC101 (58%), while nal-IRI and IRI showed no effect. Pharmacokinetic studies revealed that nal-IRI increased the retention, circulation time and tumor levels of IRI and its active metabolite SN-38. The addition of nintedanib or DC101 had no effect on plasma or tumor levels of IRI or SN38. In *in vitro* cell viability assays in mutationally different GAC cells, SN-38 had a dose-dependent growth inhibitory effect that was more pronounced than single-agent IRI. Importantly, combinations of SN-38 or IRI with antiangiogenic agents demonstrated additive effects on cell proliferation inhibition.

CONCLUSION: nal-IRI showed greater antitumor efficacy than IRI, and its antitumor effects can be enhanced by antiangiogenic agents suggesting that this combination has potential for improving clinical GAC therapy.

Citation Format: Niranjan Awasthi, Margaret A. Schwarz, Changhua Zhang, Stephan Klinz, Florence Meyer-Losic, Benjamin Beaufils, Arunthathi Thiagalingam, Roderich E. Schwarz. Antitumor efficacy of a liposomal formulation of irinotecan in preclinical gastric cancer models: Augmenting its response by antiangiogenic agents [abstract]. In: Proceedings of the Annual Meeting of the American Association for Cancer Research 2020; 2020 Apr 27-28 and Jun 22-24. Philadelphia (PA): AACR; Cancer Res 2020;80(16 Suppl):Abstract nr 553.

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August 2020
Volume 80, Issue 16 Supplement
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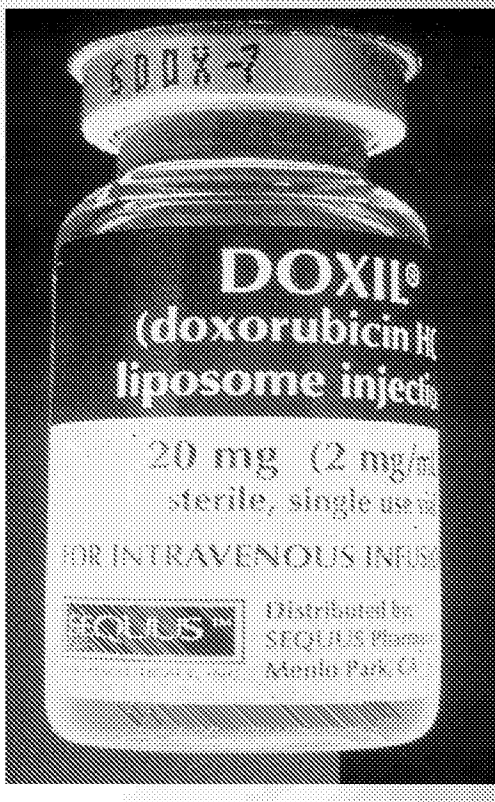
Development of liposomal drugs And Nano-Drugs: From academic research via incubators and startups to FDA and EMA approved products

Part I: Science and Technology

Professor Yechezkel (Chezy) Barenholz,
Laboratory of Membrane and Liposome Research,
The Hebrew University – Hadassah Medical School,
Jerusalem, Israel

Barcelona NanoMed
March 4-5 2014



Doxil: 80 – 100 nm SSL remote loaded with doxorubicin via ammonium sulfate gradient. 18 years to Doxil 1st FDA approved nano-medicine (11.95)

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Today Agenda

- General difficulties in current drug development
- NMII MLV for osteoarthritis treatment
- Doxil the first FDA approved drug and its MOA (in short)
- Why 3 years after Doxil relevant patent expiration FDA approved only one generic product, Lipodox?
- Lessons learned for the development of novel nano-drugs
- LC100 new generation liposomal doxorubicin with less side effect and better efficacy than Doxil
- Scientists as entrepreneurs: a user's guide & personal experience

Drug development: from basic research to approved drug

The current chances

It was shown that for every 1,000 compounds that reach testing, only 5 make it to advanced clinical trials and less than 1 is ultimately approved by the U.S. Food and Drug Administration (FDA).

Namely chances for success are very low, the driving force for development in spite the failure is the large reward in case of success.

Ideas to increase of success chance will be discussed

How to improve the chances?

By orienting Research and R & D programs to focus on:
1. Leading emerging targets in specific therapeutic areas

2. Methods of breaking through the deficiencies in target-based drug discovery and drug performance.

3. Discovering better lead molecules and their optimal delivery for targeting unmet needs

4. Achieving proof of principal that can translate into human clinical trials

5. Good coordination between all involved in the development including good cross talk between basic and applied research!!! and between university researchers and TTO (technology transfer office) regarding I.P. and "marketing package" as well as the right strategic/financial partner(s).

Each of these points require having rational decision making processes, which will be demonstrated.

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Barenholz Lab applied research and its status

- **"Theoretical" and general aspects of DDS, science and technology**
- **QC methologies**
- *Cancer therapy, Doxil LTI (Sequus) to ALZA to J n J & LC100 Lipocure)*
- ***Vaccinology (NasVax)*** → ↘
- ***Inflammatory and autoimmune diseases (Omri to Lipocure MS, RA, Lupus,*** *on the way to clinical trials* ***)***
- ***Infectious diseases therapy***
- **Lipid-based signal transduction**
- ***Local anesthetics*** *(Lipocure on the way to a clinical trial)*
- ***Osteoarthritis (cartilage lubrication and reduction of wear, Moebius Medical (****Finished clinical trial)*
- **Gene delivery (mostly basic)**
- **Environmental (LipoGreen)**

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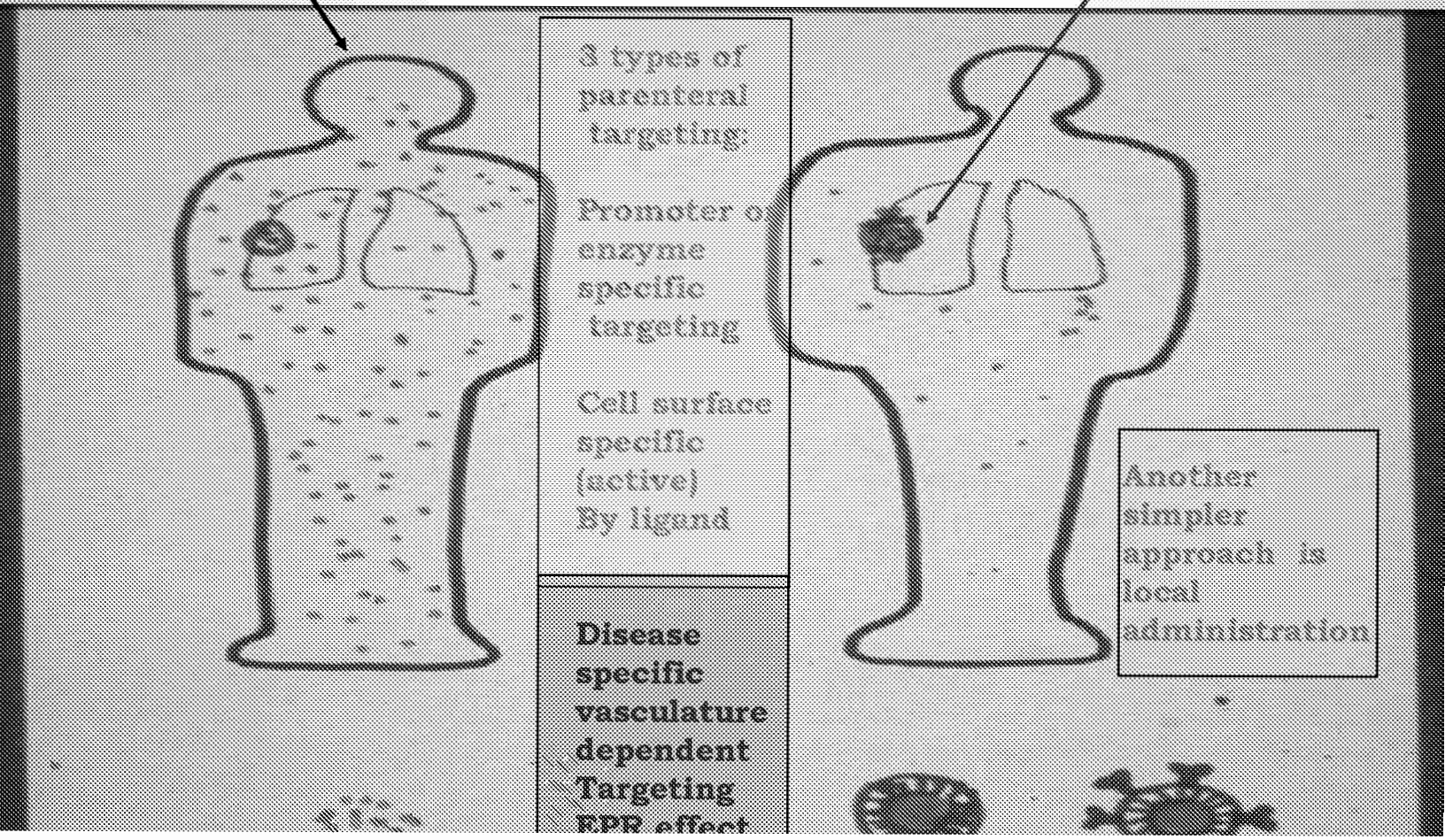
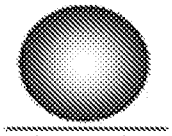
What is all about? Why nano-drugs? Most current anticancer drugs are highly efficacious in cell culture however in vivo and especially in humans their performance is not good enough due to low efficacy and high toxicity, a result of inferior body distribution. Successful "magic bullet approach" (P. Ehrlich concept) is expected to change the body distribution thereby improving dramatically the drug performance.



How to select the best nano-drug delivery systems (DDSs) from big list of available systems

Nanoparticles

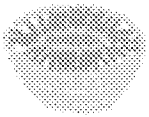


50-200 nm

Polymeric micelles

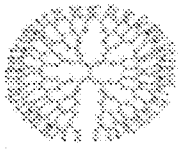
20 nm

Liposomes



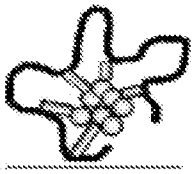
40 nm - 400 nm

Dendrimers



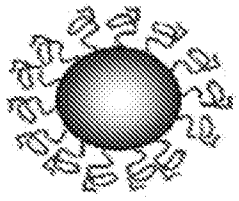
1-10 nm

Polymer-drug conjugates

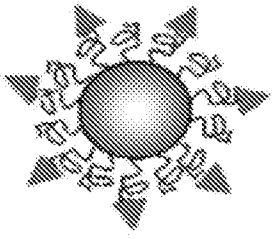


6-15 nm

PEGylated nanoparticles



Liganded PEGylated nanoparticles



Definition of a nano-drug

In agreement with definitions of Nano-technology
In order to be referred to as nano-drugs the particles loaded with drugs have to be superior to larger particles carrying the drug as well as to the drug given "as is" in a none-particulate form

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Danhier et al., J Control Release 2010

Liposomes are different from most other nano particles and carbon nanotubes

- Liposomes are:
- Biocompatible
- Biodegradable
- Not immunogenic
- Familiar to the scientific community
- Have known pharmacokinetics and biodistribution
- Have known metabolism

Therefore from Toxicology point of view they have advantages on most other nanoparticles and nanotubes for medical/pharmaceutical application and for consumer products including cosmetics and cleantech

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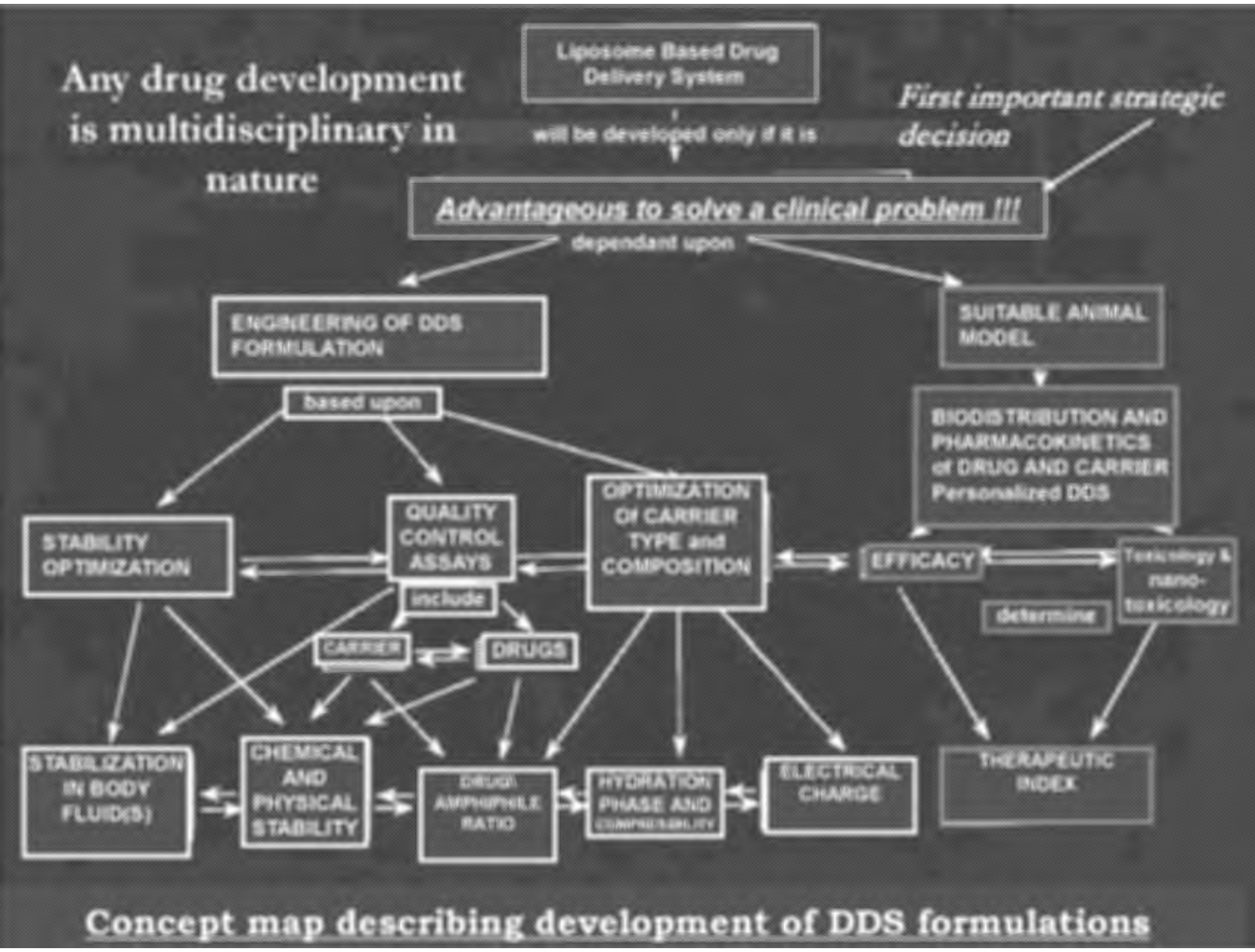
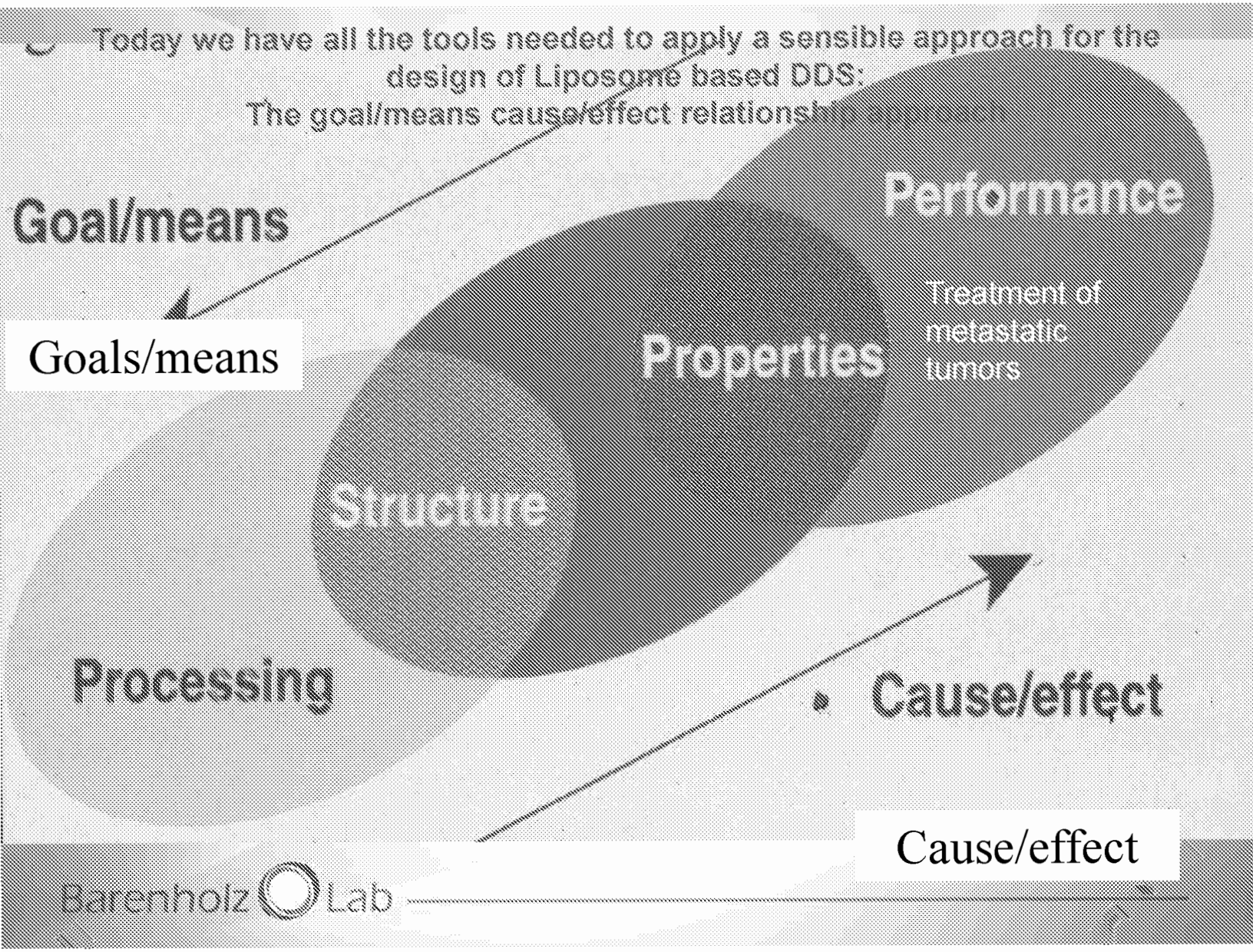


Concept map describing development of DDS formulations


Today we have all the tools needed to apply a sensible approach for the design of Liposome based DDS:
The goal/means cause/effect relationship
Goal/means
Goals/means
Performance
Treatment of metastatic tumors
Properties
Structure
Processing
Cause/effect
Cause/effect
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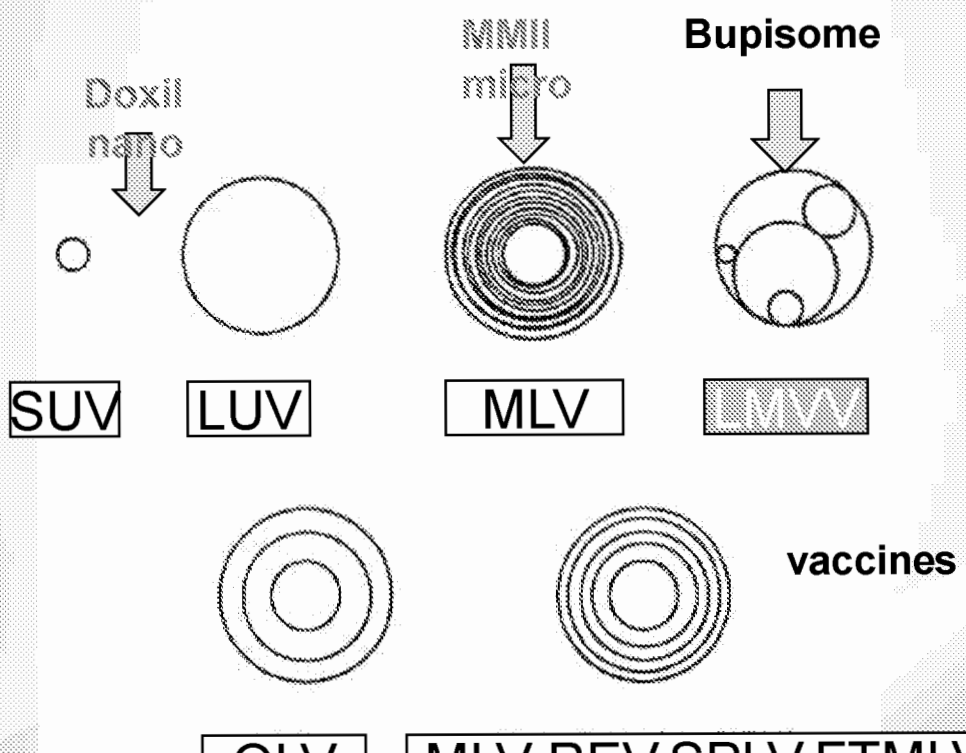
Liposomes

Are classified by their size and lamellarity.

Today we will deal with 2 liposomal drugs: the anti cancer nano-drug Doxil for systemic administration and the micro-medical device NMII for treatment of osteoarthritis



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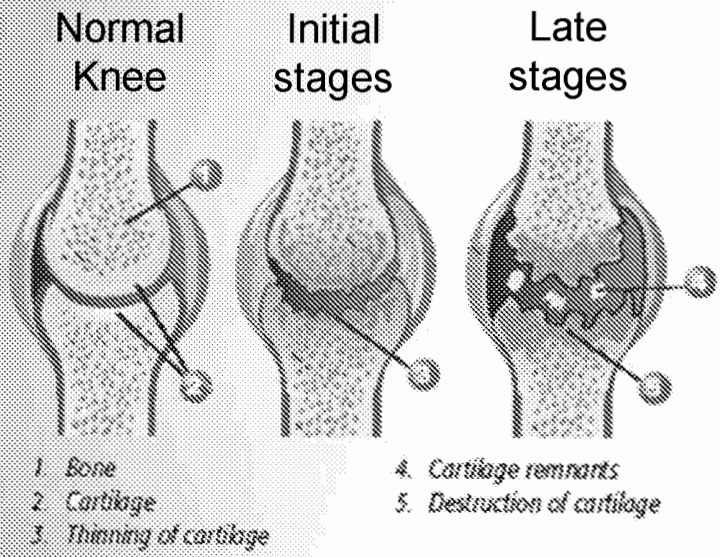
Osteoarthritis (OA) Treatment

NMII MLV Licensed to Moebius Medical LTD (incubator)

Osteoarthritis a major disease >1% of the population

The Issue: Cartilage destruction due to wear increases friction further accelerate wear and leads to pain & Inflammation (osteoarthritis = OA)



Treatments

Corticoids (oral) - ↓ inflammation

HA (I.A.) - ↑ viscosity

Liposomes – (I.A.)↑ boundary lubrication

We suggest that local liposome treatment may affect directly the disease cause by improving lubrication thereby and reducing cartilage wear !!!

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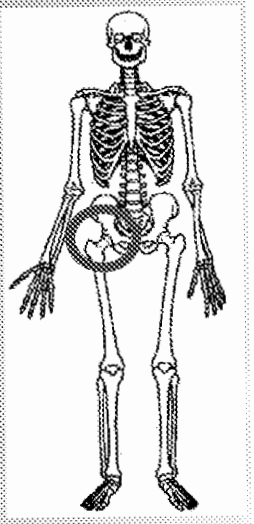
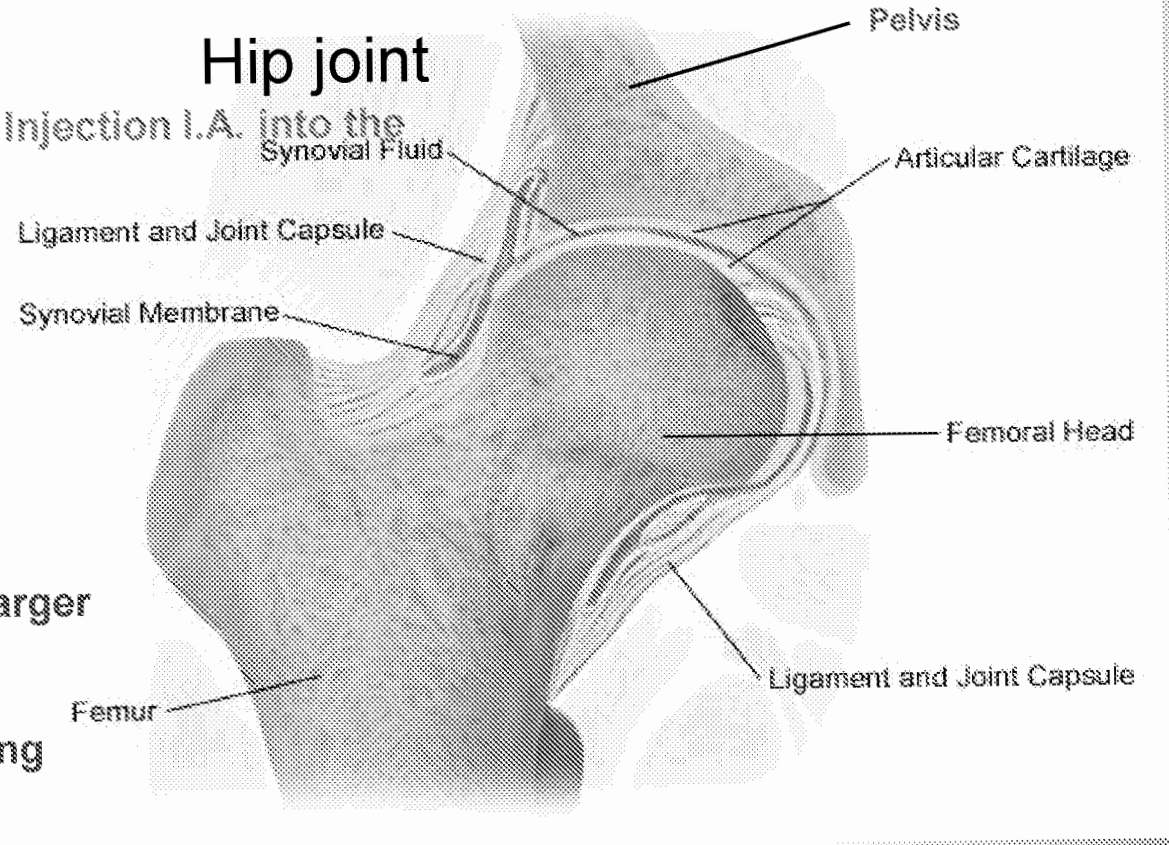
Use of liposomes in joint tribology



Hip joint

Injection I.A. into the



Size has to be larger than 250 nm as as the synovial membrane having 250 nm pores!

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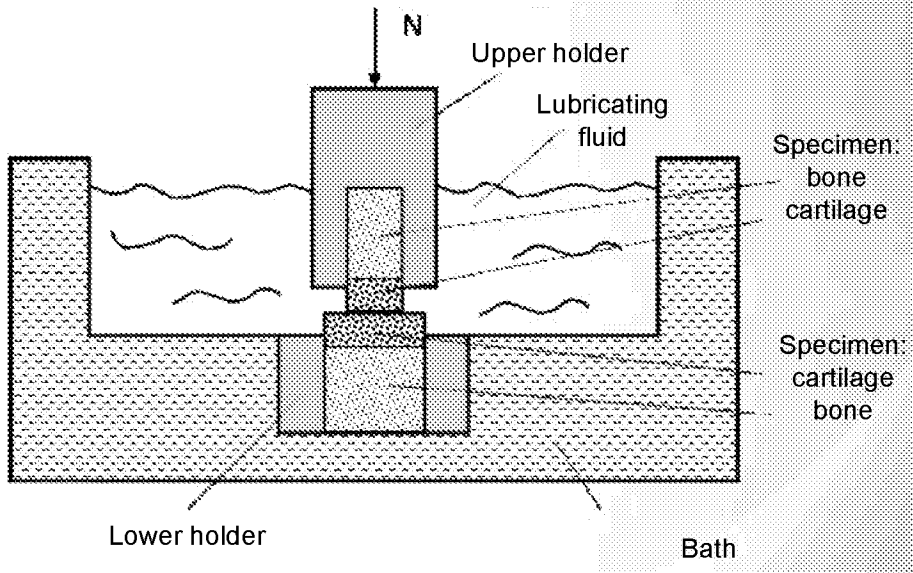
Screening for the optimal liposomes as lubricants and wear reducers

(Lipid composition and liposomes structure)

An ex-vivo cartilage-on-cartilage setup was developed in order to compare the lubrication and wear reducing capacity of different potential lubricants



Etsion Barenholz and coworkers, Wear 2010
Barenholz,Etzion and Coworkers Langmuir 2010

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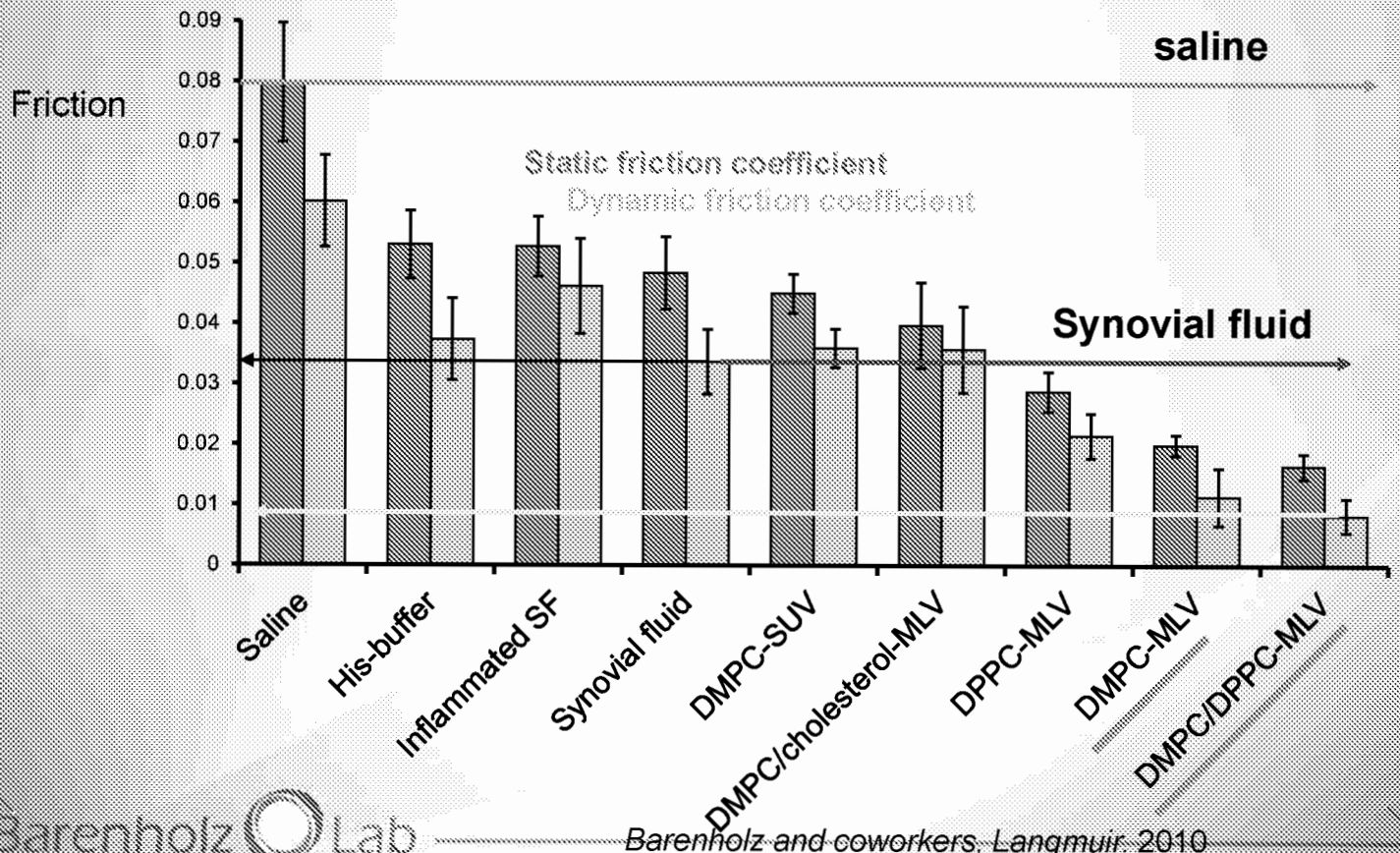
Finding the Optimal Liposomes – Lubrication: DMPC/DPPC- and DMPC-MLV are superior lubricants of human cartilage



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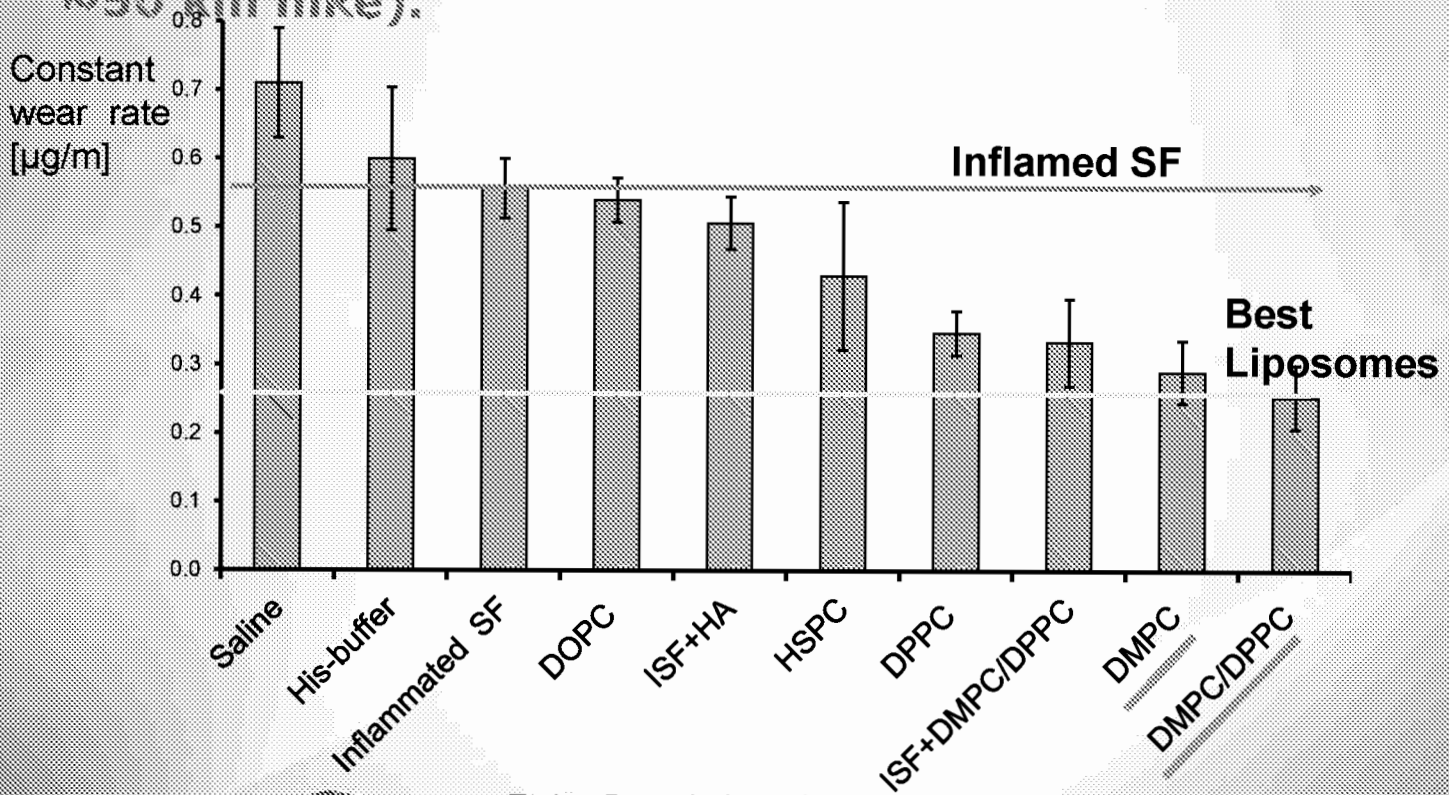
Barenholz and coworkers, Langmuir 2010

Finding the Optimal Liposomes – Wear Reduction

The liposomes which are the best lubricants DMPC/DPPC and DMPC MLV are also the best cartilage wear reducers (under condition which imitate long ~50 km hike).



Etsion Barenholz and coworkers, Wear, 2010

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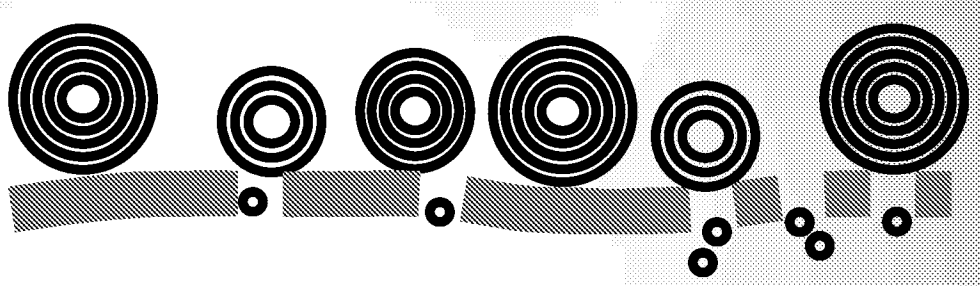
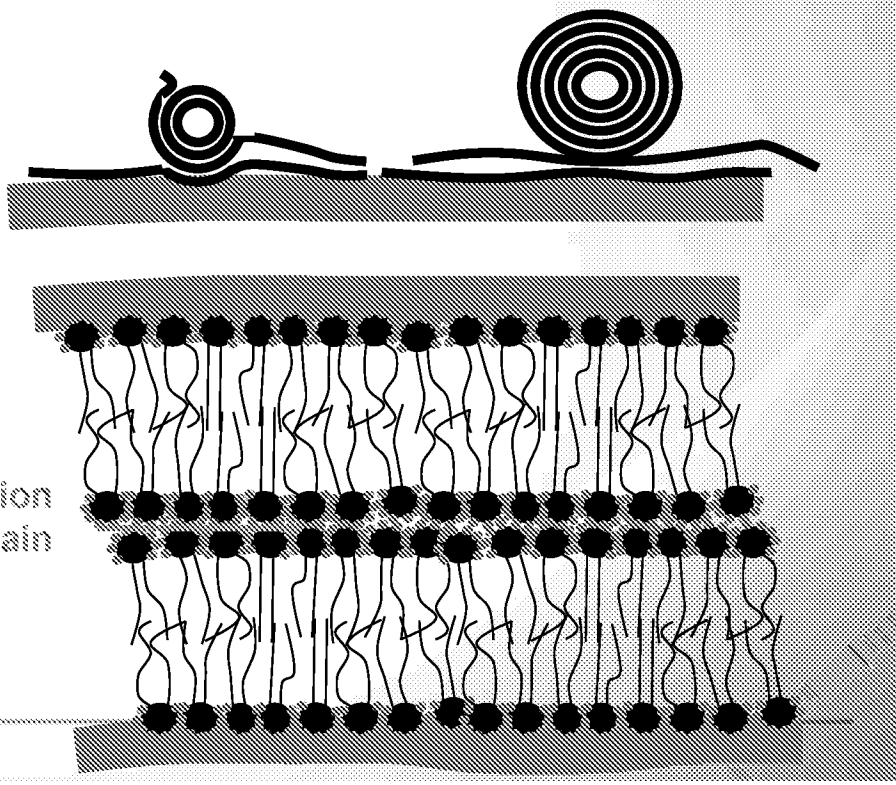
MoA: Hydrophilic Lubrication (fits J. Klein concept)

multi-lamellar vesicles (MLV) remain on and near cartilage surface, while small unilamellar vesicles (SUV) penetrate deeply into cartilage



Hydrophilic Lubrication:

MLV, with phospholipid bilayers in the LD phase, readily delaminate by pressure deposition on opposing cartilage surfaces. Each of these 2 bilayers have highly hydrated 10-12 H_2O per PC head-groups (blue) which serve as nano ball-bearings, thereby facilitating very low friction joint motions



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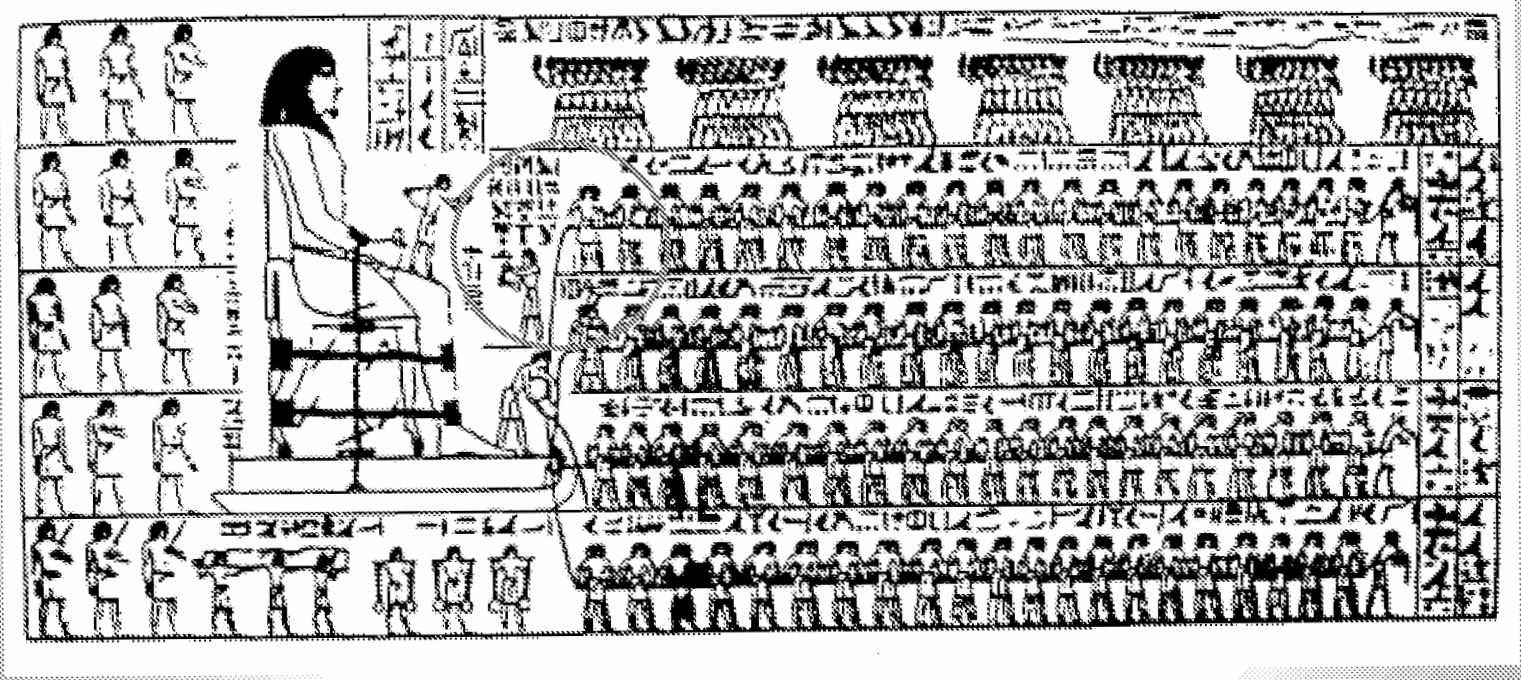
Based on biology hydrophilic lubrication may be better than the well known (to mechanics) hydrophobic lubrication. The Importance of wet (hydrated) surface is long known!

Old Egyptian description of how to overcome friction

(Prof' I. Etsion, Technion)



Transporting an Egyptian colossus from the tomb of Tehuti-Hetep 1880 B.C.

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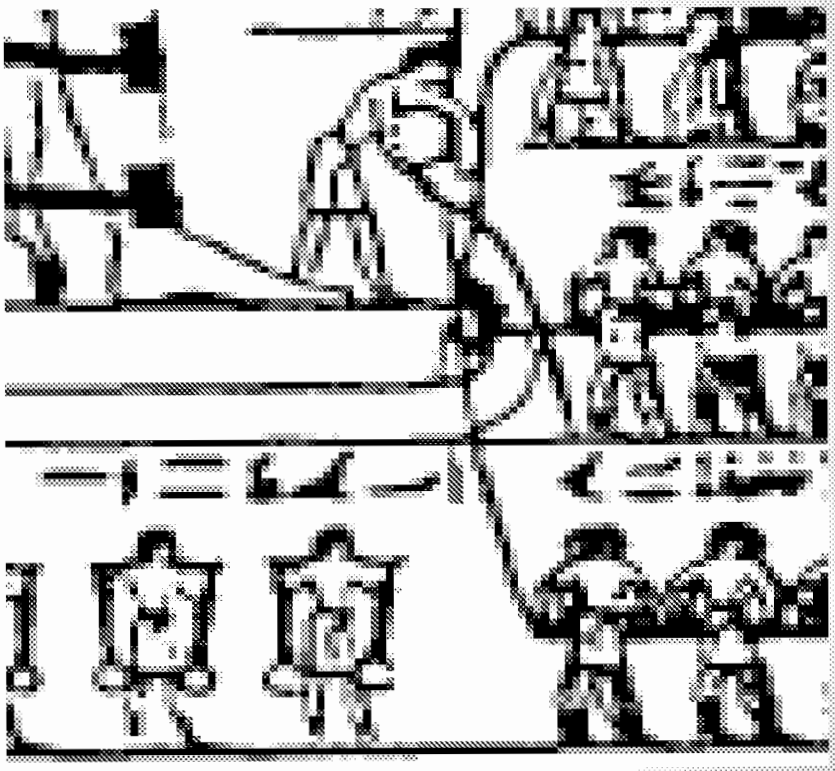
Zooming on Lubricating by wetting



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FIM as a Proof of Concept of OA Treatment

Phase I/IIa at Hadassah Medical Center in Jerusalem, Israel
Concluded in December 2012, by Prof Aryeh Kandel

- **40 participants** (av. age 64 years) suffering from symptomatic moderate osteoarthritis
- Randomized
- Compared / head to head standard of care (HA - Durolane) with **NMII** product (DMPC/DPPC MLV)
- Double-blind
- Single injection
- Follow-up 90 days

PRIMARY OBJECTIVE **Safety and tolerability**

SECONDARY OBJECTIVE **Pain reduction and functionality improvements**

The clinical trial was sponsored by Moebius Medical (Dr Yaniv Dolev , CEO) who licensed the technology from Yissum, HUJI (Barenholz) and Technion (Etsion)

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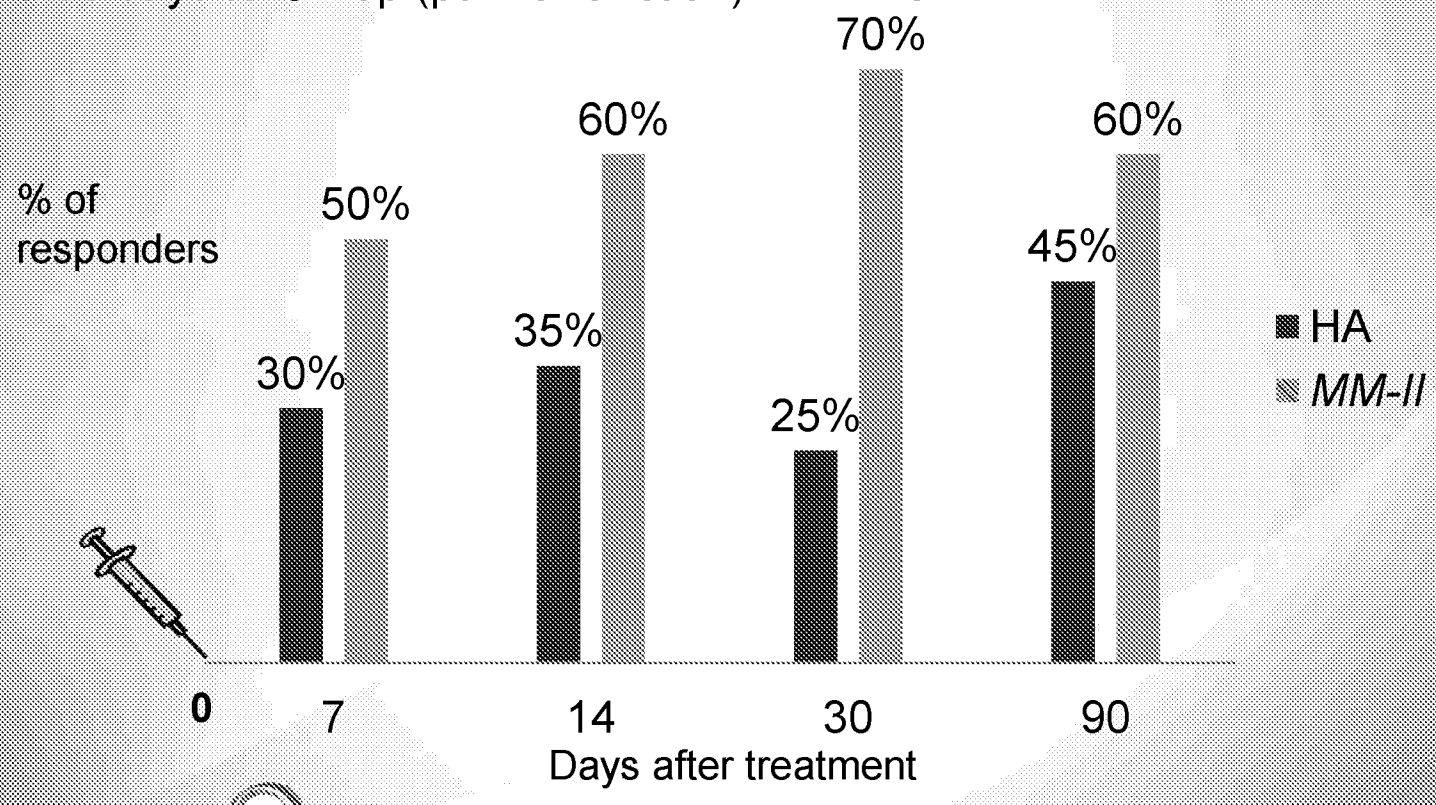
Proof of concept clinical trial highlights

OMERACT-OARSI criteria / index of responders to one treatment and 90 days follow up (pain & function)* NMII vs HA



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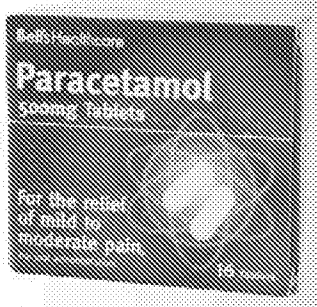
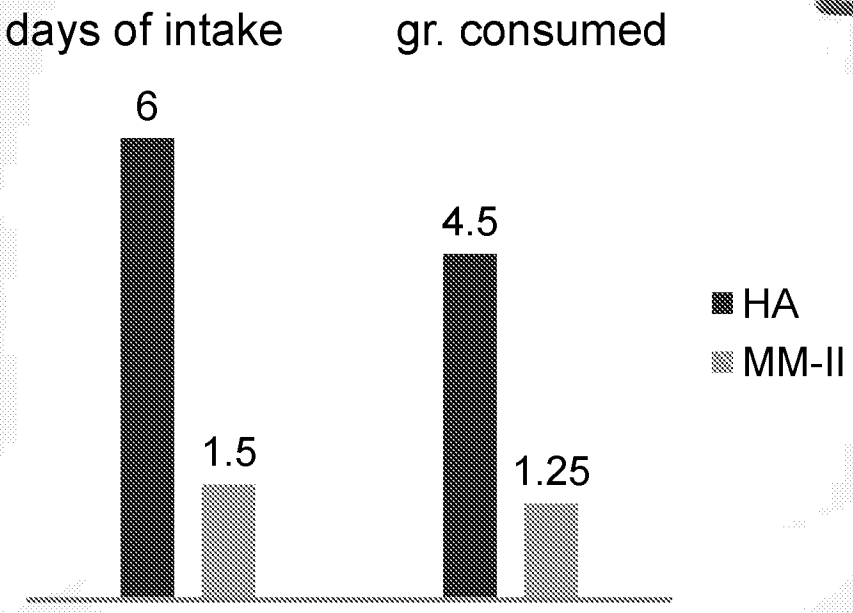
Proof of Concept Clinical Trial Highlights

Rescue Medication used between visits by the participates during the study





* median, ITT population

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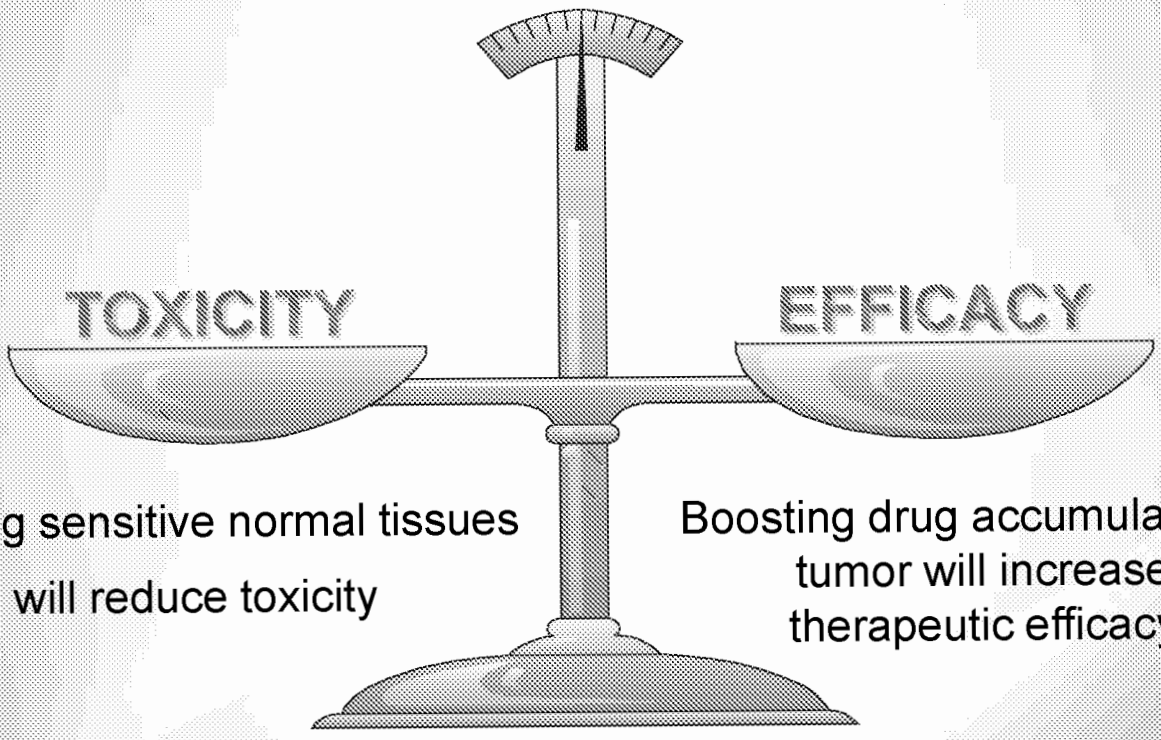
Doxil is an anticancer chemotherapeutic nano-drug aims to improve therapeutic index

INCREASING the THERAPEUTIC INDEX



Sparing sensitive normal tissues will reduce toxicity

Boosting drug accumulation in tumor will increase therapeutic efficacy

Thanks to Alberto Gabizon for this slide

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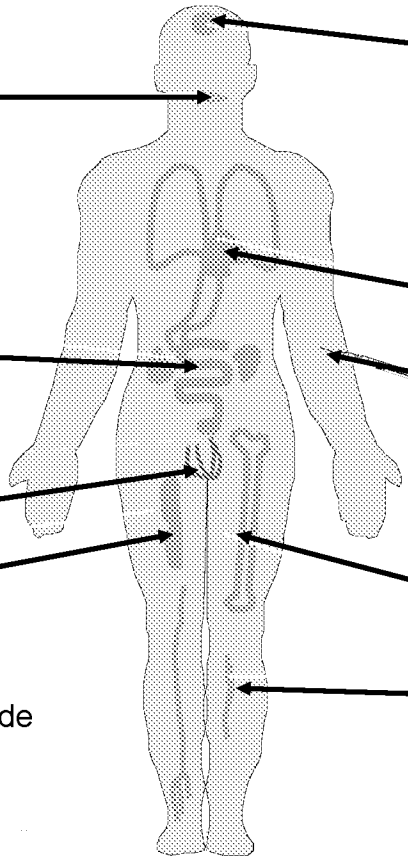
Side effects of doxorubicin



Mucositis

Alopecia

Cardio toxicity

Nausea/vomiting

Local reaction

Sterility

Fatigue

Myelosuppression

Phlebitis

Thanks to Alberto Gabizon for this slide

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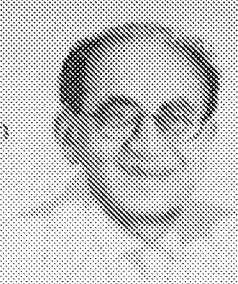
Rakesh K. Jain is Andrew Werk Cook Professor of Tumor Biology and director of the Edwin L. Steele Laboratory for Tumor Biology in the radiation oncology department of Massachusetts General Hospital and Harvard Medical School. He is a member of the National Academy of Sciences, the National Academy of Engineering and the Institute of Medicine, one of only 20 people ever to have been elected to all three bodies.



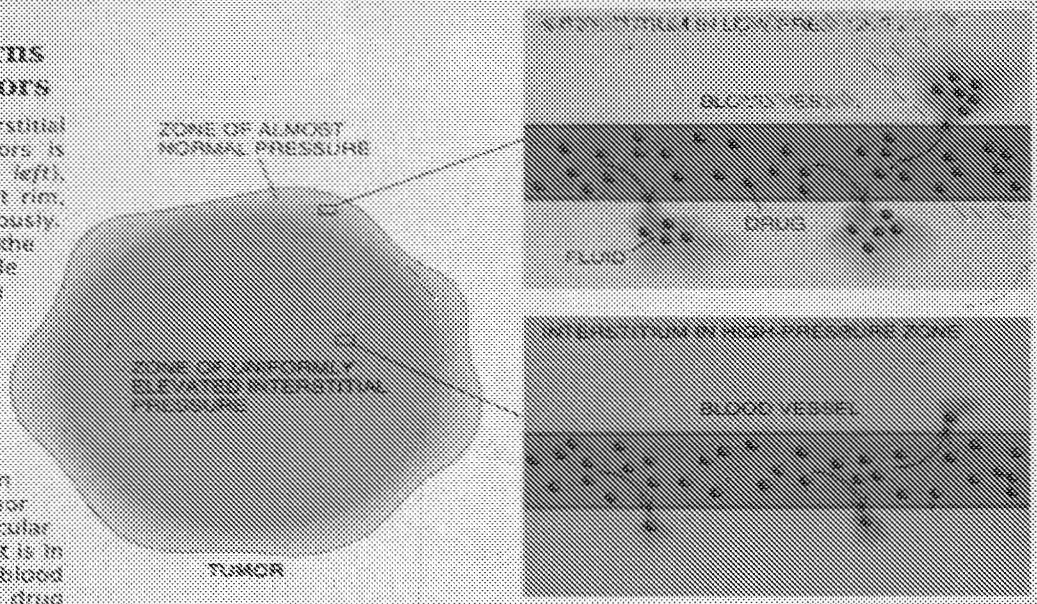
"To improve treatment, researchers need to not only gain a better understanding of cancer genetic underpinnings but also consider the physical forces in tumors"

R.K Jain in Scientific American, February 2014 p33-39

We are saying that : one also has to consider taking advantage on tumor tissue unique metabolism

Pressure Patterns in Human Tumors

Pressure in the interstitial tissue of solid tumors is uniformly elevated (*top left*), except in the outermost rim, where it drops precipitously. The elevated pressure in the inner zone can impede movement of large drug molecules into the matrix from the blood stream—for a simple reason. Large molecules travel mainly by convection, flowing in fluid from a high- to a low-pressure region. In the outer zone of a tumor (*top detail*), the vascular pressure is higher than it is in the interstitium, and so blood fluid (*gray*) laden with drug molecules (*blue*) seeps (*arrows*) into the interstitium. In the inner zone (*bottom detail*), the interstitial pressure is about equal to that in the blood vessels; hence, convection virtually ceases. High interior pressure, which was predicted by a mathematical model, has now been found in humans (*table*) as well as in animals. The pressure readings listed are measured in millimeters of mercury.



INTERSTITIAL PRESSURE IN HUMAN TUMORS

TYPE OF TISSUE	NUMBER OF PATIENTS	MEAN PRESSURE
[illegible]	[illegible]	[illegible]
NORMAL SKIN	3	0.4
[illegible] CARCINOMA	1	30.0
CERVICAL CARCINOMAS	26	32.8
[illegible]	[illegible]	[illegible]
HEAD AND NECK CARCINOMAS	27	19.0
[illegible]	[illegible]	[illegible]
METASTATIC MELANOMAS	13	14.3
[illegible]	[illegible]	[illegible]

One of the major obstacles to tumor efficacious chemotherapy is the physics of tumors. **(Jain and Coworkers, Sci. Amer.)**

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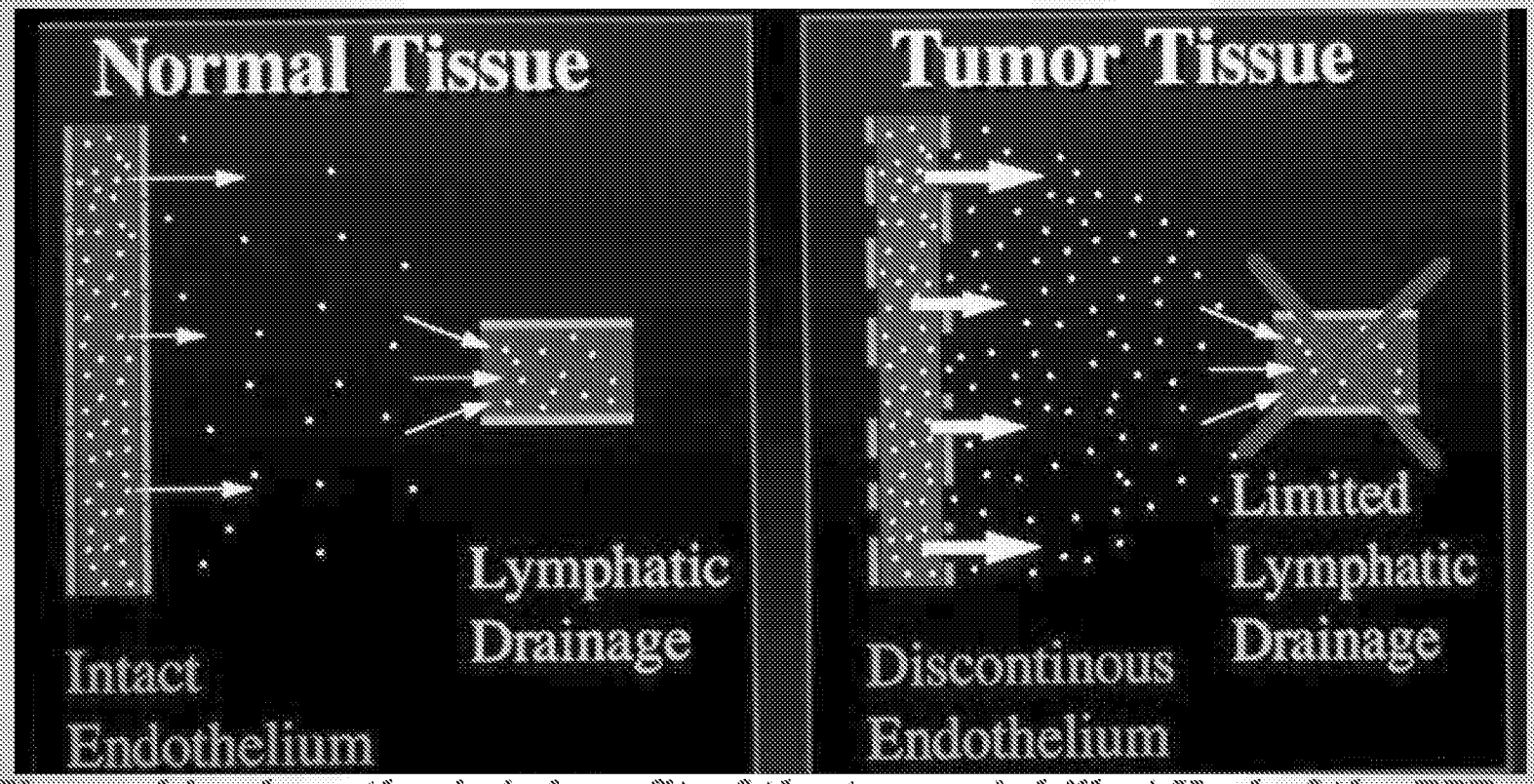
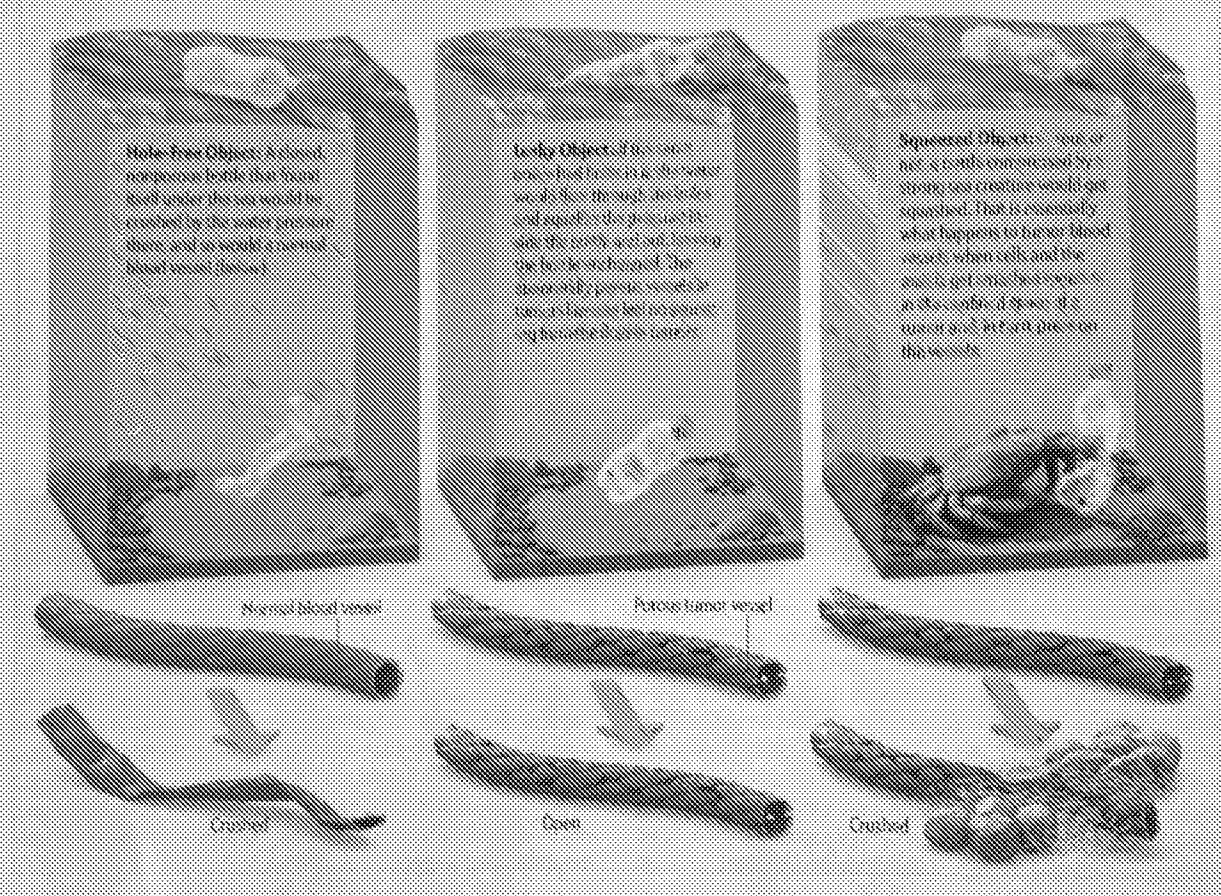
How can we take advantage of the "enhanced permeability and retention" (EPR) effect in tumors, the tumor "Achilles Heel" which explains nSSL selectivity into tumors



The working hypothesis is benefit of the tumor Achilles' Heel of the EPR effect which support use of nano <100 nm long circulating liposomes (nSSL) (adapted from: Maeda & Matsumura 1986)

Why Only Solids Squeeze Tumor Vessels

People are often surprised to learn that only the rigid walls in tumors squeeze blood vessels shut; the fluid inside does not play a role in the effect. The author uses an analogy to a plastic soft-drink bottle dropped into the ocean.



R.K Jain Sci. Amer Feb 2014 33-39

Efficacious systemic cancer and inflammation chemotherapy by liposomes loaded drug requires enough such liposomes reaching the disease site with therapeutic drug levels, releasing the drug there. These can be achieved by optimizing the cross talk of lipid biophysics, nanotechnology, and biology.
[For doxorubicin a relatively large dose of 50 mg/m2 dose is needed]

This requires:	Solutions:
Stable and high (but enabling release) drug encapsulation	**Remote loading by ammonium sulfate encapsulation enables to deliver enough drug to the tumor** which can be released there at therapeutic levels
Steric barrier to slow down interaction with blood components and RES	**2K PEG-DSPE grafted lipopolymer** which increases circulation time and reduced RES uptake so enough liposomes can reach the tumor site
Extravasation into the tumor sites	**Size < 120 nm** permit extravasation in the tumor (EPR effect) and increase of circulation time

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Design of a liposomal DDS requires the following decisions: should be based on lipid biophysics, physical-chemistry and nanotechnology principles and these fields cross talk with disease biology

- **Liposome type? Nano liposomes are preferred for systemic use and large liposomes (MLV or LMVV) for local applications**
- **Desired zeta and surface electrical potential?**
- **Desired size distribution?**
- **desired in vivo release kinetic order (zero or other orders) and rate?**
- **Desired lipid composition? Steric barrier, Lipid phase? (LD, SO, LO)**
- **The preferred drug? (here mainly physicochemical considerations)**
- **Drug loading method? Passive? Remote (active)?**
- **Drug selection and/or design by Data Mining using many drug features. Creating an "trainning set, and evaluation by "test set" Zucker et al JCR 2009, Cern et al JCR 2012, JCR 2013)**
- **Method of removal of non encapsulated drug?**
- **Liposomal storage conditions? Electrolyte versus no electrolyte? pH?**
- **Administration approach (oral, local, intravenous, topical, other)?**

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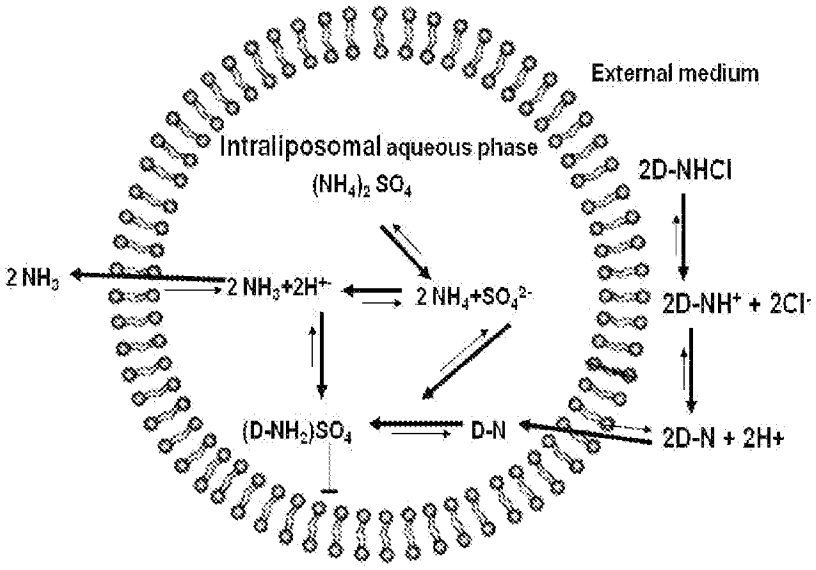
Remote loading by trans-membrane ammonium gradient is an essential component in Doxil clinical success



The sulfate counter ion lead to crystallization of DOX-sulfate which stabilizes drug loading

Trans membrane ammonium ion gradient **is acting like a nano-pump and driving force for the uptake of doxorubicin into the intraliposome aqueous phase. There due to the excess of sulfate ion and protons it precipitates in a stable and reversible way as doxorubicin sulfate crystals allow for large drug accumulation without increasing of intra-liposome osmotic pressure**

It also allow for drug release at the tumor site as will be discussed later

. Un-ionized drug base (D-N) or acid (D-COOH) crosses the liposomal membrane and is trapped inside by its ionization and insoluble salt formation with the intraliposome counterion. **Active loading benefit from the nano volume of nano-liposomes.** Haran et al 1993, Zucker et al 2009, Cern et al 2012, Avnir et al 2008, 2011; Barenholz et al , US patents 5,192,549, 5,316,771 (1994

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Small angle X-ray diffraction demonstrates formation of DOX crystals-sulfate in the presence of sulfate anion and as result of ammonium sulfate gradient induced remote loading into SSL (to form Doxil) **(Lasic et al 1992)**

Sample	Reflections (Å)
Doxorubicin-HCl	20.0, 10.1, 8.5, 7.6, 6.6
Crystalline	5.9, 5.3, 4.8, 4.5, 4.3
Empty liposomes	83, 55, 41.5
Loaded liposomes	27
3% Dox solution	none
3% Dox-$(NH_4)_2SO_4$	27
3% Dox-Na_2SO_4	27

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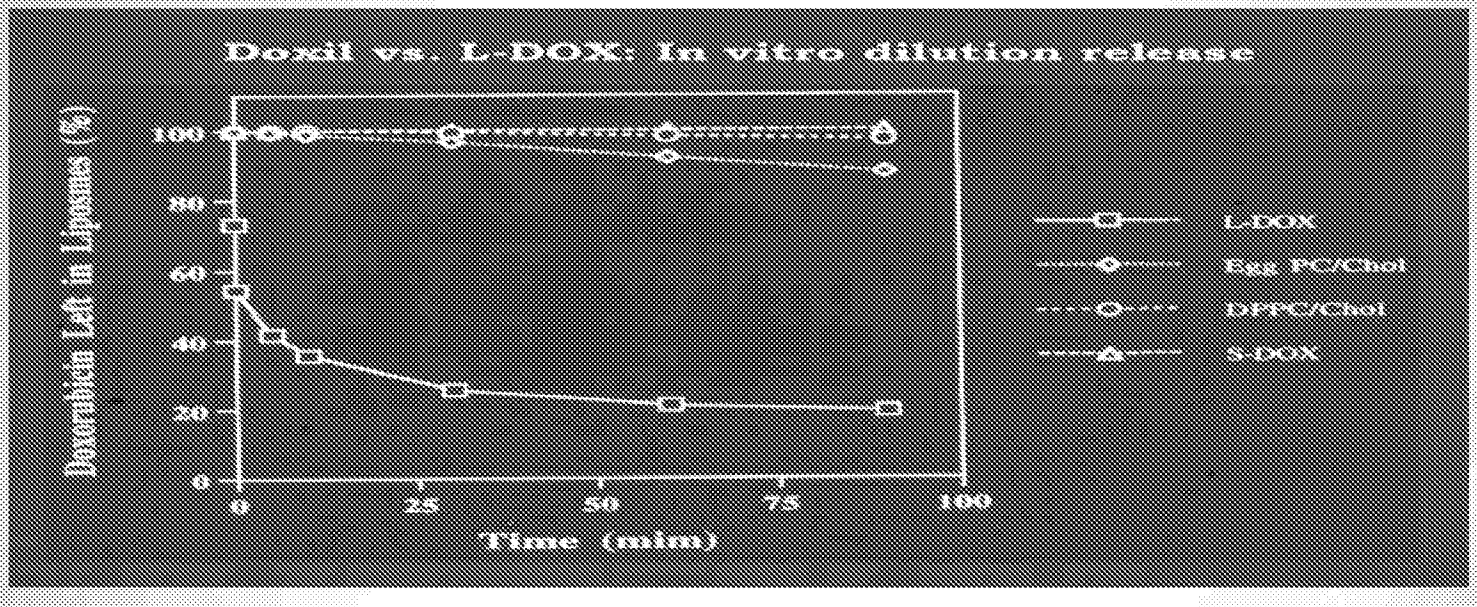
Stability of doxorubicin loading into liposomes: Comparing passive versus active (remote) trans-membrane ammoniun sulfate gradient driven loading



Dilution (up to 10,000 fold) imitate the stability of drug loading upon infusion to human plasma. The first ml infused is diluted 3500 fold.
In such test we can discriminate between the effects of loading method (active vs passive and the contribution of liposome lipid composition

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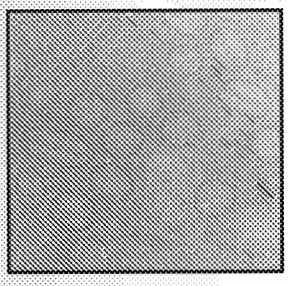
Relevance of Nanoism

Active Loading

Effect: Improving active loading of amphipathic weak bases and acids.

Mechanism: The smaller the liposome intraliposome aqueous phase the easier it is to form ammonium sulfate gradient for loading amphiphatic weak bases [Haran et al 1993 others, IP: Barenholz and Haran US patents 5,192,549 (1993), 5,316,771 (1994), others] applied for doxorubicn (DOX), tempamine (TMN), bupivacaine(BUP), others
or calcium acetate gradient for loading amphiphatic weak acids [Clerc and Barenholz 1995 and US patent 5,939,096 (1999), others]. Both gradients are efficient to induce intraliposome drug loading and drug precipitation which correlates with high drug to lipid ratio and with loading stability. Applied for methyl- prednisolone hemisuccinate sodium salt (MPS = Solu-Medrol ^{R}).

Doxil® Experience



1. An example of successful passive targeting of liposomes to tumors

2.First patented liposomal drug

3. First nanomedicine reaching the clinic. FDA approval 11.95

4. Annual Sales rate > $700 millions 2010

INDICATIONS AND USAGE

DOXIL is an anthracycline topoisomerase inhibitor indicated for:

- **Ovarian cancer (1.1)**

After failure of platinum-based chemotherapy.

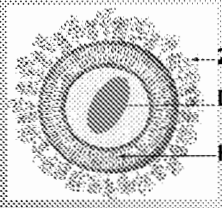
- **AIDS-related Kaposi's Sarcoma (1.2)**

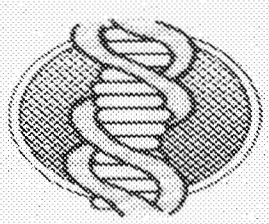
After failure of prior combination chemotherapy or intolerance to such therapy. Results are based on objective response rate, no results are available from controlled trials that demonstrate clinical benefit.

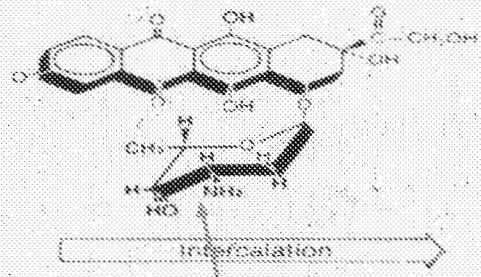
- **Multiple Myeloma (1.3)**

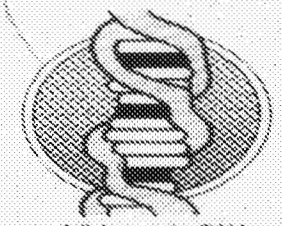
In combination with bortezomib in patients who have not previous bortezomib and have received at least one prior therapy

1 ml of the Doxil dispersion has $2.3x10^{14}$ liposomes and each liposome contain ~10000 molecules of doxorubicin.









DOXORUBICIN DUE TO THE PRIMARY AMINE OF ITS MANOSE AMINE IS AN AMPHIPHATIC WEAK BASE AND THEREFORE CAN BE REMOTE LOADED INTO LIPOSOMES HAVING AN AMMONIUM ION GRADIENT

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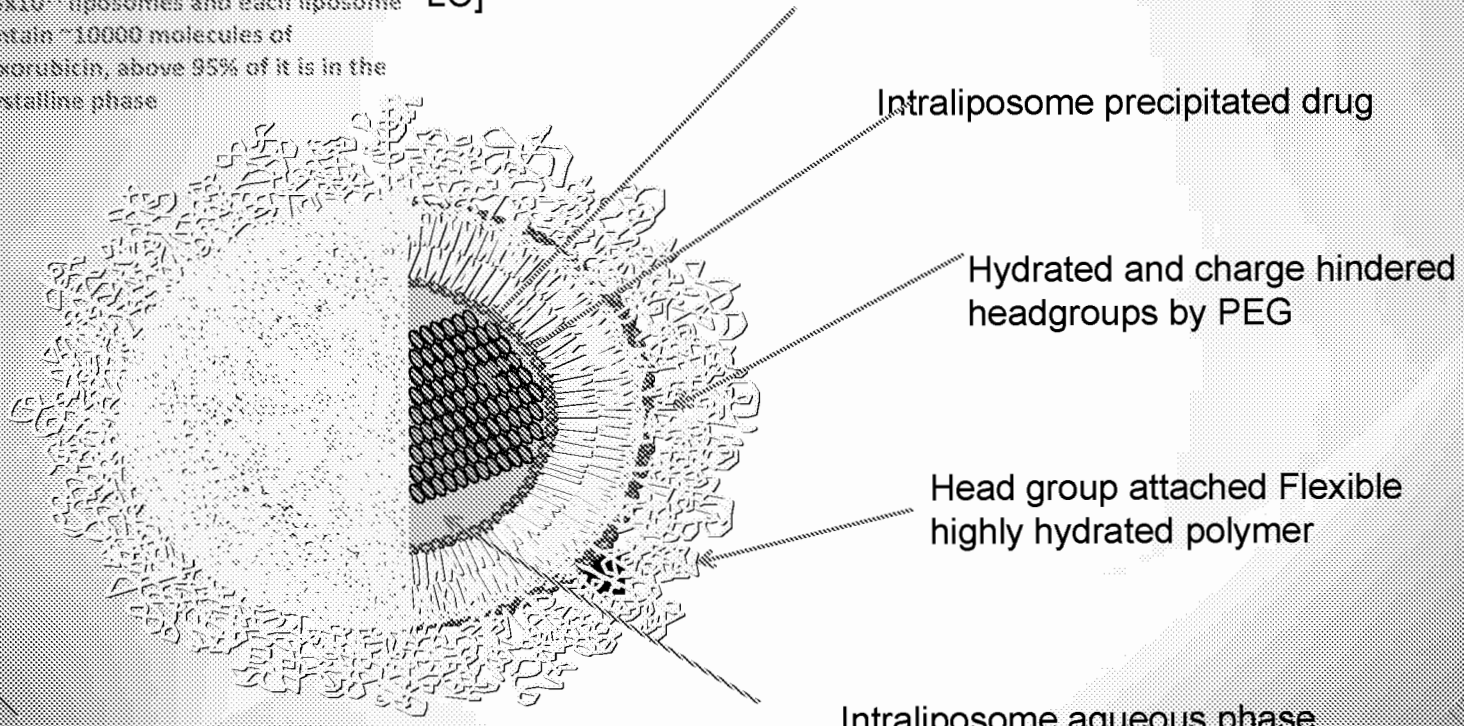
Doxil®: Structure-Performance Relationships (Every detail matters)

1 ml of the Doxil dispersion contains 2.3×10^{14} liposomes and each liposome contain ~10000 molecules of doxorubicin, above 95% of it is in the crystalline phase



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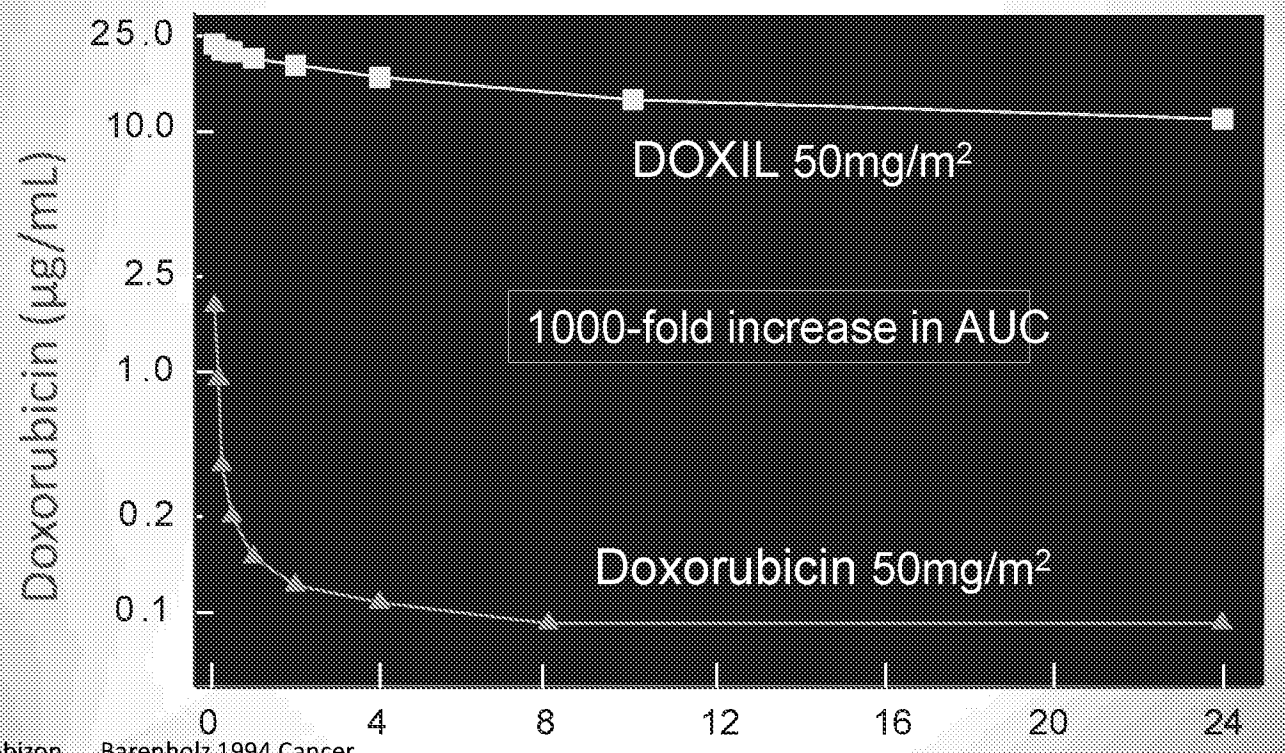
Doxil® Prolonged PK and being nano allow for tumor accumulation



Gabizon .....Barenholz 1994 Cancer Research

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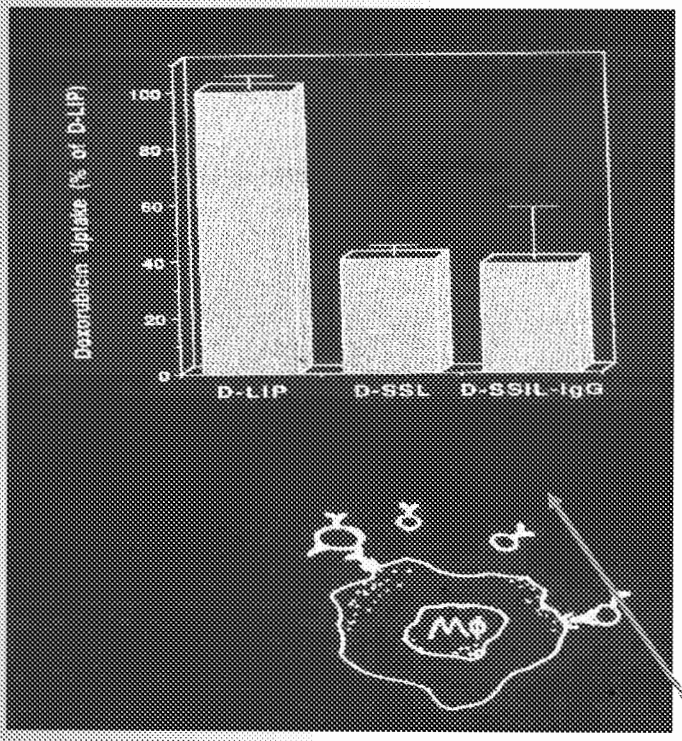
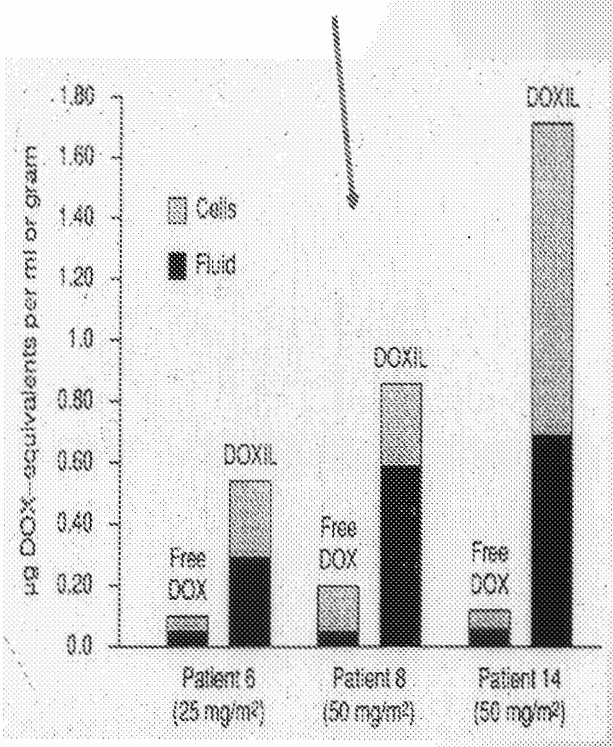
The first proof for human tumor passive targeting and accumulation of Doxil's doxorubicin due EPR effect in cancer patients. Demonstrating superior bio-distribution of Doxil versus free doxorubicin (Based on Gabizon & Barenholz, Cancer Research 1994)





Doxil being nSSL show much lower uptake by macrophages leading to its prolonged and superior doxorubicin pharmacokinetics in all animal species tested including humans (Emanuel et al Pharma Res 1976)

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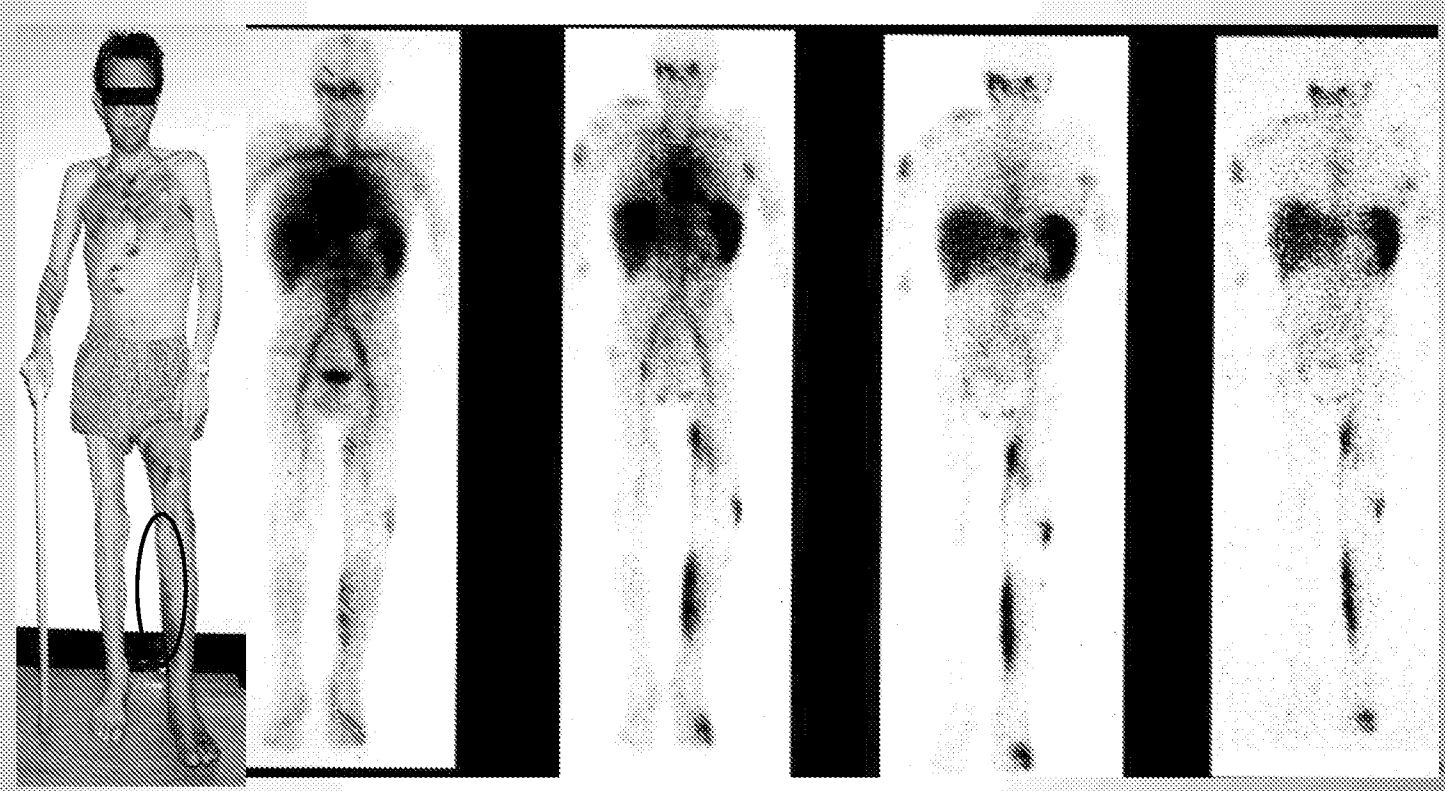
Serial Gamma Scintigrams of KS Patient after Pegylated Liposomes Containing ^{111}In-DTPA (remote loaded)



Personalized DDS

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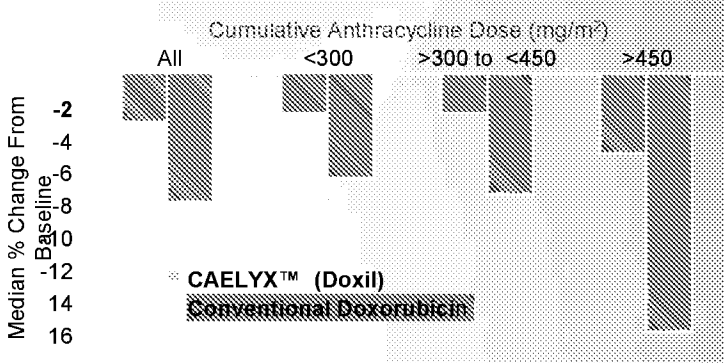
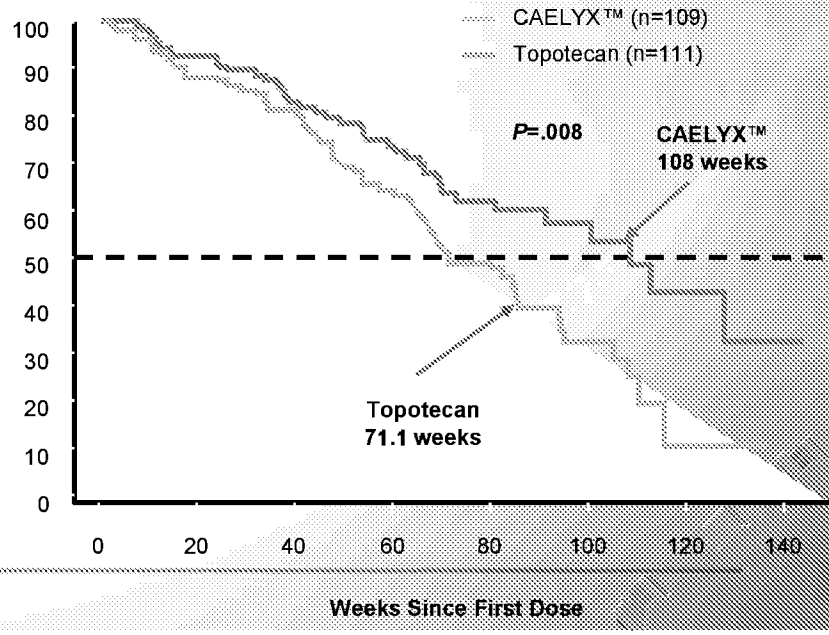
Doxil side effects are lower than of doxorubicin

	Doxorubicin	Doxil
Vesicant effect	+++	+/-
Infusion reaction	-	+
Nausea/Vomiting	++	+/-
Myelosuppression	+++	+ (no gr. 4)
Stomatitis/Mucositis	++	+++
Hand-Foot (PPE)	-	+++
Cardiotoxicity	+++	+
Alopecia	+++	+
Max. Tolerated Dose	60 mg/m^2	50 mg/m^2
Dose Intensity	20 mg/m^2/wk	12.5 mg/m^2/wk
Max. Cum. Dose	450 mg/m^2	Undefined >650 mg/m^2

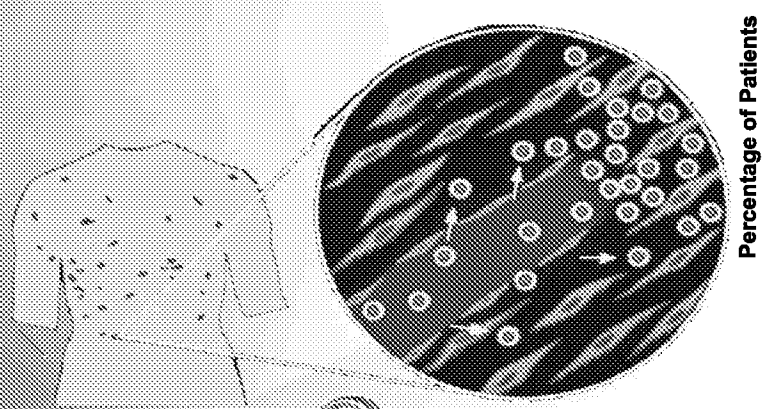
LVEF show that cardio-toxicity of cumulative drug dose is lower for Doxil than for doxorubicin



In ovarian cancer Doxil is superior over topotecan



Doxil Mechanism of action: EPR effect and what next?



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What is the MoA of doxorubicin release of Doxil at tumors ? (2)

There are few options to explain tumor interstitial doxorubicin release of Doxil

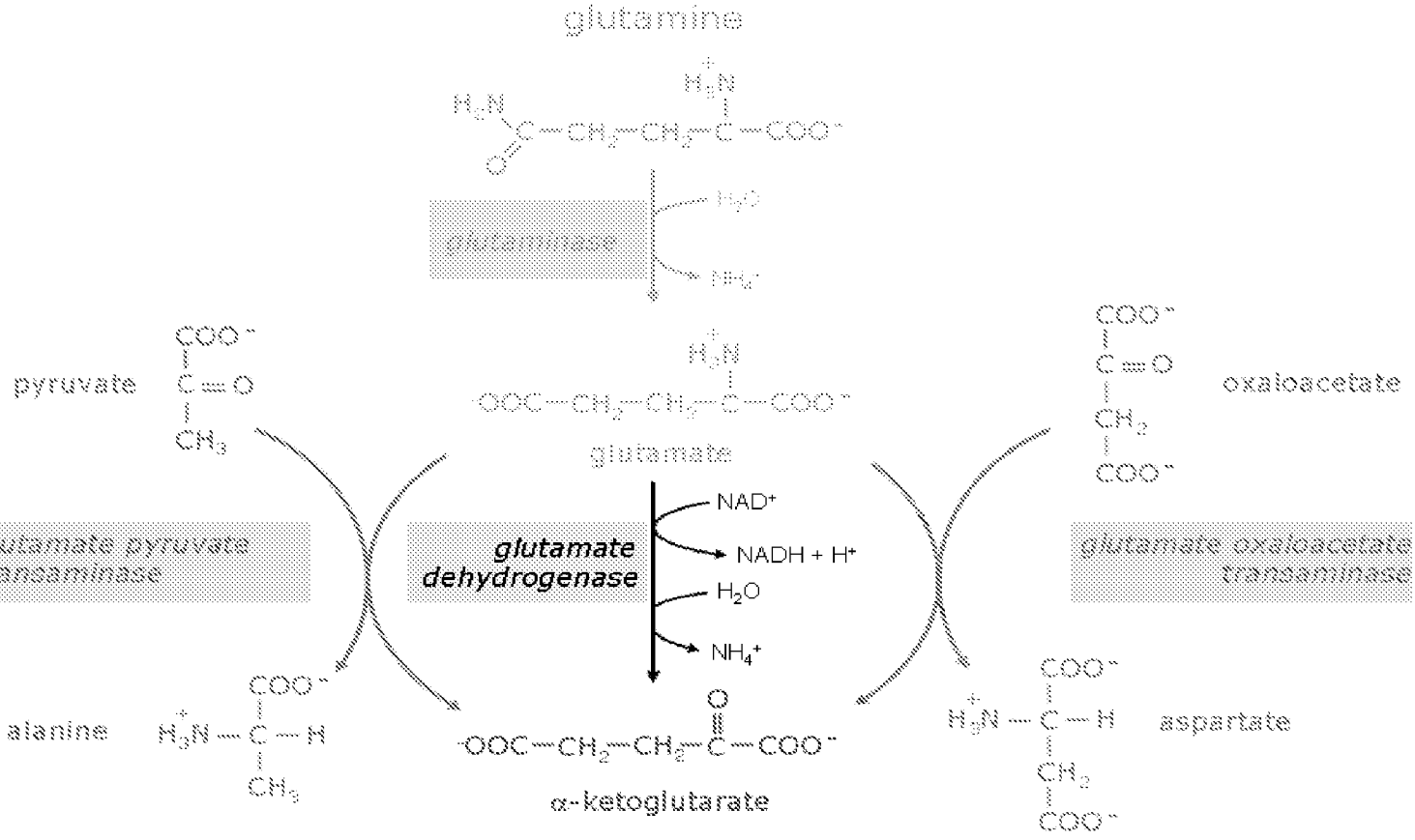
- Breakdown of liposomes by phospholipases such as secretory PLASE A2 ?

This is not applied to Doxil liposomes:

1. Due to their high mole% of cholesterol (Mouritsen, K. Jorgensen, Pharm. Res. 15 (1998) 1507–1519: Andresen et al Prog. Lipid Res. 2005).
2. Also confirmed by the lack of therapeutic effects and cis platin release of Stealth cisplatin (ref. in previous slide)

- Therefore it must be assumed that some factors typical (or unique) to tumor interstitial induce doxorubicin release of Doxil at the tumor sites.
- A very attractive option is our hypothesis that ammonium/ammonia produced due to the tumor unique glutaminolysis (Moreadith and Lehninger JBC, 259, 6215-6221, 1984; Eng et al Science Signaling 3, 1-9, 2010) induces the doxorubicin release of Doxil either by collapse of the ammonium gradient or other mechanisms. This presentation is focused on the evaluation of the mechanism by which ammonium/ammonia induce doxorubicin release of Doxil

Glutaminolysis in tumor cells is a response by which tumor cells overcome inhibition of Krebs cycle aconitase by ROS and creating α ketoglutarate to move the highly important Krebs cycle forward.



From Glutaminolysis in Wikipedia

RESEARCH ARTICLE

CELL BIOLOGY

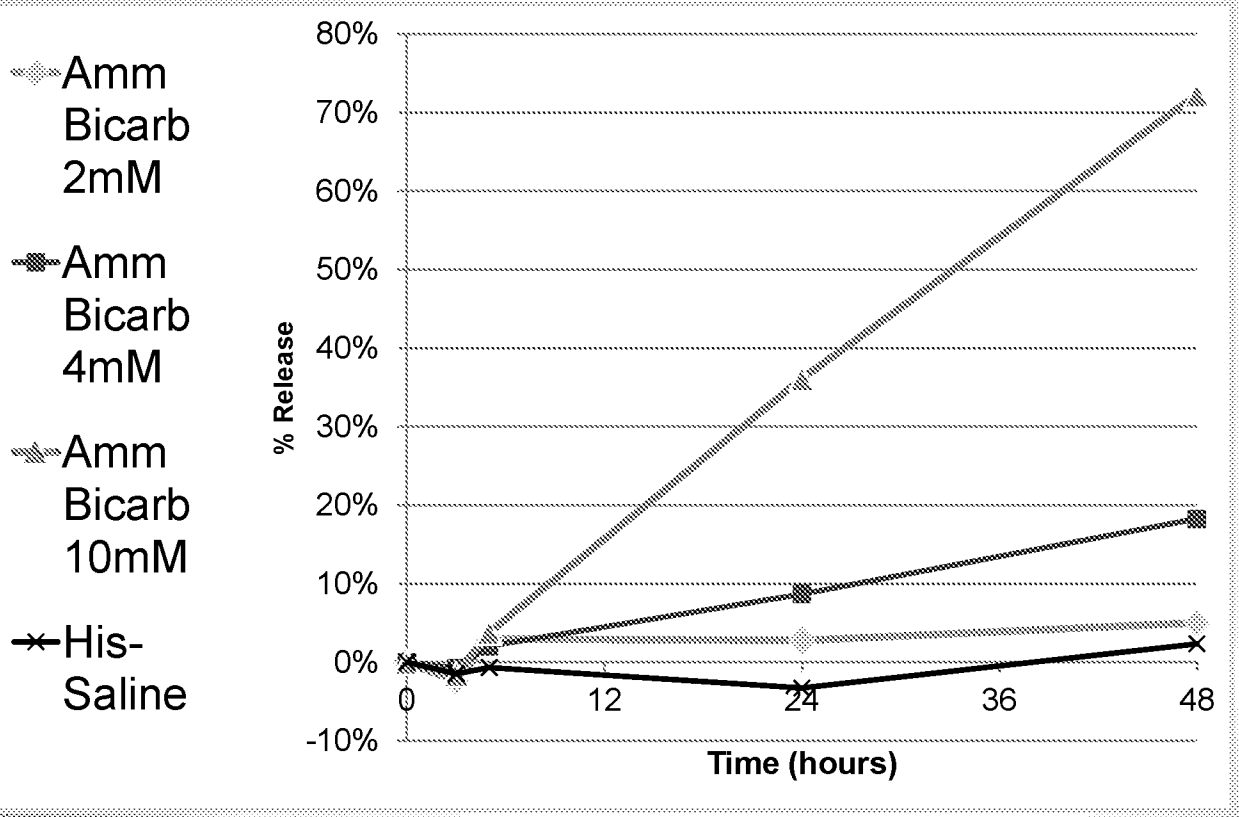
Ammonia Derived from Glutaminolysis Is a Diffusible Regulator of Autophagy

Christina H. Eng,[1] Ker Yu,[1] Judy Lucas,[1] Eileen White,[2,3] Robert T. Abraham[1]*

(Published 27 April 2010; Volume 3 Issue 119 ra31)

Autophagy is a tightly regulated catabolic process that plays key roles in normal cellular homeostasis and survival during periods of extracellular nutrient limitation and stress. The environmental signals that regulate autophagic activity are only partially understood. Here, we report a direct link between glutamine (Gln) metabolism and autophagic activity in both transformed and nontransformed human cells. Cells cultured for more than 2 days in Gln-containing medium showed increases in autophagy that were not attributable to nutrient depletion or to inhibition of mammalian target of rapamycin. Conditioned medium from these cells contained a volatile factor that triggered autophagy in secondary cell cultures. We identified this factor as ammonia derived from the deamination of Gln by glutaminolysis. Gln-dependent ammonia production supported basal autophagy and protected cells from tumor necrosis factor–*a* (TNF-*a*)–induced cell death. Thus, Gln metabolism not only fuels cell growth, but also generates an autocrine- and paracrine-acting regulator of autophagic flux in proliferating cells.

At pH 6.8 in the presence of bicarbonate a substantial release occurs already at physiological ammonium concentrations of tumor microenvironment



Physiological concentration of ammonium at tumor site

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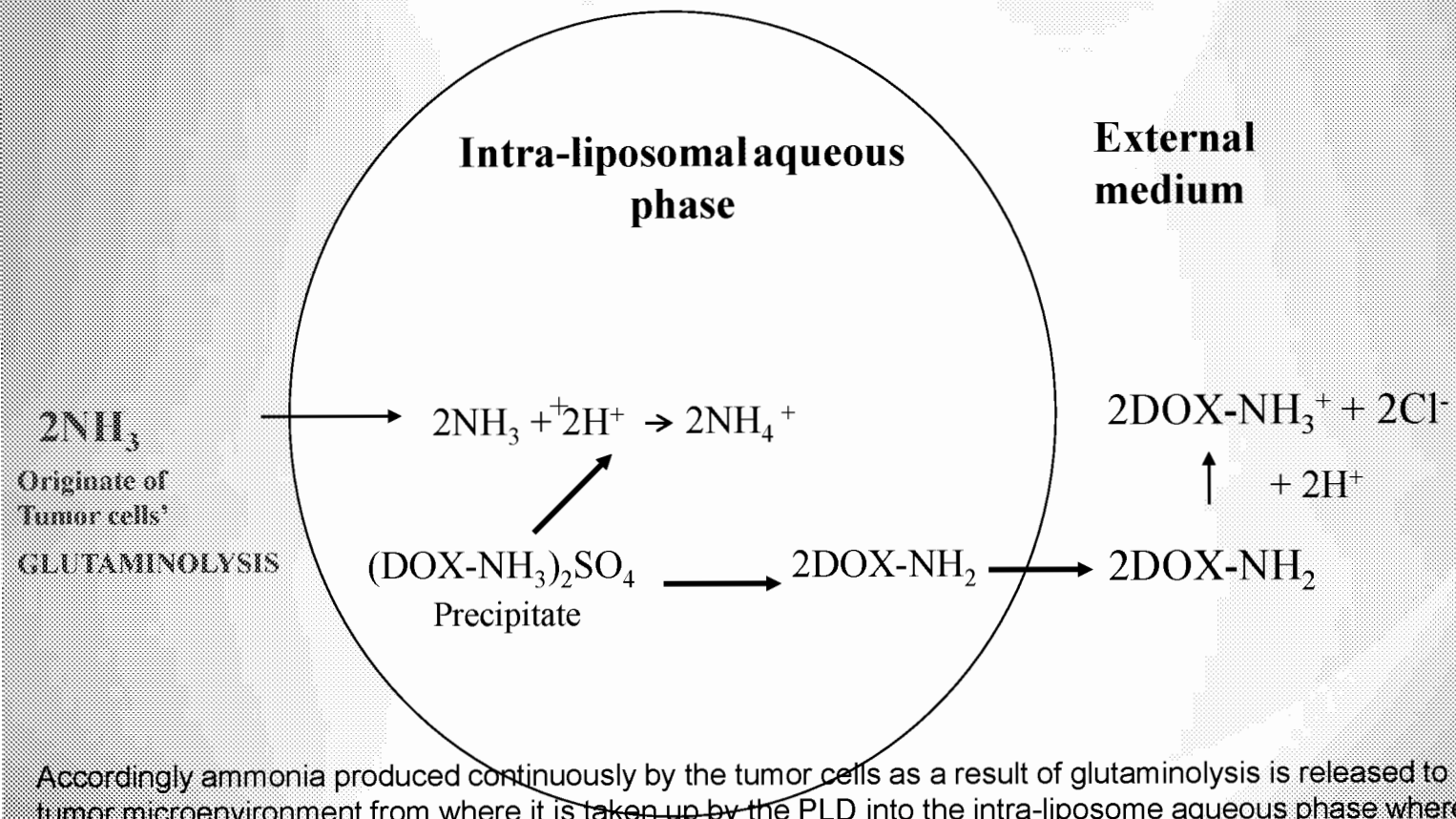
MoA of ammonia induced doxorubicin release from PLD.



Accordingly ammonia produced continuously by the tumor cells as a result of glutaminolysis is released to the tumor microenvironment from where it is taken up by the PLD into the intra-liposome aqueous phase where it get protonated by a "proton transfer reaction" from the protonated doxorubicin. The resulted un-protonated uncharged doxorubicin is than released from the liposomes enable it to be taken up by the tumor cells.

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Ammonia form in tumor is reaching a tumor tissue concentration of 5mM (Eng et al 2010). The ammonia results of the of glutaminolysis which is a unique pathway specific to tumor cells.

Wikipedia describes glutaminolysis as follows:

Glutamine is the most abundant amino acid in the plasma and an additional energy source in tumor cells especially when glycolytic energy production is low due to a high amount of the dimeric form of M2-PK. Glutamine and its degradation products glutamate and aspartate are precursors for nucleic acid and serine synthesis.
Glutaminolysis is insensitive to high concentrations of reactive oxygen species (ROS).
Due to the truncation of the citric acid cycle the amount of acetyl-CoA infiltrated in the citric acid cycle is low and acetyl-CoA is available for de novo synthesis of fatty acids and cholesterol. The fatty acids can be used for phospholipid synthesis or can be released.[21]

Doxil®- Short History

Advantages:

- Doxorubicin in liposomes (Doxil®) has much lower cardio-toxicity and most other side effects than conventional doxorubicin.
- Overall Doxil improves patient compliance and quality of life.

Indications:

- Approved by the FDA (1995) and world wide (as Caelyx) for:
  - AIDS-related Kaposi's sarcoma (KS), Nov, 1995
  - Relapsed ovarian cancer, 1999; after platinum-based treatment, 2005
  - Metastatic breast cancer with cardiac risk, Europe,2003
  - Multiple myeloma in combination with VELCADE® (bortezomib), 2007

History and I.P.

- Doxil is based on 2 patent families (1988/1989) those of Liposome Technology Inc. (LTI) on the pegylated liposomes, and those of Yissum (Barenholz and Haran) on drug loading Licensed to LTI which was acquired by ALZA which was acquired by J & J. Both patent families were expired before April 2014.
- Doxil is (was) produced by Ben Venue Laboratories in the United States for Janssen Products LP, a subsidiary of Johnson & Johnson for global distribution. 2011 onward Doxil Shortage, August 2013 Doxil production stopped.
- February 2013 FDA approved the Lipodox of Sun Pharma 1st generic Doxil

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Doxil®- Reasons for Success

1. High and stable remote loading that enable release in the tumor
2. Steric stabilization by the pegylated lipopolymer
3. Nano ~100nm size which enable benefit from EPR effect)
4. High Tm based LO phase of the lipid bilayer which enables to achieve zero order slow release
5. **We do not know the MoA of doxorubicin release from Doxil at the tumor site?**
6. **We also do not know what is the optimal release rate?**

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Doxil Teams' Acknowledgment

Department of Oncology
Hadassah — Hebrew University Hospital

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Laboratory
Dorit Goren
Aviva Horowitz
Michel Shemla
Dinah Tzemach
Zohar Yehoshua
Neuro-Oncology: Tali Siegal *and team*

Clinics
Raphael Catane
Roland Chisin (*Nuclear Medicine*)
Rut Isacson
Bella Kaufman
Eugene Libson (*Radiology*)
Tamar Peretz
Aaron Sulkes
Beatrice Uziely
Nursing Team of Oncology Ward and Day Care
Pharmacy Services — Cytotoxic Unit

Other Locations

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and Colleagues, USC, Los Angeles
(Clinical Research)

Demetrios Papahadjopoulos
and Colleagues, UCSF, San Francisco
(Basic Research)

Department of Biochemistry
Hebrew University — Hadassah Medical School

Chezy Barenholz

Major Participants
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Lily Bar
Rivka Cohen
Gilad Haran

Others
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Eli Kedar
Elijah Golotin
Stephane Clerc
Arie Degan
Shula Druckman
Noam Emanuel
Oren Tirosh

SEQUUS Pharmaceuticals
(Liposome Technology Inc. — LTI)

Bob Abra
Tony Huang
Danilo Lasic
Frank Martin

and their teams

Why two years after expiration of Doxil® patents and with drug sales exceeding $ 700 millions and at least 12 companies that try to make it there is still only one FDA approved Doxil equivalent generic pegylated liposomal doxorubicin (Lipodox of Sun Pharma was very recently approved in US (Not yet in Europe)

Understanding the difficulties will help a lot in the development of new nano-drugs

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It seems generic Doxil is a big challenge

The lack of FDA approved generic Doxil is also the opportunity to develop an improved formulation which will overcome Doxil main drawbacks but will retain Doxil advantages.

For this we have firstly to identify Doxil main drawbacks

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Doxil side effects are lower than of doxorubicin

	Doxorubicin	Doxil
Vesicant effect	+++	+/-
Infusion reaction	-	+*
Nausea/Vomiting	++	+/-
Myelosuppression	+++	+ (no gr. 4)
Stomatitis/Mucositis	++	+++
Hand-Foot (PPE)	-	+++
Cardiotoxicity	+++	+
Alopecia	+++	+
Max. Tolerated Dose	60 mg/m^2	50 mg/m^2
Dose Intensity	20 mg/m^2/wk	12.5 mg/m^2/wk
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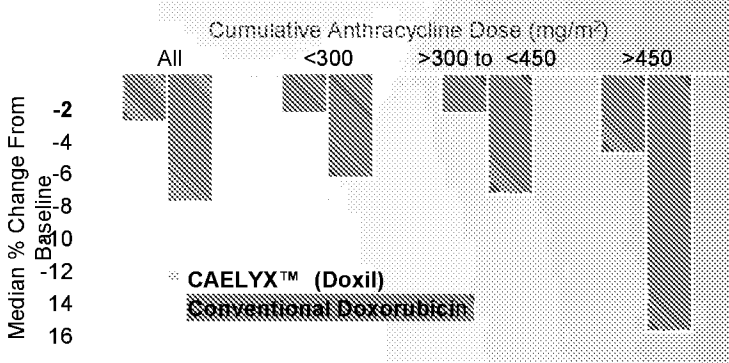
LVEF show that cardio-toxicity of cumulative drug dose is lower for Doxil than for doxorubicin



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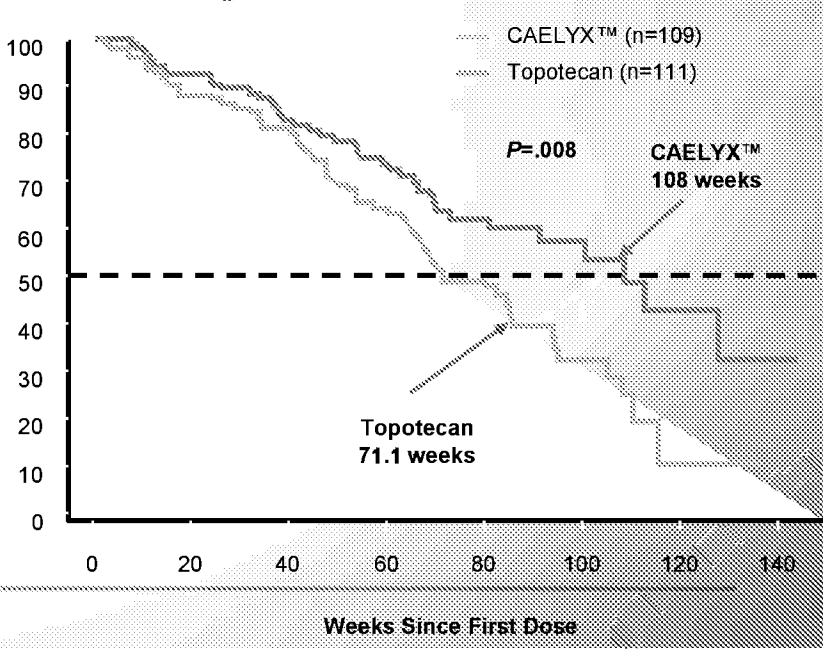
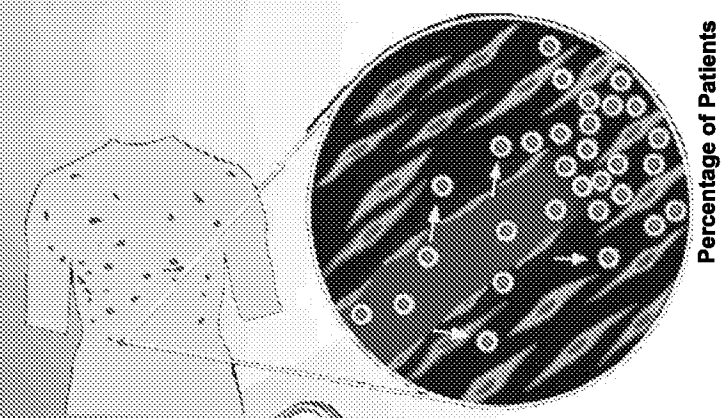


Doxil Mechanism of action: EPR effect and what next?



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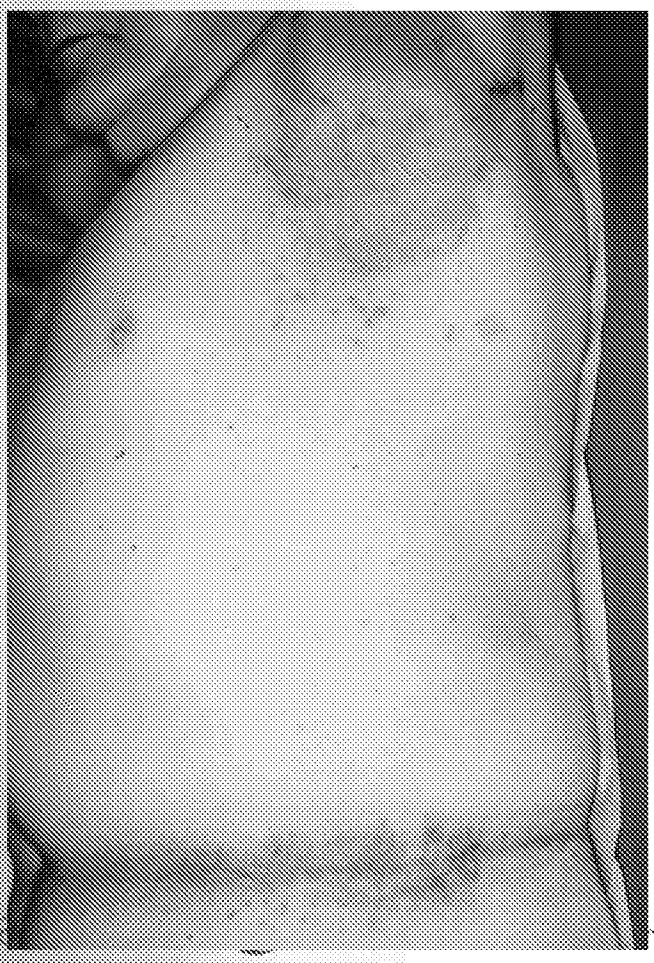
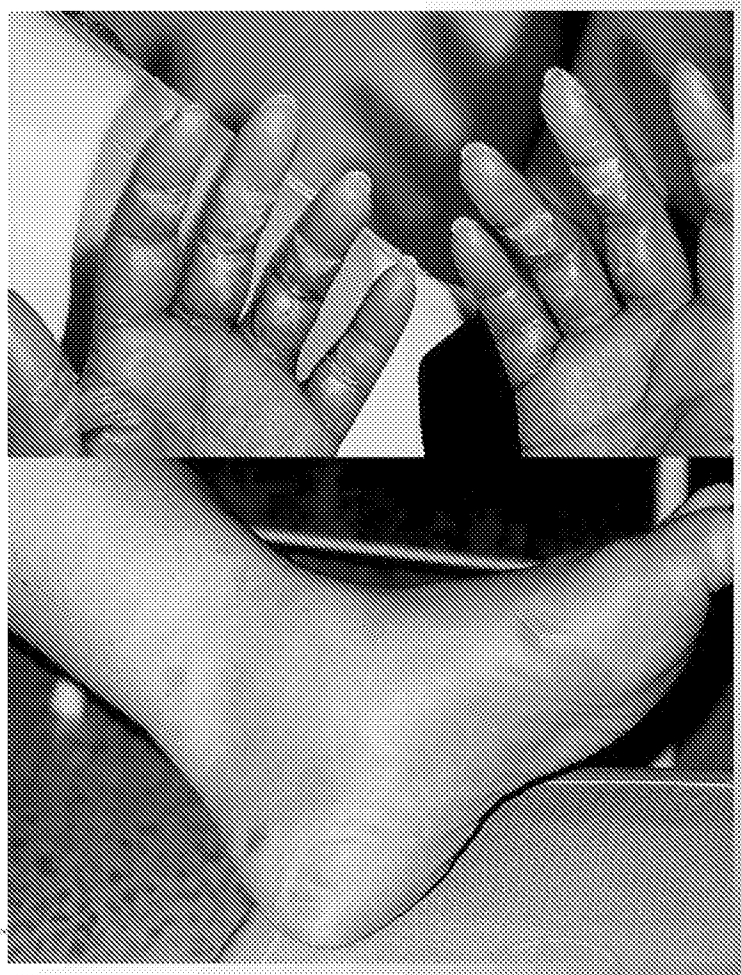
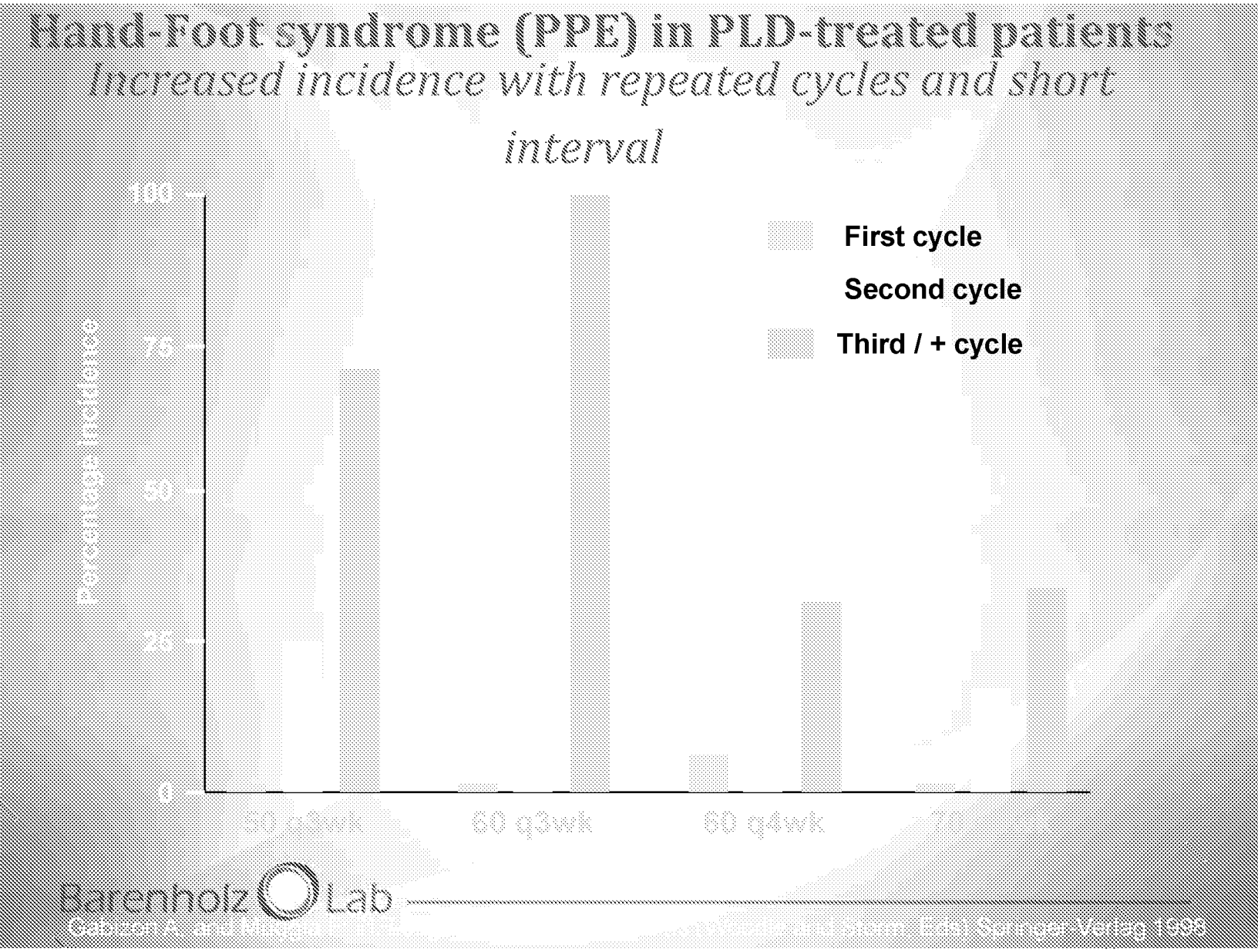
Hand-Foot Syndrome (Palmar-Plantar Erythema)
A major issue of patient compliance





Hand-Foot syndrome (PPE) in PLD-treated patients

increased incidence with repeated cycles and short interval



What is expected of an improved liposomal doxorubicin?

- It should have all the clinical benefits of Doxorubicin and Doxil™ concomitantly with much better safety profile regarding unwanted side effects
- Over all it should improve cancer patient compliance and quality of life without compromising therapeutic efficacy

Our LC-100 a novel (patent protected) liposomal doxorubicin nano-drug with is expected to meet the above 2 conditions

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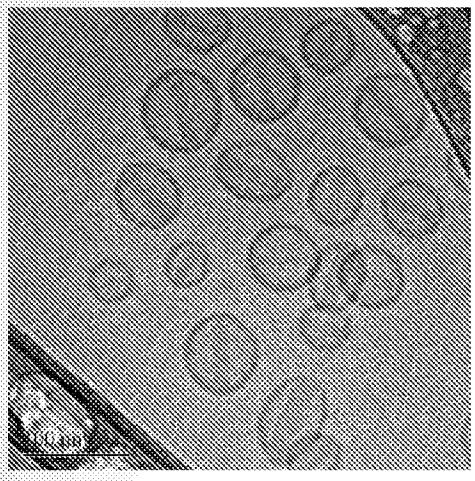
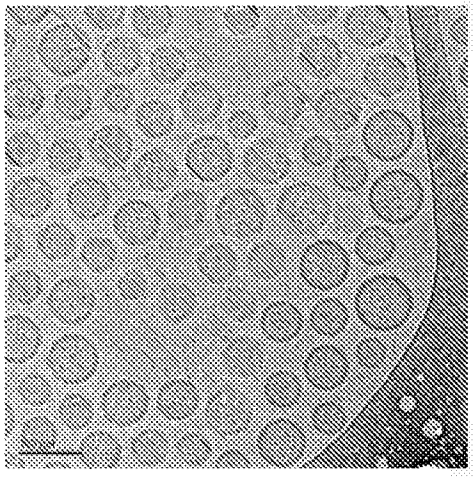
LC100 features

A Long circulating sterically stabilized Doxorubicin liposomes (like Doxil liposomes) but with an improved safety profile



Doxil™ (Dox -Sulfate)
Scale bar: 100nm



LC-100 (Dox NEW)
Scale bar: 100nm

LC 100 are spherical liposomes and their intraliposomal doxorubicin does not show a crystal formation as one can seen in Doxil

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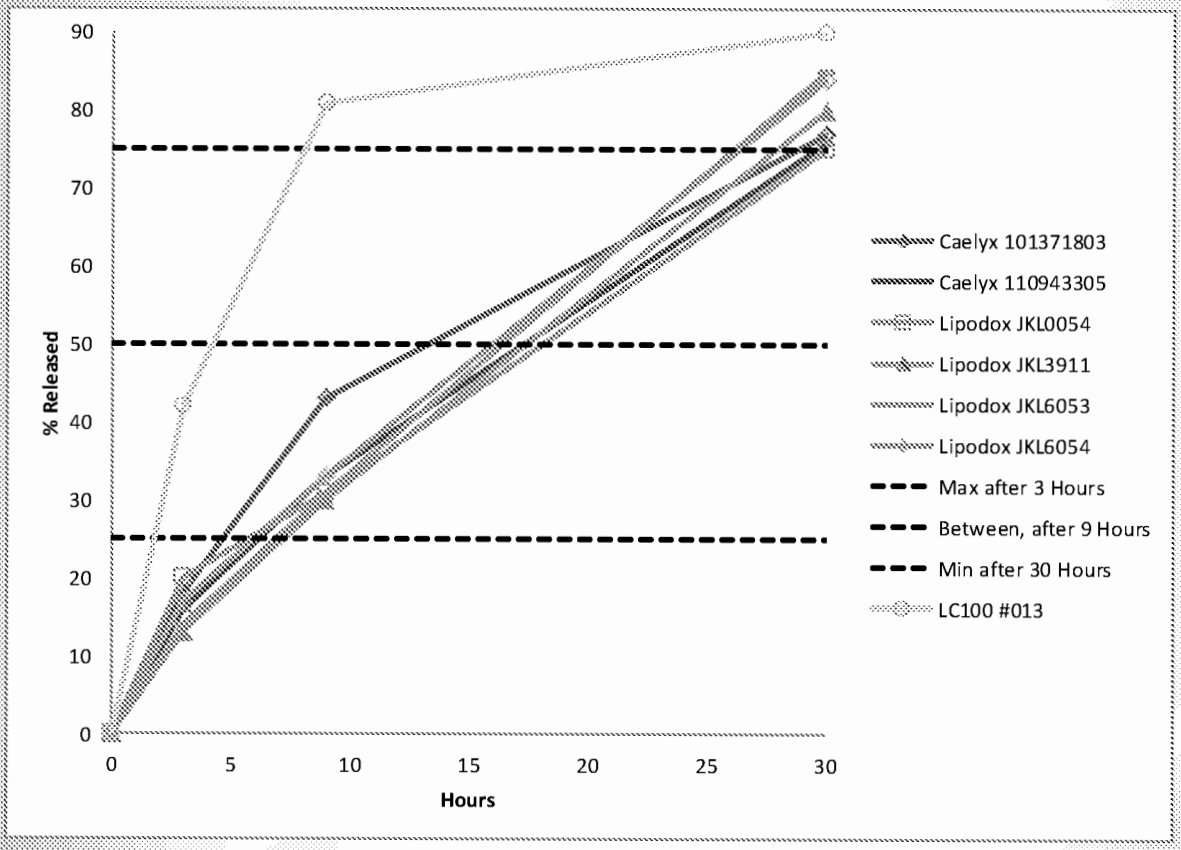
Dissolution (PLD release kinetics) evaluation of PLD

Comparing Doxil and Lipodox™ to LC100 at tumor microenvironment conditions (at 37°C)



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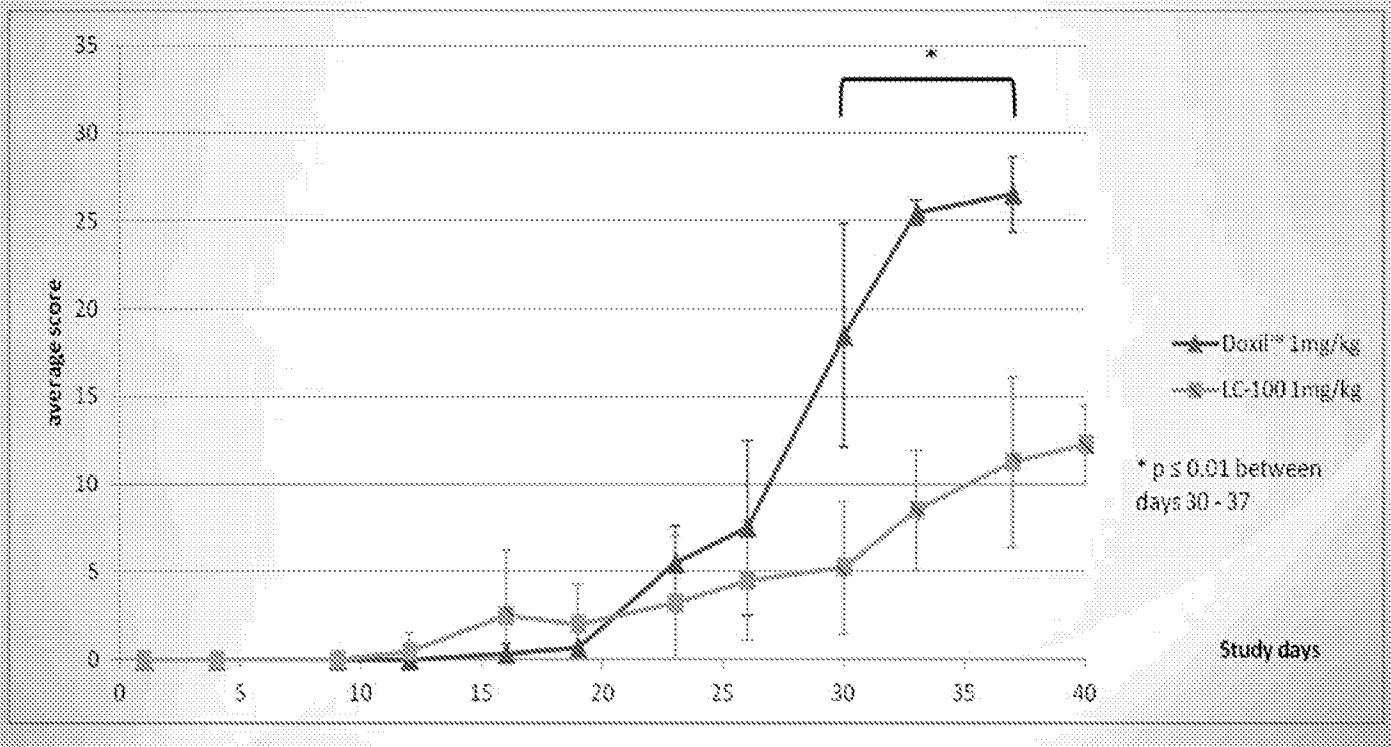
LC-100 versus Doxil™ - reduced rat Hand and Foot (PPE) toxicity (as accumulative score)



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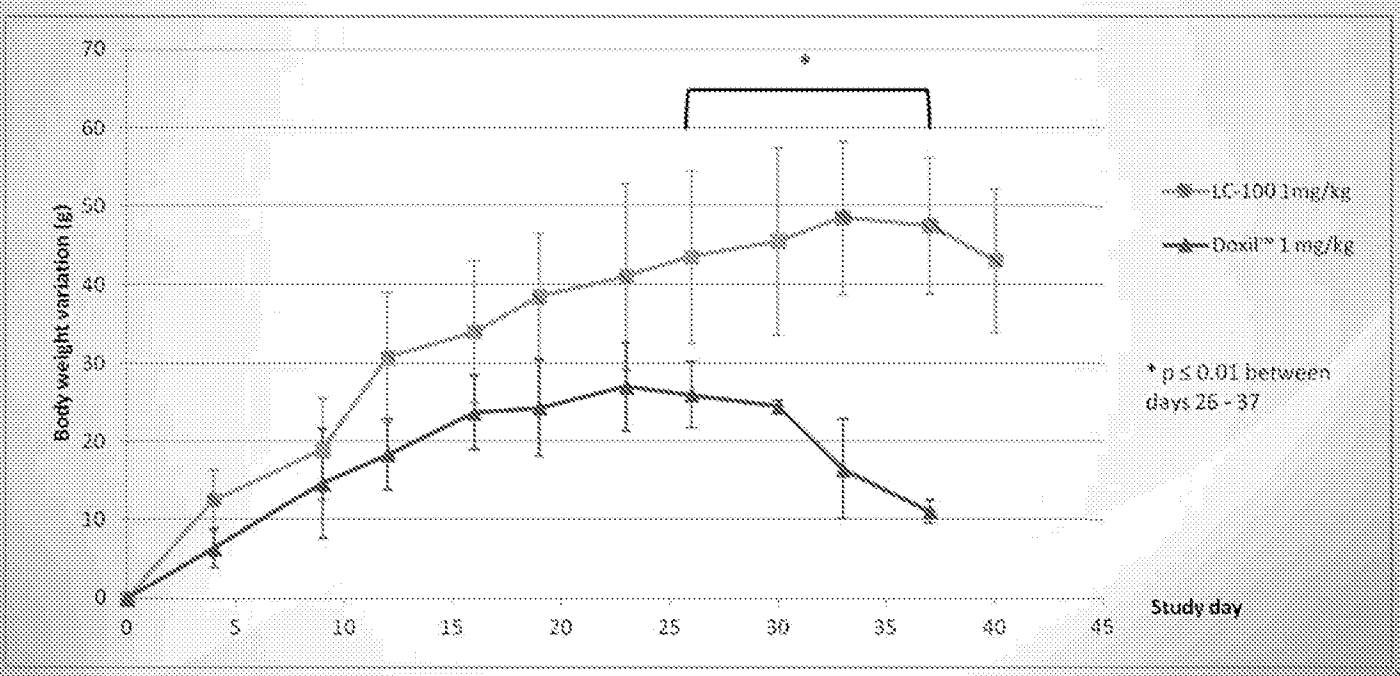
LC-100 versus Doxil™ - Effect on Body Weight gain (general toxicity)

(our novel pegylated liposomal doxorubicin nano-drug) showed better growth based on body weight gain in comparison to Doxil™ treatmer



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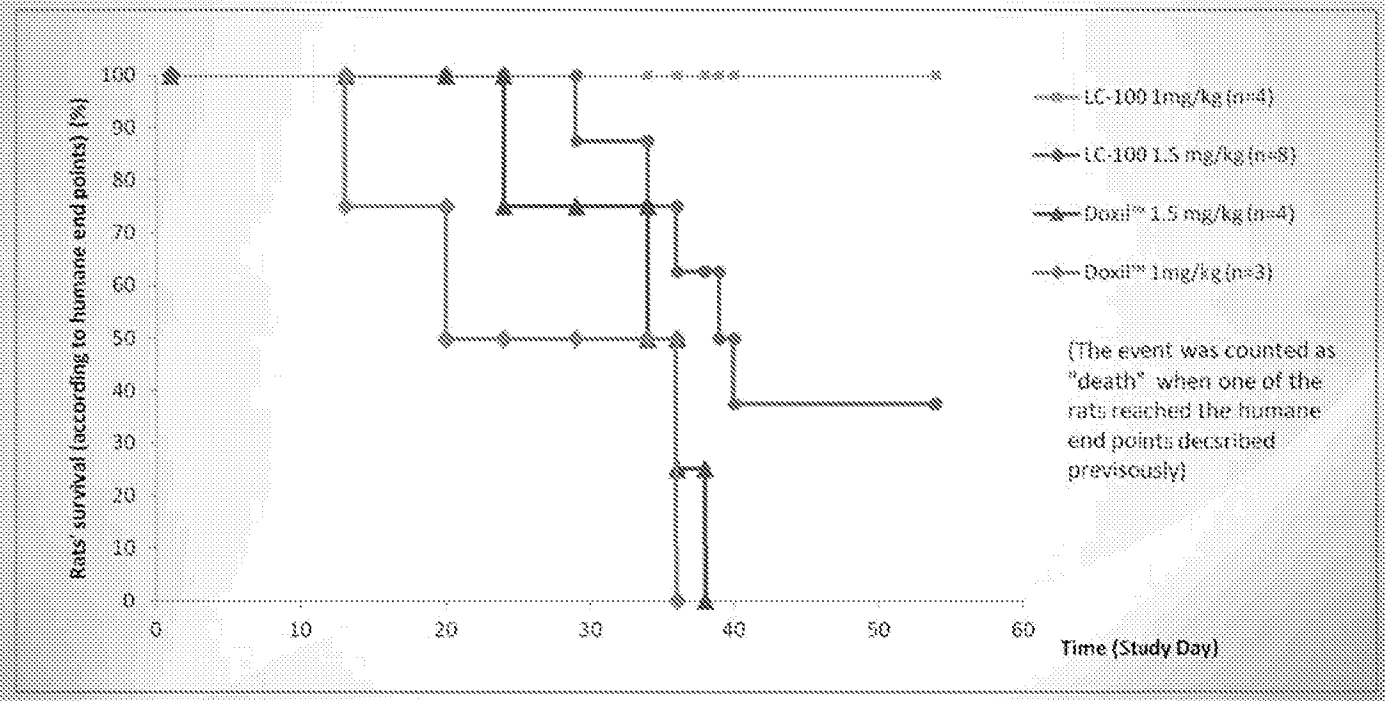
Survival of Rats "humane end points"

LC-100 (our novel pegylated liposomal doxorubicin nano-drug) treatment showed much better "QUALITY OF LIFE" as compared to Doxil™ treatment



"Humane end points": signs of severe pain (usually associated with a scoring above 20), excessive porphyrin secretion from the eyes and/or nose, excessive aggressiveness, severe signs of infections, etc..!!

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Conclusions

- LC-100 at repetitive injection of 1mg/kg twice weekly over 12 weeks has an improved safety profile over Doxil™
  1. lower "Hand & Foot Syndrome" score
  2. Lower General Toxicity (body weight)
  3. Both should translate into better "quality of life"

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LC-100 Product Development Status

- Current CMC status:
  - Established product specifications and production process
  - Stability for over one year
  - Closure selection and specifications
  - Developed in-vitro release method
- Status pre-clinical (Comparison with Doxil):
  - PPE rats model demonstrating superiority over Doxil
  - General Toxicity demonstrating superiority (Body Weight)
  - Supportive mice PK mice studies with similar tumor drug accumulation
  - Similar (or even slightly better) therapeutic efficacy in the two tested mice models
  - On the way to phase I/IIa clinical trials in ovarian and breast tumors

Special Thanks

to my LC 100 team

Dr Doron Friedman

Tal Berman

Jackie Toledo

Yaelle Felsen

Michael Raslin

Wolf Rajchenbach

Alexander Lyskin

Lisa Silverman

And Janos Szebeni *my partner in the development of Doxebo used to reduce Doxil induced Complement activation*

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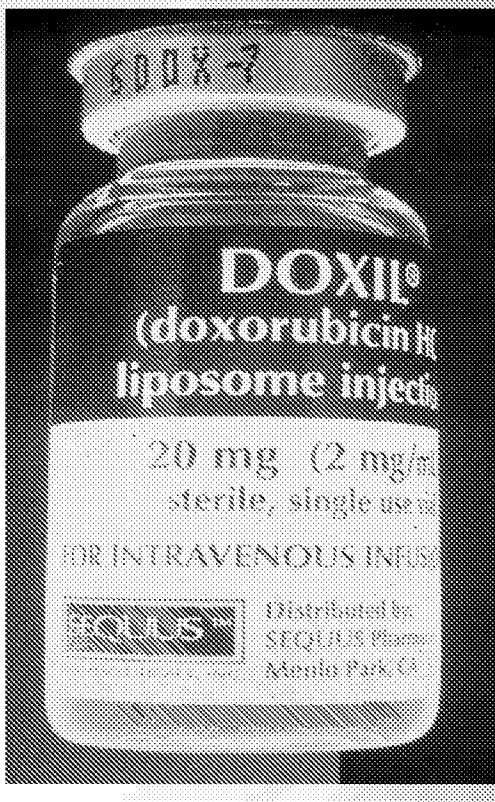
Development of liposomal drugs And Nano-Drugs: From academic research via incubators and startups to FDA and EMA approved products

Part II: entrepreneurs and entrepreneurships

Professor Yechezkel (Chezy) Barenholz,
Laboratory of Membrane and Liposome Research,
The Hebrew University – Hadassah Medical School,
Jerusalem, Israel

Barcelona NanoMed
March 4-5 2014



Doxil: 80 – 100 nm SSL remote loaded with doxorubicin via ammonium sulfate gradient. 18 years to Doxil 1st FDA approved nano-medicine (11.95)

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Today Agenda

- General difficulties in current drug development
- NMII MLV for osteoarthritis treatment
- Doxil the first FDA approved drug and its MOA (in short)
- Why 3 years after Doxil relevant patent expiration FDA approved only one generic product, Lipodox?
- Lessons learned for the development of novel nano-drugs
- LC100 new generation liposomal doxorubicin with less side effect and better efficacy than Doxil
- Scientists as entrepreneurs : a user's guide

  35 years of personal experience

The Harvard MBA/Dentist Joke

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Tech Transfer from Barenholz Lab HUJI

- *Cancer therapeutic, Doxil licensed to LTI (Sequus), a medium size company (1985) to ALZA to J & J (a very large Pharma)*
- *LC100 a new generation improved "Doxil" licensed to Lipocure a medium size start-up with capacity to produce clinical materials)*
- ***Vaccinology Licensed to NasVax as incubator later a start up***
- ***Inflammatory and autoimmune diseases LC200 licensed to Omri transferred to Lipocure (MS, RA, Lupus,** on the way to clinical trials)*
- ***Local anesthetics** ultra long local anesthetics licensed to Lipocure on the way to a clinical trial)*
- ***Osteoarthritis (cartilage lubrication and reduction of wear licensed as a medical device to Moebius Medical an incubator** Finished successfully proof of concept clinical trial , on the way to the pivotal study*
- *Large scale production of cytotoxic and non cytotoxic liposome based drugs was licensed to Ayana LTD*
- **Cleantech: Use of "cheap" liposomes to clean the environment was licensed to LipoGreen an incubator**

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Drug development: from basic research to approved drug

The current chances

It was shown that for every 1,000 compounds that reach testing, only 5 make it to advanced clinical trials and less than 1 is ultimately approved by the U.S. Food and Drug Administration (FDA).

Namely chances for success are very low, the driving force for development in spite the failure is the large reward in case of success.

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The Motivation to the development of good nano-drugs is obvious from looking at Doxil® Sales on

- Annual sales rate exceeded $700 M in H1 2011, before Ben Venue production shutdown by FDA
- Cost of Doxil® course of treatment
  - 50 mg/m^2 every 4 weeks
  - $4000/50 mg vial ($80/mg)
  - $8,000/month ($ 96.000 per year)

Based on adding new (now evaluated) indications sales may reach $ 1.0 Billion

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Doxil® market is expected to increase due to many new indications in clinical trials

Drugs	Indication	Phase
DOXIL + temsirolimus (mTOR inhibitor)	recurrent sarcoma	I/II
DOXIL + LY573636 (mitochondrial apoptosis)	advanced solid tumors	I
DOXIL	before mastectomy in invasive breast cancer	I
DOXIL + vorinostat & bortezomib	relapsed or refractory multiple myeloma	I
DOXIL + dexamethasone & lenalidomide (thalidomide derivative)	newly diagnosed multiple myeloma	II
DOXIL + ixabepilone (microtubule stabilizer)	advanced ovarian, fallopian tubem or metastatic breast	I/II
DOXIL + bortezomib, cyclophosphamide, dexamethasone	multiple myeloma	I/II
DOXIL + bortezomib, dexamathasone, lenalidomide	relapsed/refractory multiple myeloma	II
+ ~40 additional clinical trials		

Efforts to Make Generic Doxil®

Name	Company	Comments
Myocet®	Enzon Pharmaceuticals/Cephalon	Non-pegylated, approved in Europe for breast cancer
TLC Lipo-Dox®	Taiwan Liposome Company/TTY Biopharm	Non-pegylated, launched in Taiwan in 2001 for AIDS-Kaposi sarcoma, breast cancer and ovarian cancer
Doxisome	Taiwan Liposome Company	In planning of bioequivalence studies as Doxil®/CAELYX® substitute
Lyodox	Amronco Life Sciences Ltd.	Sold in Latin America. poor quality
Doxoget	Getwell pharmaceuticals	Sold in Indi. Poor quality
Lipodox	Sun Pharma	Sold in India, failed EMA and FDA recently FDA approved
Generic Doxil®/CAELYX® Early stage	Teva Pharmaceutical Industries, Ltd.	Israeli-based generic company; in development
Generic Doxil®/CAELYX® early stage	Gedeon Richter PLC	Hungary-based generic company; in development

However as can be learned from the FDA draft guidelines of 2010 on generic Doxil this is not easy

- :
  - Have the same drug product composition
  - Manufactured by an active liposome loading process with an ammonium sulfate gradient and,
  - have equivalent liposome characteristics including liposome composition, state of encapsulated drug, internal environment of liposome, liposome size distribution, number of lamellar, grafted PEG at the liposome surface, electrical surface potential or charge, and in vitro leakage rates.
- Requires *In-Vitro and* clinical Studies

http://www.fda.gov/downloads/Drugs/.../Guidances/UCM199635.pdf

Why three years after expiration of Doxil® patents and with drug sales exceeding $ 700 millions and at least 12 companies that try to make it there is still only one FDA approved Doxil equivalent generic pegylated liposomal doxorubicin (Lipodox of Sun Pharma was very recently approved in US (Not yet in Europe)

Understanding the difficulties will help a lot in the development of new nano-drugs

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Technical Hurdles

- Liposomes with the same physicochemical characteristics can have different therapeutic outcomes and toxicities, because measurements of bioequivalence measure only the average properties
- Small changes in manufacturing processes, such as lipids, excipients, equipment, exact method of preparation, or facilities can result is significant changes to therapeutic outcome or toxicities
- The PK/PD of therapeutic nanoparticles is complex

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FDA guidelines draft, 2010

http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM199635.pdf

Require a complex CMC and simple clinical trials

Identity in CMC which is based on detailed physico-chemical parameters

Equivalent composition:

Chemical: drug-to-lipid ratios, amounts of free and encapsulated drug, percent drug encapsulation, lipid bilayer phase transitions, excipients

Liposome characterization

liposome size distribution and morphology as demonstrated on multiple batches and samples of test and reference products (number of lamellae, lipid bilayer phase transition and X ray diffraction pattern, entrapped volume)

Internal liposome environment

drug loading using an ammonium sulfate gradient, internal pH, magnitude of the pH gradient across the membrane, equivalence in the doxorubicin sulfate level, presence and structure by SAXS, WAXS and cryo-TEM of DOX-sulfate precipitate inside the liposomes

Equivalent surface properties

electrical surface and zeta potential, PEG layer thickness, equivalent concentrations and size of grafted PEG at the surface, equivalent PEG-lipid chemistry to prevent premature cleavage of the PEG from the liposome surface

Equivalent drug release rates in a variety of conditions that result in equivalent drug delivery to target (tumor) cells

different physiologically relevant solutions, e.g., human plasma, a range of pH values, a range of temperatures, under low frequency ultrasound

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Clinical Requirements

- A single-dose, two-way crossover pharmacokinetic study, in ovarian cancer patients whose disease has recurred or progressed after platinum-based chemotherapy
- AUC and C_{max}
- Vd_{ss} and Cl
- Not easy as it require a BE in both encapsulated and free doxorubicin may require many patients which are not easy to get

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Failures of Generic Doxil® (QbD)

- Smaller liposomes (75 nm, 300 mM ammonium sulfate)
  - identical lipid composition
  - identical PK parameters in normal mice
  - rapid drug release for the 75 nm liposomes
  - Doxil liposomes had higher AUC and C_{max} in S-180 sarcoma-bearing mice
  - 75 nm liposomes were more active, therapeutically
  - 75 nm liposomes had greater toxicity (decreases in body weight)

Cui J et al. J Control Release. 2007 Apr 2;118(2):204-15.

- 6 different PEGylated liposomal doxorubicin formulations were compared to Doxil® in a murine breast cancer model, and in tumor-free monkeys
  - counter ion in the loading process was changed from sulfate to dextran sulfate (DSAS)
  - same doxorubicin plasma PK as Doxil
  - greater decreases in tumor volume
  - 3.2-fold increased aspartate transaminase levels (heapto-toxicity)
  - 5.0-fold increased cardiac troponin I levels (cardiac toxicity)
  - increased bone marrow hypocellularity (bone marrow toxicity)
  - increased kidney toxicity

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Rao NVS et al. Cancer Chemother Pharmacol. 2010 Nov;66(6):1173-84.

Basic *vs* Applied Research: Major points to remember

There is a statement made by the famous French scientist L. Pasteur that "there is no Basic Science and Applied Science but only Good and Bad Science" which is still 100% correct today.

However differences between the two do exists with the good Basic Science being a pre-requisite for a good Applied Research. These differences are important for the optimization of the both R & D and the drug development processes.

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The main differences between the basic and applied research are that applied research require to deal with:

Long term stability and large scale production issues

I.P. and "regulation" (a lot of paper work according to requirements of agencies such as FDA)

In applied research which aims to develop product "the best may the enemy of the good" and in order to save money and time the good is enough to get to an approved product.

In applied research ego should not play a major role

Requirements of Industry from Scientists and drug developer at the 21 century are much larger than at the end of the 20th century.

I.P. and know-how related decisions

The I.P. and Know-How are our actual assets

Only I.P. but not know-how can be protected, however:

1. **Patents are very costly. Many times saving I.P. expenses ends up in great losses and even total loss.**

2. **Patents has to be made so what is licensed can be controlled and will not be too broad limiting other options of the inventor.**

3. **Many times it will be advantageous (in spite cost) to split patent according to specific applications so each of the applications will not be to broad and can be licensed to a separate company.**

4. **When is the best time to file a patent application?**
   Patent will be in force 21 years after date of filing of the provisional application

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Other important points to remember

Every Scientist believe his project is unique and he forget that Industry has many options.

Scientist has to understand industry and investors needs and language

Scientists, University, and TTO require knowing well the competition, and understanding the needs and limitations.

External help to the Scientist is a must especially on I.P. and regulatory issues.

approaching the right people in Industry can make make the "click". (Connections, connections, connections)

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Important roles of thumb:

The I.P. clock has a limit of 21 years (from patent filing), therefore shortening development time or postponing as long as possible mean a lot of money

Development of nano-drugs is multidisciplinary Therefore productive sharing is a must.

100% of Zero is equal exactly to Zero!!!

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Major Strategic Decisions to be Taken
How to select a strategic/financial partner ?
What are the options?

1. **Big Pharma (royalty agreement and maybe upfront payment plus research and consulting). The main advantages: good and long term financial and professional support. Disadvantages: rigidity and competition with many other company projects, also the inventor may have only a small or no say in the development program.**

2. **A middle size Pharma or existing start-up. Advantages and disadvantages are the opposite to the situation exists for Big Pharma. The scientist project may become the main focus of the company**

3. **Building a start-up around the TTO I.P. (royalties and/or equity, consulting, research)? See NasVax example. Always short on money wasting IP time**

4. **Starting with n incubator (Mobeius OA project), hardly enough for feasibility studies unless larger that required investment was made (Polypid)**

5. **Trying to take if forward to include production, fits only very specific projects**

The complex triangle involved in a productive applied science

Three sides of a triangle: Industry, (and/or financial enterprise); University plus Technology Transfer company (TTO); and the Scientist (researcher) constitute the three sides combine to perform a project and commercialize it.

Although all three parties share a common interest: the project success, they also have many opposing interests.

Relationships between the 3 parties are therefore complex and common interests varied, like: University (TTO) – Scientist; Industry – Scientist; University (TTO) - Industry etc.

Each of the 3 sides should perform what he knows best, but help other sides on all need (good interaction and collaboration)

In this short presentation I will discuss the role of each side and demonstrate it on my own >20 years of experience

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Roles of Each of the 3 Parties

Role of Industry (strategic/financial partner) :

To supply the financial support of the R & D program; being responsible (or sharing responsibility with Scientist) on all what involved in clinical trials or equivalent steps (such as beta sites),

Finish R & D of the drug prototype and the final form of the drug

To finance Scientist research and I.P.

Roles of Each of the 3 Parties

Role of Technology Transfer Company (TTO):

To represent Scientist (and University);

To deal on behalf of the scientist and University with all legal and administrative issues of the project.

To make sure all I.P. (patentability and "freedom to operate" issues were dealt with properly

To make sure that Scientist get all his needs to perform his duties in the project.

To make sure all I.P. issues were dealt with properly

To maximize interests of Scientist and University in a fair way

Roles of Each of the 3 Parties

Role of scientist/researcher

To perform the research, proof of concept, and feasibility studies. These may require minimal R & D to produce a prototype according to the needs

To help with supplying the basic information required to respond to I.P. issues

To keep good records of research methods and results

To supply Industry with all the needs for advancing the program smoothly

To interact with and be open to advice from Industry

The 3 parties in my case are:

The Technology Transfer Company: Yissum Ltd

R & D company of the Hebrew University Jerusalem Israel

The Scientist: Professor Yechezkel Barenholz Ph.D.

Head Laboratory of Membrane and Liposome Research at the Hebrew University – Hadassah Medical School, Jerusalem, Israel

The Industry: for Doxil - LTI (Sequus), ALZA, J & J

For other projects: Various, including large Pharma, Biotech companies, Start-ups, and Incubators.

TTO - Scientist Relationship

What I got from TTO during Doxil development?

1. Help (financial and "package design") with feasibility studies (**NO**)
2. Help on IP issues (patentability and freedom to operate) to find suitable patent attorney **(YES)**
3. Help in finding funding (**NO**)
4. Help in contractual arrangements with respect to IP issues, research support and benefits **(YES, partial)**
5. **Guidance on how to keep records (NO)**
6. Help in presentations (**NO**)
7. Criticism!!! **(NO)**

Today Yissum is better on most items but the scientist has to be on guard all the time


HANK
OU!
From Jerusalem

Journal of Controlled Release 160 (2012) 117–134

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review

Doxil® — The first FDA-approved nano-drug: Lessons learned

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ARTICLE INFO

Article history:
Received 10 February 2012
Accepted 21 March 2012
Available online 29 March 2012

Keywords:
Liposomal development
FDA approval
Anticancer therapy
Doxorubicin

ABSTRACT

Doxil®, the first FDA-approved nano-drug (1995), is based on three unrelated principles: (i) prolonged drug circulation time and avoidance of the RES due to the use of PEGylated nano-liposomes; (ii) high and stable remote loading of doxorubicin driven by a transmembrane ammonium sulfate gradient, which also allows for drug release at the tumor; and (iii) having the liposome lipid bilayer in a "liquid ordered" phase composed of the high-T_m (53 °C) phosphatidylcholine, and cholesterol. Due to the EPR effect, Doxil is "passively targeted" to tumors and its doxorubicin is released and becomes available to tumor cells by as yet unknown means. This review summarizes historical and scientific perspectives of Doxil development and lessons learned from its development and 20 years of its use. It demonstrates the obligatory need for applying an understanding of the cross talk between physicochemical, nano-technological, and biological principles. However, in spite of the large reward, ~2 years after Doxil-related patents expired, there is still no FDA-approved generic "Doxil" available.

Contents

1. What led to Doxil® development: OLV-DOX

Development of Doxil was initiated in Israel and the USA ~14 years ago when it became evident in a "first in man" (FIM) clinical trial by

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doi:10.1016/j.jconrel.2012.03.020

Gabizon and Barenholz that a "first generation" liposomal doxorubicin did not justify further clinical development despite an elevation of drug MTD (rev. in [1]). In this FIM trial we used negatively charged, medium-size oligolamellar liposomes (OLV) composed of two low-T_m (fluid) phospholipids [the zwitterionic egg-derived phosphatidylcholine (EPC), the negatively charged egg-derived phosphatidylglycerol (EPG)], and cholesterol. In these OLV the doxorubicin was membrane associated and passively loaded during the lipid hydration. This liposomal doxorubicin (DOX) is referred to as OLV-DOX (for more information on the OLV-DOX formulation development, characterization, performance, and clinical experience see [2–17], and reviewed in [1]).

In this FIM we also determined the patients' plasma PK of doxorubicin and of phosphatidylglycerol (PG), a phospholipid that is not normally present in human plasma and therefore was used as the liposome marker of the OLV-DOX. From the ratio between the DOX PK and the PG PK we calculated the drug release rate in human plasma in vivo [14,15]. We also determined the OLV biodistribution (BD) by imaging of ^{111}In-remotely-loaded OLV (^{111}In-OLV, [14]). These studies clearly demonstrated that the clearance of DOX when delivered as OLV-DOX is a composite of two processes: (i) clearance of liposomes containing DOX by the RES, predominantly liver and spleen, but not the liver tumor, which is avoided by these OLV; and (ii) clearance of free DOX released fast from liposomes in plasma. The analysis, which includes PK of total drug (DOX), liposome-associated DOX, and liposome markers (PG and ^{111}In-OLV), suggests that both processes operate in human patients and that factors such as the patient's liver function may affect their relative contribution [14,15].

These PK, BD, and imaging data suggest that the reduced clinical toxicity of OLV-DOX results from a somewhat lower peak level of free drug and possibly some changes in the tissue distribution of the liposomes, with a partial shift toward drug accumulation in the RES at the expense of other tissues. The main limitations of the therapeutic strategy based on OLV-DOX, as revealed by this study, are the significant drug leakage and preferential RES uptake.

These shortcomings are probably the result of the basic inferior formulation physicochemical characteristics given below.

(i) Drug location in the liposome bilayer as opposed to encapsulation in the liposome aqueous interior. Bilayer-associated drug may be more accessible to be released to the plasma upon dilution and to associate with plasma proteins [10,11,15] This process is determined by the drug membrane/medium partition coefficient, which in the case of doxorubicin is not high enough to retain the drug during the major dilution the OLV-DOX undergo as a result of intravenous slow infusion to humans [12,15,18,19]. We demonstrated that the discrepancy between the successful therapeutic efficacy in mice and the failure in the human studies is a result of the very large difference in plasma volume (compare, ~1 mL in mice with >3500 mL in humans and in mice and human size). The association of doxorubicin with liposomes is related to the liposome membrane/aqueous medium (plasma) partition coefficient (Kp). Therefore, slow infusion of the liposomes will result in an immediate very large dilution of 3500-fold for each mL that reaches the plasma, compared with only a 5-fold dilution with the i.v. bolus injection of the same liposomes to mice. The fast free drug clearance from plasma keeps this huge dilution effect active throughout all the time of the infusion [12,15,18–20]. The burst of drug leakage shortly after injection into patients (Fig. 4 in [14]) is compatible with the dilution release effect.

(ii) The presence of a high mole fraction of PG in the liposome bilayer may accelerate uptake by the RES [13]; it may also induce complement activation [21–23].

(iii) The liposome size is too large to allow for extravasation in extrahepatic tissues [24] and to take advantage of the enhanced permeability and retention (EPR) effect that was first described by Matsumura and Maeda [25] and reviewed by Maeda et al. [26]. This effect may allow for selective accumulation of nanoparticulates in tumors due to tumor (but not normal healthy tissue) being rich in porous blood capillaries that are permeable to particles of 100 nm and smaller. In addition, the tumor tissue is poor in lymphatic drainage, which enables prolonged retention of the nanoparticles there, followed by local (tumor) drug release and/or for the liposomes to be taken up by the tumor cells. Therefore, the fact that the same dose-limiting bone marrow toxicity was observed with OLV-DOX and with doxorubicin administrated as is (standard care) is not surprising and can be assigned to the large extent of fast drug leakage from circulating liposomes.

In view of the OLV-DOX fast plasma drug release and the changes in tissue distribution and bioavailability, it is uncertain whether the somewhat increased tolerated dosage of OLV-DOX (over free, non-liposomal DOX) will result in an enhanced antitumor activity. The liposomes used in this clinical study are cleared fast by the RES of liver and spleen and to a lesser extent by the bone marrow. These human studies suggest that the mechanism of antitumor activity of OLV-DOX is complex, and presumably results from exposure of tumor cells to drug leaking from circulating liposomes and drug released from the RES. Obviously, drug leakage from circulating liposomes is undesirable since it resulted in unwanted cardiotoxicity. Regarding drug release from the RES, the clinical conditions most likely to benefit from this approach are limited. This approach should not work for treatment of solid tumors, as in most solid tumors drug exposure in relation to dosage may be suboptimal. The OLV-DOX is expected to be highly sensitive to factors such as RES/liver function, site of tumor involvement, and proximity of tumor cells to RES cells.

The failure of this OLV-DOX used in humans had some basic "take home lessons" that led us to the development of a liposomal doxorubicin formulation that should be less toxic and more efficacious than free DOX in humans. The failure of OLV-DOX served as the main driving force and as the basis for Doxil® development.

Our failure with OLV-DOX supported the 1980s' overall low expectation of liposomes as a broad spectrum drug delivery system. This disappointment was summarized in an almost "lethal" paper (to the medical application of liposomes) in *Cancer Research* by Poste et al. [27], which states categorically that: "The inability of liposomes to escape from continuous capillaries and their rapid uptake by circulating and fixed phagocytic cells calls into question the feasibility of using liposomes to 'target' drugs to cells in extravascular tissues".

This and Poste's 1983 publication [28] were "catastrophic" to the medical application of liposomes as it led the scientific community as well as the major grant agencies, the pharmaceutical industry, and the venture capital community to lose interest in this field. It took 10 more years and a few real clinical successes for the field to recover and gain back some trust that enabled the development of more than a dozen FDA-approved liposomal drugs from 1995 to the present.

In planning our advanced liposomal anticancer drug, Liposome Technology Inc. (LTI), Gabizon, and I decided to stay with doxorubicin as the cancer chemotherapeutic agent of choice as most of our considerations (medical, scientific, and practical) for the selection of this drug [1] were still valid. Doxorubicin, like many other anthracyclines, is produced by one of the *Streptomyces* bacteria (*Streptomyces peucetius* var. *caesius*). It was discovered in the 1960s near the Adriatic Sea, which explains the source of the brand name Adriamycin, and showed significant anticancer activity [29–31]. Doxorubicin acts on the nucleic acids of dividing cells by two main mechanisms of action. Firstly, it inhibits DNA and RNA synthesis by intercalating between base pairs of the DNA strands, thus preventing the replication and transcription in rapidly-growing cancer cells. This mechanism is based on the chemistry and physics of the doxorubicin molecule (its positively charged mannose amine that binds efficiently to the negatively charged nucleic acid phosphate diester groups and the excellent fit of the drug anthroquinone planar ring structure for intercalation into the double-stranded DNA). All together, these structural features lead to high affinity of the drug to double stranded nucleic acids in a way that is not dependent on cell metabolism. The high affinity to

DNA in vivo is easily measured physically from the fast quenching of the drug fluorescence upon its binding to the nuclear DNA. The DOX primary amino group, when combined with the drug amphiphacy, makes this drug a good candidate for remote loading into pre-formed liposomes, a property which enabled the successful development of Doxil (see below, and rev. in [32–34]). Secondly, doxorubicin inhibits the enzyme topoisomerase II, preventing the relaxing of super-coiled DNA, which is an additional way for blocking DNA transcription and replication. An additional major biological effect is that doxorubicin forms iron-mediated free radicals that cause oxidative damage to DNA, proteins, and cell membrane lipids. Especially sensitive to this effect are the mitochondrial membranes due to their high level of the negatively charged phospholipid diphosphatidylglycerol (cardiolipin), to which doxorubicin has higher affinity than to other phospholipids. This higher affinity to cardiolipin (which resembles the affinity to DNA) was the basis of Rahman and coworkers' [35–38] selection of cardiolipin as their formulation's negatively charged lipid. This oxidative induced damaging effect is now considered one of the main reasons for doxorubicin's toxicities and side effects. The fact that heart muscle is enriched in mitochondria explains in part the drug's high cardiotoxicity. Standard care treatment by conventional doxorubicin is done by i.v. administration of a relatively high drug dose, in the range of 10 to 50 mg/m^2 (rev. in [39–42]).

The most important clinical consideration for doxorubicin selection as a chemotherapeutic is that this drug is considered one of the most effective anticancer drugs ever developed, and therefore it became one of the main "first line" anticancer drugs almost from its discovery and it remains so till today. It is effective against more types of cancer (including leukemias, lymphomas, and breast, uterine, ovarian, and lung cancers) than any other class of chemotherapy agents [39–41].

However, like most other chemotherapeutic drugs, doxorubicin has toxicities and side effects attached to its use. Its most dangerous toxicity is the cumulative dose-dependent cardiotoxicity (irreversible congestive heart failure), which considerably limits drug usefulness (upper accumulative dose allowed is 550 mg/m^2). Its other side effects include severe myelosuppression, nausea and vomiting, mucocutaneous effects (stomatitis, alopecia, severe local tissue damage, and hyperpigmentation of skin overlying veins used for drug injection) [40,42–44].

It is the combination of doxorubicin's clinical use for such a broad spectrum of tumor types and the very large number of patients treated with it, together with its major deficiencies of dose limiting and accumulating dose limiting toxicities that made it very appealing and attractive to us (and many others) for selecting doxorubicin as the drug of choice for delivery by liposomes.

The scientific supportive reasons were that the drug's chemistry and physicochemical properties were well established, as were drug stability [17,45], and drug ADME (Absorption, Distribution, Metabolism, and Excretion) was common knowledge [46].

Our practical reasons that led us to select doxorubicin as the drug of choice include its distinct spectral (absorbance and fluorescence) properties, which allow easy and accurate quantification of doxorubicin level, its chemical degradation, its state of aggregation, as well as changes in its local environment (pH and level of hydrophobicity). Doxorubicin's reasonably high molar extinction at 486 nm (12,500 OD/M) allows for its quantification spectrophotometrically, and when combined with diode array HPLC enables following certain doxorubicin degradation products. Another major practical advantage of doxorubicin is its long wavelength (>550 nm), and high quantum yield fluorescence emission. The use of fluorescence detection increases the limit of detection by more than 100 fold. The fluorescence excitation and emission spectra are distinguished from each other and both are sensitive to the environment (pH, salt, etc.). This enables following doxorubicin PK and BD for long periods of time [12,15,47–51].

For the above reasons, we were not the only ones to select anthracyclines as the anticancer drugs of choice. All 3 liposome-dedicated US start-up companies were competing in developing 3 different liposomal athracycline formulations. Vestar (later changing its name to Nextar) in Pasadena, CA, developed DaunoXome, which was sold by Gilead Pharmaceutical and recently acquired by Galen. The Liposome Company (TLC) in Princeton, NJ, developed Myocet (now sold by Zenous Pharma Sopherion Therapeutics). Gabizon and I with Liposome Technology Inc. (LTI) in Menlo Park, CA, developed Doxil, which was produced by the Ben Venue CMO plant in Ohio, USA, until the production site was shut down after FDA/EMA testing at the end of 2011 (see more details in Section 7 below). Doxil was sold in the USA by LTI, which in 1996 changed its name to Sequus, which was bought by ALZA. The latter was bought by Johnson & Johnson, which until the recent shortage sold Doxil (= Caelyx) worldwide.

None of the above three liposome companies (Nextar, TLC, and LTI) survived. Currently, Doxil is by far the most successful product of these three.

By 1987, for the reasons described above, it became evident to us that our OLV-DOX formulation would not become a viable product. Based on the lessons learned from this failure in the clinical trial, we came up with guidelines that were expected to overcome at least most of the deficiencies demonstrated by our OLV-DOX (see above). The combination of the 200–500 nm size distribution and the negatively charged and "fluid" liposomes resulted in fast uptake of these liposomes by the RES; (namely, there was no RES avoidance). Not less important is the fact that in the liver the liposomes were not taken up by tumors, but by the RES macrophages [14]. Finally these liposomes reached the liver with a very low level of drug due to the fast drug release upon i.v. injection [14,15]. This fast release is explained by fast dilution induced release of most of the drug load in human plasma as discussed above. In addition such large liposomes could not take advantage of the extravasation typical of nano-particulates (having long circulation time) via the porous blood vessels of the tumor tissue. This unique microanatomy can be used as the Achilles heel of the cancer tissue for selective accumulation of macromolecules and nanoparticles in the tumor tissue (also referred to as passive tumor targeting). The latter unique extravasation was described first by Matsumura and Maeda [25] and referred to as the enhanced permeability and retention (EPR) effect.

The microanatomy of tumor blood vessels and its relevance to tumor therapy were studied extensively also by Jain and coworkers [52] while Bassermann [53] described the changes in vascular pattern of tumors and surrounding tissues during different phases of metastatic growth. Jain and coworkers also point out the high interstitial pressure in tumors (but not in healthy tissues), which reduces the diffusion of low molecular weight drugs from blood vessels into the tumors, thereby reducing the therapeutic efficacy and increasing toxicity of the chemotherapy [54].

The upside of the OLV-DOX development and clinical trial was that we were encouraged and confident that if we overcame the major obstacles discovered during the clinical trial the development of a viable liposomal doxorubicin formulation will be feasible. Actually "with the food came more appetite" as we decided to look for a totally alternative liposomal doxorubicin formulation which will be able to reach most metastatic solid tumors and will not be limited to liver-residing tumors.

2. Development of Doxil

2.1. Liposomal doxorubicin: the desired product profile

We decided that in order for the liposomal doxorubicin product to become an anticancer FDA and EMA approved drug the product should be characterized by the following features:

The liposomes used should be at the nano-scale (nano-liposomes) so they will be able to take advantage of the EPR effect and extravasate from the blood vessels at the tumor into the tumor tissue. Such liposomes can be considered as a "Nano-Drug".

However going nano imposes a major challenge of achieving a sufficient level and stability of drug loading. This issue is related to

the very small (nano) volume of the nano-liposomes. For doxorubicin this may be an especially difficult obstacle to overcome due to the high dose needed to achieve therapeutic efficacy (routine treatment by i.v. doxorubicin is 10 to 50 mg/m^2 [39–42,44]).
In addition, the physicochemical properties and especially the low doxorubicin solubility are unfavorable to achieve sufficient passive loading into a 100-nm liposome's intraliposome aqueous phase. Therefore novel drug loading approaches were needed.

- In order to be efficacious the liposomes should reach the tumors loaded with a therapeutically high enough drug level.
- The drug PK and BD should be controlled by the nano-liposomes, namely the liposomal drug should demonstrate a highly prolonged plasma circulation time which is determined by the nano-liposomal carrier's prolonged circulation time in order to enable drug tumor accumulation.
- Drug should be available to tumor cells either by drug release at the tumor site or by the drug loaded nano-liposomes being internalized by the tumor cells.

Table 1 describes the requirements we found necessary to achieve therapeutically efficacious passively targeted drug-loaded liposomes for tumor treatment.

2.2. How and where Doxil was developed

To achieve all the above and a viable liposomal doxorubicin product, LTI used and combined two different novel ideas that matured into two novel technologies that resulted in two very different and independent patent families. The first one deals with the drug loading into nano-liposomes in a way that meets all the above needs, and the second, enables prolonging nano-liposomes' plasma circulation time and RES avoidance. Both technologies were not tried before.

In order to save time, all 4 aspects described in Table 1 were investigated in parallel at 4 different locations: LTI labs at Menlo Park, CA, by LTI scientists; Papahadjopoulos' lab at UCSF by Gabizon (continued later at Gabizon's lab at Hadassah University Hospital in Jerusalem); Terry Allen's lab at the University of Alberta in Canada; and at my lab at the Hebrew University-Hadassah Medical School in Jerusalem.

LTI, Terry Allen, and Alberto Gabizon/Dimitri Papahadjopoulos worked on achieving liposomes having extended circulation and RES avoidance, which due to being at the nano-range size can take advantage of the EPR effect. The EPR effect was expected to result in a selective nano-particulates extravasation from the tumor capillaries to the tumor tissue. The liposomes with prolonged circulation time and RES avoidance were termed by Dr. Frank Martin of LTI "Stealth®" liposomes and this unique property of liposomes was referred to as "Stealthness", which means unseen or unrecognized as particulates by the RES.

At the same time I and my student Gilad Haran (now a Professor at the Weizmann Institute) developed a novel remote and stable loading method of amphipathic weak bases such as doxorubicin into nano-liposomes. This method met all the expectations described in Table 1 above [48,55,56] as it enabled the Doxil nano-liposomes to reach the tumor site loaded with sufficient level of drug and drug release that are needed to achieve therapeutic efficacy in humans [1,50,51]. This loading enabled intratumoral drug release and was not a "dead end" as was the case for "Stealth cisplatin", which did not enable drug bioavailability to the tumor cells, (rev. in [1,19,20,34,50,51]).

Table 1
The requirements to achieve therapeutically efficacious passively targeted drug loaded liposomes and means to fulfill them. For relevant references to Table 1 see Barenholz [34].

	Main requirements to achieve therapeutically efficacious passive targeting of liposomes to cancer tissues	Physicochemical and biophysical solutions used to meet the requirements
	Requirements	Solutions
1.	Extended circulation time in intact form in the human plasma	Development of sterically stabilized liposomes (SSL) composed of high T_m phospholipids, cholesterol, and a lipopolymer such as ^{2000}PEG–DSPE
2.	Sufficient levels and stable loading of drug in order for long circulating nano-liposomes to reach disease site with liposomes loaded with drug at a level needed to achieve therapeutic efficacy [$t_{1/2}$ of drug release in blood should be longer than circulation $t_{1/2}$]	Use of pH or ammonium ion gradients for remote (active) loading of amphipathic weak bases or acids into long-circulating nano-liposomes
3.	Extravasation into diseased tissue (tumor)	Using small enough (<120 nm, preferably <100 nm) nano-liposomes in order to efficiently extravasate through the gaps in the tumor vasculature (taking advantage of the EPR effect)
4.	Getting active drug into target cells	Releasing drug from liposomes through selective drug leakage at site due to diseased tissue properties, or using: collapsible ion gradient, or liposomes sensitive to secretory phospholipases, or by applying physical means such as heat [thermosensitive nSSL or use of radiofrequency (RF)] or ultrasound or by internalization due to active targeting.

2.3. Remote loading of doxorubicin into nSSL to form Doxil

2.3.1. The need for remote loading

For liposome formulations designed for metastatic tumor treatment, intravenous (i.v.) administration is the only option. This requires the use of nano-liposomes for which high and stable (during storage and in circulation) loading are a must. This was not an easy task, due to the combination of very small nano aqueous volume of the nano-liposomes and the high dose of doxorubicin (~50 mg/m^2) needed to achieve therapy. To overcome these obstacles the intra-liposome drug concentration has to reach the range of hundreds of mM. However this is impossible to reach by passive loading due to the poor drug solubility. When the loading is poor, so will be the drug/lipid ratio. This means that either therapeutic levels of drug cannot be reached or therapeutic use of such liposomes will require administering very large amounts of lipids. In addition, when the loading is inefficient there is a great loss of the active agent and a need to remove unloaded drug. Therefore, the use of liposomes as a vehicle becomes inefficient as well as uneconomical.

A careful analysis of the available loading approaches that existed at that time (1986/1987) revealed clearly that the remote (active) loading approach is the only option to achieve a viable formulation, and in many cases the only way to achieve the desired intra-liposome drug concentration, usually defined as drug to lipid mole ratio [48] and rev. in [1,19,20,34].

Deamer and coworkers [57,58] were the first to demonstrate remote loading of amphipathic weak bases (such as catecholamines) by a pH gradient. This approach was extensively used by Cullis and coworkers for many amphipathic weak bases including doxorubicin [59]. Their studies on doxorubicin remote loaded into liposomes by pH gradient led to the development of Myocet by The Liposome Company (TLC) in Princeton.

We in Jerusalem used another remote loading approach, which is based on a transmembrane gradient of ammonium sulfate: $[(NH_4)_2SO_4]_{liposome} \gg [(NH_4)_2SO_4]_{medium}$ that acts as the driving force for the efficient and stable remote loading of amphipathic weak bases into preformed nano-liposomes [20,34,48,55,56]. This drug loading approach is based on the strategy of fabricating nano-liposomes that exhibit a transmembrane intra-liposome high/extra-liposome medium low ion gradient, which acts as the driving force for the remote loading of amphipathic weak base drugs. Amphipathic weak acids can be remote loaded by a similar approach in which the driving force is a transmembrane gradient of calcium acetate (rev. in [32–33]).

Since the application of this approach to Doxil, which was initiated in 1987/1988, [48,55,56,60], our remote loading approaches for loading amphipathic weak bases or acids were successfully employed for other drugs

[20,32–34,48,61,62]. The transmembrane ion gradients can be described as nano-chemical loading engines pre-fabricated into the liposomes, which then exhibit the desired pH and/or ion gradient. These nano-engines are achieved by using salts composed of either weak bases (e.g., ammonium sulfate) or weak acids (e.g., acetic acid). The degree of ionization of these compounds is pH dependent, their ionized species (i.e., ammonium and acetate) have a very low permeability coefficient and octanol-to-buffer partition coefficient and therefore they do not, or only very slowly, transverse the liposome lipid bilayer, while their un-ionized species have high permeability and octanol-to-buffer partition coefficients (exemplified by ammonia gas and acetic acid) and therefore these un-ionized species can diffuse relatively fast across the lipid bilayer and reach the intraliposome aqueous phase (rev. in [20,32–34]). The magnitude of the intraliposome high/external medium low transmembrane gradient of such ions is the driving force for remote loading, as they can be exchanged with amphipathic drugs (weak acids with the liposomal acetate ion and weak bases with the liposomal ammonium ion). The counterion of the gradient-forming ion (e.g., sulfate in the case of ammonium or calcium in the case of acetate gradient) can be selected so that it will control the state of aggregation and precipitation/crystallization of the drug-counterion salt in the intraliposome aqueous phase, thereby contributing to control the efficiency and stability of remote loading, as well as drug release rate at various temperatures [20,63,64].

It is important to note that the successful application of this nano-chemical engine benefits from the very small trapped aqueous volume of nano-liposomes (i.e., 2.209E + 5 nm^3 for a 37.5-nm radius liposome), which supports faster and higher accumulation, as well as intraliposome precipitation of drug-counterion salt in crystalline or non-crystalline (amorphous) forms.

A very important question is how to select amphipathic weak base drugs that can be remote loaded, and especially, remote loaded by transmembrane ammonium sulfate gradient. The answer to these questions requires drug classification.

2.3.2. Drug classification: relevancy to the development of drug delivery systems (DDS)

In 1986 we looked for a simple way to classify drugs by their physicochemical features in a way that will enable the formulators to predict for which DDS they are most suitable and which drug loading approach should be used to load the drug into the DDS [18,19], and in the case of liposomes as the DDS of choice, to predict if the desired drug is suited for a remote loading approach. At that time, with very little available information, we came up with an oversimplified approach and classified all agents into 3 categories based on their oil/buffer and octanol/buffer partition coefficients (Kp). Category I, which fits mainly the oil phase of emulsions and microemulsions, are molecules having high oil/buffer Kp, which are considered highly lipophilic; these molecules do not fit liposomes as their carrier. Category II molecules, having low oil/buffer partition coefficient and medium to high octanol/buffer Kp; are amphipathic in nature. Category III molecules, having very low values in both partition coefficients, are, by definition, water-soluble. For some of the molecules, those which are amphipathic weak acids or bases, the classificanon between groups II and III is pH dependent, as these molecules can be ionized and charged or non-ionized and not charged. Only at the pHs when ionized, these molecules are at least to some degree water soluble [18–20,32–34]. Although the use of octanol/water partition coefficient to determine suitability of molecules to reside in a lipid bilayer is controversial, it is well established that it is indicative of agent transmembrane diffusion rate and permeability coefficient (as discussed by Stein [65]), and therefore it is relevant to loading efficiency, loading stability, and the drug release profile [65,66].

2.3.3. Remote loading optimization

Liposomes' remote loading by transmembrane gradients is one of the best approaches to achieve the high enough drug level per liposome required for the liposomal drug to be therapeutically efficacious. This "breakthrough" which enabled the approval and clinical use of nano-liposomal drugs such as Doxil, has not been paralleled by an in-depth process understanding that allows predicting loading efficiency of drugs. In our collaboration with Amiram Goldblum and his team at the School of Pharmacy of Hebrew University, we have been applying data-mining algorithms on a databank based on our laboratory's > 20 years of liposome research experience on remote loading of 9 different drugs combined with information on basic physical and physicochemical descriptors that include, not only the partition coefficients (logP and/or logD), but also details on apolar and polar surface areas of the desired molecule (and the ratio between the two surface areas), its pKa, and logD at different pHs, as well as characterization of the liposome membrane used. All these data enabled us to build the first model that relates drug physicochemical properties and loading conditions to loading efficiency [32]. This study was a first computation-model-based attempt to enable selection of candidate molecules for remote loading and optimizing loading conditions according to logical considerations. However the small size of our "training set" (9 molecules only) forced us to use a simplified approach to database analysis using the j48 decision tree classification tool in Weka 3.4 software, validated using 10% leave-group-out (LGO) cross validation [32]. In a more recent study [33] we extended the training set to > 60 molecules used in 366 loading experiments performed in many laboratories worldwide. This extended information enabled us to develop Quantitative Structure Property Relationship (QSPR) models of liposomes' remotely-loaded drugs. Both experimental conditions and computed chemical descriptors were employed as independent variables to predict the initial drug/lipid ratio (D/L) required to achieve high loading efficiency. Both binary (to distinguish high vs. low initial D/L) and continuous (to predict real D/L values) models were generated using advanced "machine learning" approaches and fivefold external validation. The external prediction accuracy for binary models was as high as 91–96%; for continuous models the mean coefficient R^2 for regression between predicted vs. observed values was 0.76–0.79. We suggest that QSPR models can be used to identify candidate drugs expected to have high remote loading capacity while simultaneously optimizing the design of formulation experiments [33].

In addition such computation-based approaches and modeling should help in designing pro-drugs suitable for remote loading.

2.3.4. Transmembrane ammonium sulfate gradient driven doxorubicin loading into nSSL

For the remote loading of doxorubicin into nSSL we applied the transmembrane ammonium sulfate gradient under conditions that $[(NH_4)_2SO_4]_{lip} \gg [(NH_4)_2SO_4]_{med}$ (lip is the nSSL and med is the extra-liposome medium). Fig. 1 describes the overall mechanism of this loading process. The drug loading is actually a base exchange

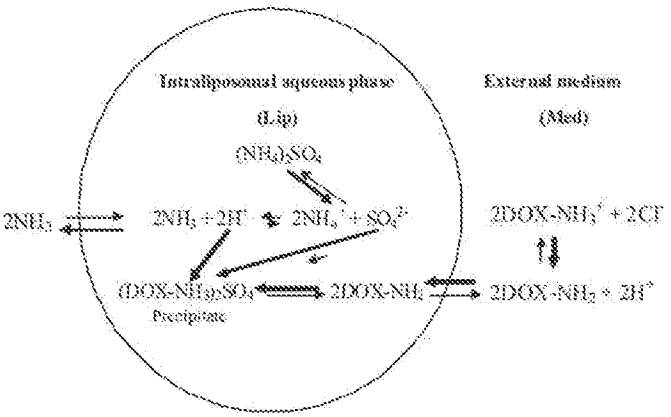

Fig. 1. Doxorubicin remote loading into nSSL exhibiting a transmembrane ammonium ion gradient. ⇔ Represents processes occurring during drug loading, → represents processes occurring during drug release.

of the amphipathic weak base drug with the ammonium ions. For doxorubicin >90% drug encapsulation was obtained.

Doxorubicin is accumulated in the intraliposome aqueous phase, where it reaches a concentration >100-fold the drug level in the loading medium (this explains why we refer to it as active loading, as it goes "against" the drug concentration gradient). Based on various spectral analyses including X-ray diffraction [48,56,61,67] almost all the encapsulated doxorubicin is in the intra-liposome aqueous phase and most of it is in the form of aggregated or crystalline $(doxorubicin)_2SO_4$ salt. The loading is actually driven by the transmembrane ammonium ion gradient.

The best way to prove the cardinal and obligatory role of the ammonium ion in the loading of amphipathic weak bases is to use the ionophore nonactine [34], which exchanges ammonium ions with potassium ions ($NH_4^+K^+$). Nonactine does not act as a proton ionophore, has no effect on a proton gradient, which is not related to the ammonium ion gradient and has no effect on remote drug loading driven by a proton gradient. In the presence of nonactine and potassium ions there will be exchange in the intraliposome aqueous phase of NH_4^+ that will be released (effluxed) while being exchanged with K^+ that will be taken up into the liposomes (influxed) so the ammonium ion gradient will collapse and the loading of amphipathic weak bases will be prevented irrespective of the counter-anion that forms the ammonium salt [being either inorganic anions (i.e., chloride, sulfate, phosphate), or organic, low molecular weight (such as citrate or glucoronate) [32,34,64] or polymeric anions (such as dextran sulfate [68], heparin sulfate or sucralfate).

Table 2 [34] demonstrates that the loading of doxorubicin is utilizing 65 to 70% of the ammonium gradient and loading releases 65 to 70% of the encapsulated ammonium. The residual transmembrane ammonium gradient is essential for loading stability. We proved that nonactine induces release of amphipathic weak bases from the nano-liposomes only if the loading was driven by a transmembrane ammonium ion gradient (Fig. 1B). If the remote loading is driven by a pH gradient which is not ammonium ion dependent, nonactine will not cause release of the amphipathic weak base (see Table 2). Nonactine therefore acts differently from the ionophore nigericin, which exchanges between H^+ and K^+. Nigericin prevents amphipathic weak base loading into liposomes for both transmembrane proton and ammonium ion transmembrane gradients (Table 2). Thus, ionophores are important analytical tools to evaluate the role of proton and ion gradients in remote loading [34]. Ionophores are also important tools to prove that the precipitation of the drug-counterion salt is not a dead end and that the drug can be released and be bioavailable and efficacious, as we proved 20 years ago for Doxil [69].

The loading stability in the case of Doxil is a result of the combination of using sulfate as the counter-anion of the ammonium cation and the liposome membranes lipid composition and temperature, which affect the level of $(doxorubicin)_2SO_4$ precipitation.

The transmembrane ammonium-sulfate-gradient-driven drug loading differs from most other remote loading approaches since it neither requires fabrication of liposomes in acidic pH, nor alkalinization of the extra-liposome aqueous phase.

Doxil is a good example of remote loading by an ammonium sulfate gradient under conditions that $[(NH_4)_2SO_4]_{lip} \gg [(NH_4)_2SO_4]_{med}$. Fig. 1 describes the overall mechanism of this loading process. For more details see [20,32,34,48,55,56,60,61,63,67].

The efficiency of loading by this method and its stability are dependent on:

(1) The large (~10^{12}) difference in permeability coefficient of the neutral ammonia (10^{-1} cm/s) and the SO_4^{2-} anion (>10^{-12} cm/s)
(2) The initial pH gradient having the $[H^+]_{lip} \gg [H^+]_{med}$
(3) The low solubility of $(doxorubicin)_2SO_4$ (<2 mM), which also minimizes intraliposomal osmotic pressure and therefore helps to keep liposome integrity
(4) The asymmetry in doxorubicin partition coefficient (K_p)
(K_p lip/external med > K_p lip/intra lip med)

Table 2
Characterization of transmembrane ammonium sulfate and pH gradients in ~100 nm nSSL before and after doxorubicin remote loading [1].

Property	Magnitude	Transmembrane proton gradient (ΔpH)
Transmembrane ammonium ion gradient determined by ammonium electrode	$[(NH_4)_2SO_4]_{liposome}/[(NH_4)_2SO_4]_{medium} \geq 1000$	
Intraliposome aqueous pH determined before DOX loading using pyranine preloaded in liposomes	<5.25, being out of the range of the measurement of pH range for pH determination by pyranine (pH 5.3–8.0)	
Determination of transmembrane pH gradient (inner low/outer high) as ΔpH		
± Before DOX loading		
By acridine orange (AO) distribution	96.4% by AO distribution into nano-liposomes	ΔpH ≥ 3.0 pH units
By ^{14}C methylamine (MA) distribution	87.5% by ^{14}C MA distribution into nano-liposomes	ΔpH ≥ 3.0 pH units
+ Nigericine	2.0% by AO distribution into liposomes	ΔpH = ~0.0
	3.0% by ^{14}C MA distribution into liposomes	ΔpH = ~0.0
+ Nonactine	4.0% by AO distribution into liposomes	ΔpH = ~0.0
	3.0% by ^{14}C MA distribution into liposomes	ΔpH = ~0.0
DOX loading		
% DOX loading	≥90.0%	
ΔpH after DOX loading	30–35% by ^{14}C MA distribution into liposomes	~1.0 pH units
ΔpH After DOX loading by ^{14}C MA distribution:		
+ Nonactine	2% by ^{14}C MA distribution	ΔpH = ~0.0
+ Nigericin	2% by ^{14}C MA distribution	ΔpH = ~0.0

Stability, size distribution, level of free drug, and ΔpH remain unaltered for more than 6 months storage at 4 °C. ΔpH for both % ^{14}C MA and % AO are based on calibration curves. For more details see [34].

(K_p oct/external med > K_p oct/intra lip med)
[18,20]

K_p is a partition coefficient between the two phases defined in the parentheses; lip = liposome membrane, med = aqueous medium either external or intra-liposomal, oct = bulk octanol phase.

The asymmetry in DOX K_p means that the K_p of DOX in the extra-liposomal medium supports influx in a direction opposite to the ammonium sulfate gradient (namely, into the liposomes), while the K_p of DOX in the intraliposomal aqueous phase acts to reduce partition into the membrane, thereby reducing the desorption rate (k_{off}). The reduction in DOX K_p in the intra-liposomal aqueous phase is driven by the ammonium sulfate remaining inside the intraliposomal aqueous phase after DOX remote loading. Therefore, it seems that ammonium sulfate plays a multifactorial role in the remote loading and retention of the loaded drug in the liposomes. For Doxil the interplay between the above four points, when combined with Doxil membrane composition and liposome size, determines liposome performance.

The huge difference in the permeability coefficients (Pd) between the neutral ammonia (Pd = 0.12 cm/s) and the sulfate anion (Pd < 10^{-12} cm/s) combined with the efficient precipitation (gelation) of doxorubicin sulfate in the intraliposome aqueous phase and the low octanol/intraliposome aqueous phase partition coefficient as well as the (above described) asymmetry of DOX K_p all play a major role in the success of Doxil. The type (low molecular weight inorganic or organic, or polymeric) and valency of counter anion that forms the ammonium salt can be used to control the release rate of the liposome remote-loaded amphipathic weak base [20,34,48,64].

The $(DOX)_2SO_4$ long and fiber-like crystals are clearly shown in the Doxil cryo-TEM (Fig. 2A). Doxorubicin intraliposome nanocrystalization does not occur when the bivalent sulfate counterion was

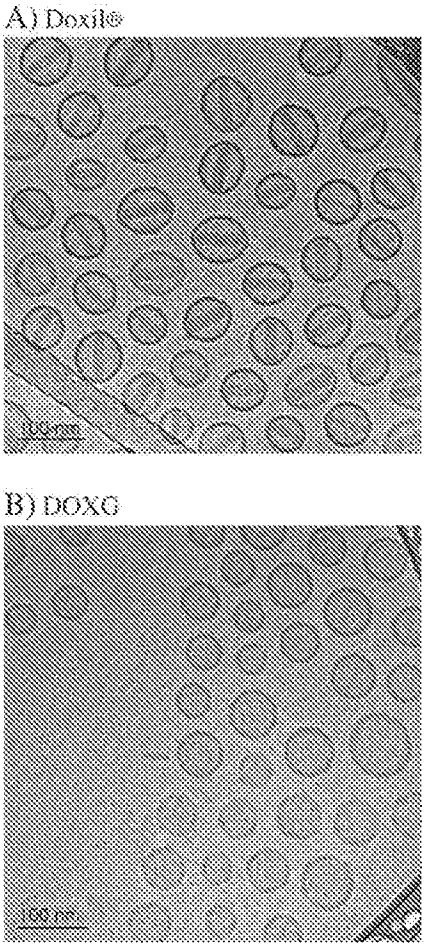

Fig. 2. Comparing cryo-TEMs of (A) commercial Doxil, which is doxorubicin sulfate remote loaded pegylated nano-liposomes and (B) DOXG, which is doxorubicin glucuronate remote loaded pegylated nano-liposomes. Doxorubicin concentration in both cases is 2 mg/mL. For more details on DOXG see [70].

replaced with the monovalent glucuronate counterion (DOXG Fig. 2B). The $(DOX)_2SO_4$ crystal formation is also strongly supported by small X-ray scattering (SAXS) measurements [67]. Accordingly, the Doxil demonstrates a unique reflection at 2.7 nm, which is identical to what was obtained for 30 mg/mL doxorubicin in either ammonium or sodium sulfate (250 mM each). This suggests that this unique reflection is a result of crystallization of $(DOX)_2SO_4$. No such discrete reflections were observed for 30 mg/mL doxorubicin in 250 mM ammonium chloride [67], or for doxorubicin glucuronate ([70] and Fig. 2B). Calculations made suggest that the $(DOX)_2SO_4$ gel is in the form of one-dimensional rods, in agreement with the cryo-TEM. Doxorubicin aggregation is also supported by the increase in 550/470 nm absorbance ratio [48]. Such intraliposome crystallization which results from transmembrane ammonium ion remote loading is not unique to doxorubicin, as the antioxidant nitroxide amphipathic weak base tempamine also shows intraliposome precipitation in the presence of sulfate as a counterion while no precipitation occurs in the presence of glucuronate as a counter-ion. However not all amphipathic weak bases behave like doxorubicin and tempamine; for example, the amphipathic weak base local anesthetic bupivacaine in the form of sulfate salt does not crystallize or precipitate [71,72]. The Doxil cryo-TEM above (Fig. 2A) demonstrates that $(DOX)_2SO_4$ rods touch the vesicle membrane, thereby forcing a vesicle shape change from spherical to non-spherical. Such shape changes do not occur for DOXG nano-liposomes (Fig. 2B), which do not show the presence of intra-liposome drug crystals and remain spherical in shape. However in spite of Doxil's shape change the Doxil liposome's membrane is robust enough to keep the liposome integrity as is indicated from the ability of the Doxil to withstand very high centrifugal forces [67]. This shape change of Doxil may be one of the factors leading to activation of complement by Doxil (see Section 3.3. and [22,23]). For doxorubicin glucuronate remote loaded pegylated nano-liposomes (DOXG) see Section 4 and [70].

Another issue, which may affect therapeutic efficacy and so far was neglected (although it may be highly relevant to drugs such as doxorubicin), is their tendency to self-aggregate at low drug concentrations (reviewed in [17]), forming oligomers of various (mostly low) mer number. Massive precipitation which can be observed by the naked eye occurs only at much higher doxorubicin concentration. The latter is highly dependent on the doxorubicin counter-ion, from >100 mM for glucuronate to 2 mM for sulfate [34,70]. The oligomerization at low doxorubicin concentration results from the stacking of the planar aromatic rings of the anthracycline due to interaction between the π electrons of the planar rings. It occurs for all doxorubicin salts. This self-aggregation is facilitated by increasing ionic strength. Doxorubicin dimers appear already at 1 μM and aggregates' size increases upon increasing doxorubicin concentration [17]. The effect of such oligomerization on therapeutic efficacy is not yet clear. However, based on simple geometric considerations, it is obvious that non-monomeric doxorubicin cannot interact with DNA in the same way as monomeric, and the exact location between the two DNA strands should differ (rev. in [17]). Therefore, the form (monomer versus oligomer) in which the drug is internalized by the tumor cell may be an important factor in drug therapeutic efficacy, with oligomers being less efficacious. The doxorubicin when released from intact Doxil would be released in its uncharged non-protonated form, but in the plasma and the interstitial medium it will be protonated and will form a chloride salt. Due to the large dilution it should be mostly monomeric and/or aggregates of low mer number and therefore the drug should retain close to full biological activity. However the question of what will be the biological activity of doxorubicin taken up by cells as part of intact Doxil in vivo still remain unanswered.

To sum up, while it is clear that the novel development of remote (active) loading driven by the transmembrane ammonium sulfate gradient for the doxorubicin was a breakthrough and one of the main reasons that enabled successful clinical use of Doxil (and its approval by regulatory agencies worldwide) there are still some open questions related to this loading approach which remain unanswered.

2.4. The role of drug release rate (k_{off})

The results of liposome loading when combined with liposome size, structure, lipid composition, and site of injection will determine the liposome bio-fate and rate of drug release in plasma and or tissues reached by the liposomes [18,19]. For example, for i.v.-administrated liposomal drug formulations, only when the drug release (determined by k_{off}) is slower than the liposome clearance (k_c) will the liposome control the drug pharmacokinetics and bio-distribution. When $k_{off} > k_c$, then the ratio k_{off}/k_c is a measure of the rate of drug release in vivo. Controlling this ratio is obligatory to achieve controlled drug release in blood or in the tissues reached by the liposomes. Therefore, this ratio also affects therapeutic efficacy of the liposomal drug. For drugs of fast clearance, when $k_{off} \gg k_c$ the benefits of use of liposomes for drug delivery will be limited for drug solubilization and dispersion but minimal or none for achieving beneficial bio-distribution and controlled drug release. In such cases the performance of the liposomal drug will be similar to that of the free drug. This was exemplified by our first generation "failed" OLV-DOX formulation (see part 1 above). An efficient and functional way to test the release rate is a functional test such as a cytotoxicity test of doxorubicin measuring its IC_{50} in cell culture (in vitro). This was well documented by Horowitz et al. [69], where it was shown that Doxil has about a 2-order-of magnitude higher IC_{50} (lower cytotoxic activity) than free doxorubicin, while as described above and reviewed in Barenholz [1], the IC_{50} value of our failed OLV-DOX was similar to the low IC_{50} value of free doxorubicin. The latter suggests a fast drug release upon the large dilution that occurs in the in vitro

test. This comparison between OLV-DOX and Doxil indicates that Doxil's robust drug retention is the reason for Doxil's high IC_{50} [69].

However, regarding therapeutic efficacy the opposite (a very low k_{off}) is as "bad" or even worse. Namely, when k_{off} is too slow and there is no liposome uptake by the target cells there will be no therapeutic efficacy even if the loaded liposomes will reach and accumulate in the target tissue very efficiently, as the free (bio-available) drug concentration at the target tissue will be too low to have therapeutic efficacy. This case is well exemplified by sterically stabilized cisplatin liposomes (Stealth cisplatin, which do not release the cisplatin [20,73–75]).

Meeting all the requirements of loading of the nano-liposomes, by itself would not be sufficient to achieve the passive targeting of enough of these nano-liposomes to the tumor site. To answer this need LTI developed the PEGylated liposomes (see below). Using doxorubicin remote loaded PEGylated nano-liposomes (Doxil®) enabled achieving a doxorubicin circulation half-life time in humans of ~90 h and doxorubicin presence in the human circulation of > 350 h [50,51]. The process leading to the development of PEGylated liposomes is described below.

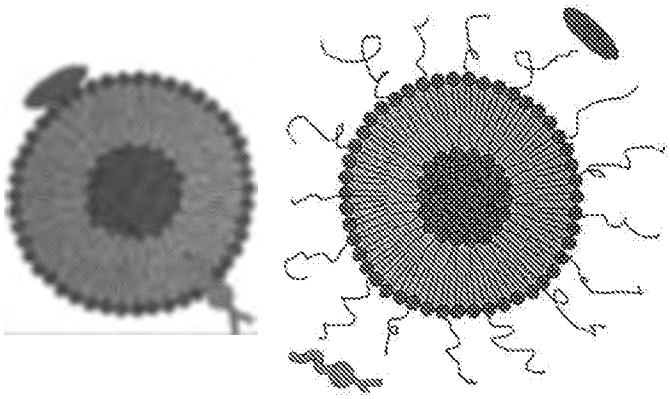

Fig. 3. A cartoon showing a comparison between a conventional liposome (left) and a sterically stabilized (PEGylated) liposome (right). The latter shows lack of insertion of plasma opsonins into its membrane. Courtesy of Dan Lasic.

2.5. Prolongation of nano-liposome plasma circulation time

Terry Allen at the University of Alberta was the first one to describe long circulating liposomal formulations. She included GM_1 ganglioside in the liposomes, which in mice is acting act as a "steric stabilizer". This means that in mice the inclusion of GM_1 reduces dramatically liposome uptake by the RES, leading to RES avoidance. This led to a prolonged plasma circulation time for the liposomes [76,77]. In 1986 LTI started to support Terry Allen's research on this topic. One year later Alberto Gabizon (in Dmitri Papahadjopoulos' Lab at UCSF) used hydrogenated phosphatidylinositol (abbreviated as HPI) as a steric stabilizer lipid [13]. LTI scientists tried another approach to achieve long-circulating liposomes of various lipid compositions that differed from those studied by Allen and Gabizon. They synthesized and studied novel lipids that were not similar to the well established natural lipid species, and instead they used pegylated phospholipids which are actually lipopolymers [78–81]. All these three groups (that were related to LTI) worked in parallel on nano-liposomes of different lipid compositions focusing on small unilamellar liposomes with narrow unimodal size distribution having a mean size of ~100 nm. These nano-liposomes were prepared by medium pressure extrusion using polycarbonate filters with defined pore size [82] licensed by UCSF to LTI. These 3 labs used different lipid compositions in order to achieve the same goal of extended circulation time, RES avoidance, and intra-tumor accumulation. Terry Allen achieved it by inclusion of sphingomyelin to rigidify the liposome membrane and GM_1 ganglioside as a steric stabilizer. Alberto Gabizon was using hydrogenated phosphandylinositol (HPI) as a steric stabilizer, which was included in nano-liposomes composed of "solid" high-T_m lipids mixed with cholesterol [13], while LTI scientists started working with DSPE-PEG; this was Annie Yau-Yang's idea combined with Carl Redmann's chemical synthesis [78,79], rev. in [80,81]. LTI was not the only group to work with PEG-DSPE; at the same time, Vladimir Torchilin and Leaf Huang and their teams joined forces and worked on it too [83], as well as Gregor Cevc's lab in Munich [84]. A comprehensive review on PEGylated liposomes is given in many of the papers in *Stealth Liposomes* [85], a book edited by Dan Lasic and Frank Martin.

The inspiration and motivation to start working with PEGylated lipids like PEG-DSPE came probably from pioneering research in the 1970s by Frank Davis, Abraham Abuchowski, and colleagues who foresaw the potential of the conjugation of polyethylene glycol (PEG) to proteins [86]. Abuchowski founded Enzon Inc., which brought three PEGylated drugs to the market. Various length (350–50,000 Da) chains of PEG polymer are available. Low molecular weight drugs were also PEGylated. However, the main PEGylated products so far are a few proteins and one liposomal formulation, Doxil® (the topic of this review article). For peptides and proteins (including antibody fragments), relatively large PEG polymers of > 5000 Da were mainly used. It was found that pegylation helps to improve safety and efficacy as well as to reduce the immunogenicity of many therapeutics [87,88]. The suggested mechanism by which PEGylation "works" is that it is a result of the alterations it produces in the physicochemical properties of the molecule to which the PEG residues are covalently attached. These may include changes in level of hydration, conformation, electrostatic binding, and hydrophobicity/hydrophilicity balance. Increasing the level of hydration of the covalently attached PEG (3 to 4 molecules of water per 1 ethylene oxide oxygen [89,90]) induces changes in structure and leads to increase in the PEG moiety's volume and bulkiness. Altogether, this results in "steric stabilization" which reduces nonspecific protein–protein interaction and nonspecific protein–cell interaction (Fig. 3). These physical and chemical changes increase systemic retention of the therapeutic agent. Also, they can influence the binding affinity of the therapeutic moiety to the cell receptors and can alter the absorption and distribution patterns. PEG polymer has only 2 reactive OH groups (one at each end of the PEG molecule) and, in order to prevent the PEG from inducing intra- and inter-cross linkages, one of these hydroxyl groups is methylated so that the proteins and lipids are PEGylated by methoxy-PEG (mPEG).

The success of PEGylated proteins was the driving force for the successful development of Doxil® as the first FDA-approved liposomal drug and nano-drug (November 17, 1995). In lipids, PEG chains of 350 to 15,000 Da were tried (equivalent to 8 to 334 ethylene oxide units), and various considerations such as the metabolism of the PEGylated lipids and the rate of secretion via the kidneys were used in the decision which PEG length to select for lipid PEGylation and to the preferred choice of a 2000 Da PEG residue.

2.6. Selection of PEGylated nano-liposomes as the basis of Doxil

Each of the labs described above that worked on long circulating liposomes has its publications and patents on its unique liposome formulations. In the early 1990s it became evident that for various scientific and practical reasons (availability, cost, species specificity, etc.), the GM_1-ganglioside-based formulation was excluded from the race [81,91]. At LTI, HPI and PEG-DSPE remained in the race. In order for LTI to decide which of the two lipids will be the one to use in humans, we performed in 1991 a critical comparative PK study in Beagle dogs [92]. Dog was selected due to its much larger plasma volume (~500 mL), which resembles humans much better in evaluating "dilution induced drug release" [14,15] than small rodents with their very small plasma volume (and therefore no major dilution).

The Beagle dog doxorubicin PK study clearly demonstrated the superiority of Doxil, which was based on a 2000 Da PEG-DSPE as a steric stabilizer, to similar nano-liposomes based on HPI as a steric stabilizer, although both liposomal formulations were much superior to free doxorubicin [92].

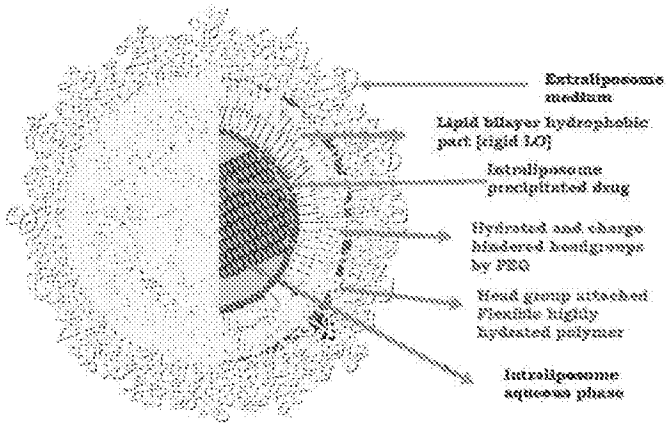

Fig. 4. A cartoon of Doxil® = PEGylated nano (<100 nm) unilamellar liposome. It is based on cryo-TEM, SAXS, WAXS, DLS, compressibility, and doxorubicin absorbance and fluorescence [48,61,67,90].

Using mice peritoneal macrophages (obtained from the ascitic fluid of mice treated with thioglycolate) in vitro, Doxil liposomes show only 40% uptake of liposomes of identical size distribution and lipid compositions but lacking the 5 mol% PEG-DSPE [93]. This reduction in liposome uptake is in direct correlation with the increase in plasma circulation time. The increase in mole% of PEG-DSPE can further reduce macrophage uptake and probably the nSSL circulation time [73,93,94]. For more details on the physicochemical effect of PEG-DSPE on nano-liposomes see [47,95].

2.7. Doxil — each component matters

In Doxil each component matters (Fig. 4) and contributes to the optimized performance!!! Doxil is an excellent example to demonstrate the essential and obligatory role of lipid physical chemistry, lipid biophysics, and nano-technology in the success of liposome-based drugs.

A calculation based on the concentration of Doxil components and on liposome size reveals that 1 mL of the commercial Doxil dispersion contains 2.3×10^{14} liposomes and each liposome contains ~10,000 molecules of doxorubicin, above 95% of which is in the crystalline phase.

3. Doxil performance in humans

3.1. Pharmacokinetics and passive targeting to tumors

In our Jerusalem 1991–1994 "first in man Doxil clinical trial" Doxil demonstrated high and selective tumor localization, published in *Cancer Research* [50]. The data (presented in Fig. 5) are the first proof for the EPR effect induced in tumors by passive targeting in humans [50]. The accumulation of Doxil in humans' tumors was further supported by direct fluorescence microscopy of patient biopsies by Gabizon and coworkers [96].

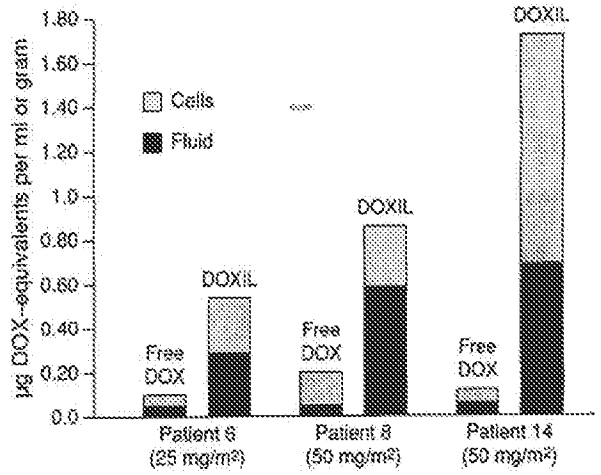

Fig. 5. Doxorubicin levels in patients' tumor biopsies, comparing free DOX and Doxil [100].

The above-cited Jerusalem pilot study includes 53 courses of Doxil (average of 3 per patient, spaced 3 to 4 weeks apart). It was aimed to determine in cancer patients the plasma pharmacokinetics and accumulation of doxorubicin in malignant effusions when given intravenously as Doxil, compared to free (non-liposomal) doxorubicin administered in what was considered then as "standard care". This study clearly showed much higher levels of doxorubicin both in tumor cells and tumor interstitial fluids after Doxil administration than after free doxorubicin administration. Using the cationic ion exchanger Dowex-50 [97,98], we found that more than 98% of the plasma doxorubicin after Doxil i.v. administration is liposome associated. Pharmacokinetics was determined for 25 and 50 mg/m^2 doxorubicin. The plasma elimination time of Doxil followed a bi-exponential curve, with half-lives of 2 and 45 h (median values), most of the dose being cleared from plasma under the longer half-life. A large difference in volume of distribution was also found (4 L for Doxil versus 254 L for free doxorubicin). Similarly, doxorubicin derived from Doxil showed a much slower rate of clearance (0.1 L/h for Doxil vs. 45 L/h for free doxorubicin).

The species of doxorubicin metabolites derived from Doxil doxorubicin in patients' urine were identical to those in patients injected with free doxorubicin; however the overall daily urinary excretion in the Doxil group was significantly reduced. Most encouraging are the results on the levels of drug at the malignant effusions, which were 4 to 16 times higher than after free doxorubicin administration. In addition, after doxorubicin administration, drug levels in the tumors peaked between 3 and 7 days post administration, which means the exposure of the tumor cell to the drug is much longer and at much higher levels than after free doxorubicin administration [50,51,99]. These data are in excellent agreement with our preclinical studies and indicate that stable remote loading of doxorubicin into long-circulating nano-liposomes serves well the objective of passive targeting of doxorubicin to tumors (rev. in [99]).

For further information on the superiority of the pharmacokinetic (PK) performance of Doxil, see [51]. That review summarizes the PK profile in humans at doses between 10 and 80 mg/m^2. The PK has one or two distribution phases: an initial phase, with a half-life of 1–3 h, and a second phase, responsible for most of the clearance, with a half-life of 30–90 h. The AUC after a dose of 50 mg/m^2 is approximately 300-fold greater than that with free drug. Clearance and volume of distribution are drastically reduced (at least 250-fold and 60-fold, respectively). These studies indicate the importance of utilizing the distinct pharmacokinetic parameters of pegylated nano-liposomal doxorubicin in dose scheduling.

3.2. Doxil bio-fate and mechanism of action

Animal data that come from many labs suggest that Doxil extravasates and accumulates as intact liposomes in tumors having "leaky" vasculature. For nano-long-circulating particulates the extravasation is probably the rate-limiting step of accumulation at the tumor tissue. Inside the tumor tissue Doxil liposomes "move" by convection and distribute through the tumor. This is the EPR effect [25,26,54,101]. Free doxorubicin, on the other hand, distributes into all tissues of the body, with tumor interstitial hypertension slowing down diffusion of the free drug from the vasculature to the tumor tissue [101,102]. The overall effect for doxorubicin administered as Doxil is that drug levels at the tumor tissue are higher than for doxorubicin administered as a free drug. In the case of Doxil almost all the plasma circulating drug is measured as liposome associated drug, in which form it also reaches the tumor. However, if there is insufficient drug release at the tumor site there is no efficacy in spite of the superior tumor localization and reduced toxicity. This was well documented in animals [49,75,103,104] and in humans [105,106] for Stealth

cisplatin, which is identical to the Doxil liposome in size distribution and lipid composition but is passively loaded with cisplatin [73,74].

However, this is not the case for Doxil, for which substantial therapeutic efficacy was shown in many tumor animal models and in humans (rev. in [1,50,99,133], and references listed therein). In the case of Doxil, doxorubicin release can be assessed by determining the presence and level of doxorubicin metabolites using HPLC [50]. The reason is that doxorubicin is metabolized only intracellularly [107], therefore the presence of doxorubicin metabolites in the cancer tissue indicates that drug was released from the liposomes and taken up by the cells where it is processed. Already in our Jerusalem 1991 Doxil FIM study [50], we demonstrated by HPLC analysis of human plasma, urine, and tumor effusions, that while in plasma the level of metabolites is very low and almost all the drug there is liposome associated, this is not the case for the urine and tumor effusions, where a relatively large fraction of the drug was transformed into doxorubicin's normal metabolites [50]. However, the mechanism of drug release and its internalization by the tumor cells is not yet known. Two different mechanisms can be suggested to explain Doxil's doxorubicin internalization into tumor cells in vivo: (i) intact Doxil liposomes uptake by cells, followed by intracellular drug release, or (ii), the doxorubicin is released in the tumor interstitial fluid from where it is taken up by the cells as a free drug. The contribution of the intact Doxil uptake by tumor cells must be minimal, as intact cisplatin Stealth nano-liposomes, which have similar lipid composition and size distribution as Doxil, does not show uptake of cisplatin by tumor cells and therefore they lack therapeutic efficacy (see references above). Therefore we are left with the second option of tumor cells' uptake of drug which was released in the tumor interstitium. Factors leading to doxorubicin release from Doxil may include collapse or partial collapse of the ammonium sulfate gradient and/or the destabilization of Doxil liposomes by phospholipases that hydrolyze the liposome phospholipids (see review by Mouritsen and Jørgenson [108]), thereby enabling faster doxorubicin release.

However, there are two major objections to the latter phospholipase-related drug release explanation. The first one is the fact there is no drug release in vivo from Stealth cisplatin which is identical in size and lipid composition to Doxil; the second is that the presence of cholesterol in the liposome membrane inhibits drastically phospholipase activity [108]. Therefore, we are left with the default, which suggests that the collapse of the ammonium sulfate gradient plays a more major role in doxorubicin release of Doxil in vivo. However the latter assumption is as yet unproven and its proof requires further in-depth investigation.

3.3. Doxil tolerability

A detailed report on Doxil (Caelyx) preclinical toxicology was summarized by Working and Dayan [133]. In our 1994 *Cancer Research* publication [50] we demonstrated that in humans overall Doxil is well tolerated and shows a distinct superiority over "standard of care" doxorubicin in most evaluated side effects. This was recently updated by Solomon and Gabizon's [99] review. In general, Doxil improves to a large extent patient daily compliance, and of special importance is the dramatic reduction of cardiotoxicity (when compared to standard care) which allows increasing the accumulated dose and thereby extending treatment duration. Recently a novel major immune modulatory effect of Doxil was discovered in patients. Treatment of ovarian cancer with the standard care first-line platinum-based chemotherapy has a very high record of efficacy at the beginning of the treatment. However, the majority of patients at advanced stages eventually have evidence of recurrent disease and are then treated with carboplatin doublets. Upon re-treatment, >15% of patients show severe HSRs to the carboplatin treatment. These reactions were fatal in a number of instances, and are mediated by IgG to platinum, explaining some cross-reactivity with cisplatin, and rarely with oxaliplatin. However, when carboplatin is given together with Doxil these reactions were not observed [109]. Moreover, of great interest is the finding in a recently published randomized trial that the doublet carboplatin/Taxol combination is significantly more often associated with carboplatin reactions than the doublet carboplatin/Doxil (Caelyx) combination [110]. Namely, Doxil seems to have an immunosuppressive effect that prevents/reduces the secondary (IgG-mediated) hypersensitivity response to carboplatin.

However, in spite of overall tolerability superiority of Doxil over doxorubicin, two side effects not typical of what is observed for the free drug standard of care treatment were observed for Doxil. The first and more dominant one results in grade 2 or 3 of desquamating dermatitis and is referred to as Palmar Plantar Erythrodysthesia (PPE) or "foot and hand syndrome". The PPE, which was already demonstrated in our first FIM study [50] and recently reviewed by Solomon and Gabizon [99] shows up as redness, tenderness, and peeling of the skin. The prevalence of this side effect limits the Doxil dose that can be given as compared with doxorubicin in the same treatment regimen. One of the bad aspects of the PPE is that its severity increases with dose and was more pronounced for 3-week intervals than 4-week intervals between treatments. So far there is no complete solution to this effect, except the above increase in interval between treatments [99]. The second effect is an infusion-related reaction that shows up as flushing and shortness of breath; it is a unique adverse immune phenomenon that Doxil, like many other nano-systems, can provoke. It is actually a complement activation-related pseudo-allergy (CARPA). CARPA is an acute hypersensitivity, or infusion reaction and called thus because of the causal role in its patho-mechanism of complement activation instead of IgE binding [111], rev. in [22,23]. CARPA can be reduced by slowing the infusion rate and by premedication. More details on Doxil tolerability can be found in [50,99], Doxil homepage (www.doxil.com), and drugs online (www.drugs.com/pro/doxil.html). There is a large probability that reducing these two side effects and especially reducing or overcoming the adverse PPE effect may improve the overall Doxil performance and extend its application.

3.4. Doxil therapeutic indications

This review will not discuss in detail Doxil clinical performance, a topic covered extensively in many publications and reports. Good starting points are the review by Solomon and Gabizon [99] and the two updated highly relevant websites, Doxil homepage (www.doxil.com) and drugs online (www.drugs.com/pro/doxil.html). The Doxil item in Wikipedia entitled "Clinical pharmacology of liposomal anthracyclines: focus on PEGylated liposomal doxorubicin" is also helpful. As of today (March 6, 2012), Doxil showed 501,000 results on Google search, 7590 on Google Scholar and 46,166 on PubMed search.

A summary of indications for Doxil approved by the U.S. FDA and/or European Medicines Evaluation Agency (EMA), with approval year is given below.

- AIDS-related Kaposi's sarcoma: superior efficacy over former conventional therapy (1995).

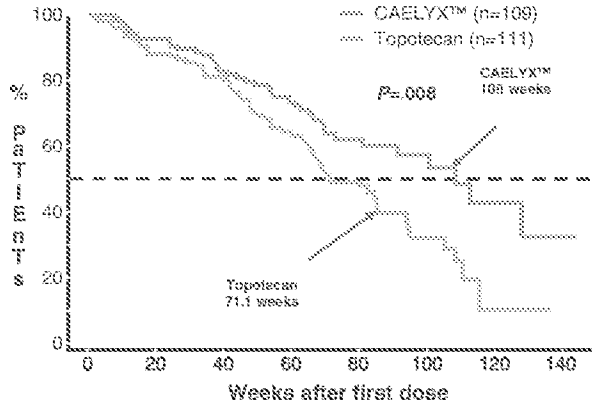

Fig. 6. Caelyx™ (=Doxil) is superior to topotecan in ovarian cancer [1].

- Recurrent ovarian cancer: superior efficacy and improved safety profile over comparator drug (topotecan) (1998), as demonstrated first by Gordon et al. [112] (Fig. 6).
- Metastatic breast cancer: equivalent efficacy and reduced cardiotoxicity compared to free doxorubicin (2003).
- Multiple myeloma: equivalent efficacy and improved safety profile compared to free doxorubicin combo. Superior efficacy in combination with bortezomib over single agent bortezomib (2007).

In addition, regarding cardiac function, Doxil demonstrates major reduction of cardiotoxicity as compared to free doxorubicin in all settings tested (2000).

4. Doxil take home lessons and what will next-generation Doxil-like liposomes look like?

In summary, the anticancer nano-drug Doxil shows superiority to free doxorubicin (standard of care) clinical performance in a variety of neoplastic conditions due to its unique EPR-related pharmacokinetics and bio-distribution, which reduce side effects (especially important is the large reduction in cardiac toxicity) and improve overall patient compliance and quality of life. These, when combined with the way the doxorubicin is remote loaded into the long-circulating nano-liposomes, improve (in certain tumors such as ovarian cancer) the antitumor therapeutic efficacy when compared with conventional doxorubicin. This explains why, of the >12 liposomal drugs approved for clinical use [113], Doxil has the most extensive clinical use.

Based on Doxil success, various novel drug formulations including modified Doxil, or other nano-drugs based on similar Stealth liposomes loaded with other drugs or with drug combinations are now at different stages of development. These novel nano-drug formulations should have reduced (or lack) the side effects of Doxil, PPE and acute infusion reactions. One approach to reduce these is to slightly reduce the half-life of the liposomal doxorubicin by replacing the sulfate counterion of the ammonium used for the remote loading with glucuronate. The use of glucuronate which has a permeability coefficient similar to sulfate but does not induce intra-liposome drug precipitation, results in somewhat shorter circulation time of the doxorubicin, but without loss of therapeutic efficacy in tumor-bearing mice, [34,70]. This relatively small but distinct effect on the PK is expected to lower accumulation of doxorubicin in the skin, thereby reducing severity of PPE. Other ways to extend and improve nano-liposome-based anticancer therapy and to have a better control of drug release (rev. in [34]) can be achieved by (a) the use of external means such as hyperthermia [114,115] or focused ultrasound [75,103,116]; (b) the use of a drug combination by remote loading of two drugs that act synergistically in one liposome [117,118] and (c) the use of a combination of two different treatment modalities, such as Doxil and interleukin-2 (IL-2), in liposome-based immunotherapy [119]. The concept of activating the host immune mechanisms to destroy residual tumor cells after chemotherapy has long been proposed. The use of the DOX-IL-2 combination stems from the fact that doxorubicin, when administered as Doxil, is much less toxic to the immune system than free drug, and therefore IL-2 delivered in liposomes following Doxil is highly efficacious. The idea behind this chemo-immuno treatment combination is that the Doxil will take care of most of the tumor burden while the immunotherapy elicited by the IL-2 will activate the intact immune system enabling it to kill the residual tumor cells [119]. The use of liposomal IL-2 results in lower toxicity of the IL-2 and prolongation of IL-2 circulation time without loss of its potency [120,121].

A very promising approach is the one used recently by Jain and co-workers [122]. Accordingly, losartan, which inhibits collagen I synthesis was used to modify the interstitial tumor environment, leading to increase in Doxil (and other nano-particulates) accumulation in tumors, thereby increasing Doxil therapeutic efficacy. For more options that were used or proposed to improve Doxil performance see Solomon and Gabizon [99].

The story of Doxil development carries two important messages. The first one is that Doxil's successful development opens the way to major improvement in tumor therapy and it served as a gold standard in the new field referred to as Nano-Medicine. The second one is that development of such a complex drug system requires having a highly multidisciplinary team that can deal in an integrative way with the expertise needed [1,19,34,100,123]. Not less important is the recognition that the understanding and optimal utilization of physicochemical principles are crucial to the successful development of such a complex drug product.

5. Doxil historical perspectives

Pre-Doxil era (liver passively targeted by liposomal doxorubicin referred to as OLV-DOX)

1979	Gabizon and Barenholz started their basic research on liposomal doxorubicin
1984	First clinical trials with liposomal doxorubicin (OLV-DOX which differs to a large extent from Doxil)
1985	LTI licensed the OLV-DOX technology and Barenholz and Gabizon 1990, 1991 I.P. on OLV-DOX
1987	Clinical trial of OLV-DOX failed
1988	Barenholz developed and Yissum, R&D Company of the Hebrew University of Jerusalem, Israel patented new concept of doxorubicin remote loading, the basis of Doxil (Barenholz and Haran 1993, 1994); patents were licensed to LTI
1989	LTI patented the Stealth concept and registered Stealth®
1989	Gabizon and LTI start to develop sterically stabilized (Stealth) liposomes
1989	LTI, Gabizon, and Barenholz start Doxil® development
1991–1992	Doxil "First in man" (FIM) clinical trial in Jerusalem
1994	Gabizon and Barenholz first major publication on Doxil clinical trials (*Cancer Research* 1994)
1995	(November 17) Oncologic Drugs Advisory Committee (ODAC) recommended FDA approval of Doxil
1998	First Doxil sales in USA and Europe
2010	(March) US patent expired

6. Doxil I.P. aspects

It is important to note that Doxil® was based on two families of patents. However, there is no direct patent on Doxil. One family covers the transmembrane-driven remote loading of amphipathic weak bases such as doxorubicin [55,56], while the second deals with contribution of the lipopolymer PEG-DSPE as a lipid component of liposome membrane for prolongation liposome circulation time and RES avoidance [78].

It took ~7 $^{1}/_{2}$ years from the submission of these two families of patent applications in 1988/1989 until Doxil's approval in November 1995. Remote loading patents were extended in the USA till March 9, 2010, and therefore Doxil enjoyed 14 years of patent protection in the USA. Currently in most countries there is no I.P. to protect Doxil®.

7. Generic doxorubicin in liposomes (Doxil-like)

The patent protection of Doxil® in the USA has been over since March 2010, and Doxil/Caelyx is selling well (over $600 million annually), so how come there is still no generic PEGylated liposomal doxorubicin (PLD)-like product approved by the FDA or EMA?

In addition to the complexity of FDA approval of generic Doxil, the current situation is even more complex. Ben Venue Laboratories (the sole supplier) has stopped Doxil production because of FDA-cited GMP deficiencies at their facilities. A shortage of Doxil has persisted since summer of 2011; in November there were reportedly ~2700 people in the US alone on a waiting list for Doxil treatment. Ben Venue spokesman Jason Kurtz said that the company does not have a time frame for when manufacturing will resume (for more information on Doxil shortage, see http://www.outsourcing-pharma.com/Contract-Manufacturing/Doxil-supplies-going-further-but-J-J-CMO-uncertainty-remains and many other websites). This shortage makes the "reward" for generic Doxil even more appealing.

The explanation for the lack of generic Doxil is that such a generic product is much more difficult to develop than a simple drug, or even than biologicals such as antibodies, because in addition to what is needed for the approval of generic low molecular weight drugs and biologicals, for approval of generic liposomal drugs, there are additional physical and physicochemical requirements needed.

The complexity of generic Doxil approval is discussed in a recent review by Jiang et al. [124] from the Office of Generic Drugs of the FDA. It is best to cite verbatim the abstract of this paper (with which I fully agree).

> *"One challenge in developing a nanoparticle drug-delivery system is understanding the critical physicochemical properties that may impact its in vivo performance and establishing analytical techniques that can adequately characterize in vitro and in vivo properties. Doxil®/Caelyx®, a PEGylated liposomal doxorubicin (PLD), is one of the leading approved nanoparticle product used in cancer therapy. In this review, we use PLD as an example to illustrate identification of key in vitro and in vivo characteristics. The following characteristics, including liposome composition, state of encapsulated drug, internal environment of liposome, liposome size distribution, lamellarity, grafted polyethylene glycol at the liposome surface, electrical surface potential or charge, and in vitro leakage, are considered critical to demonstrate the supramolecular structure of PLD and ensure consistent drug delivery to cancer tissues. Corresponding analytical techniques are discussed to determine these liposome characteristics. Furthermore, in vivo stability of the PLD can be determined by plasma pharmacokinetics of both free and liposome-encapsulated drug. A better understanding of the critical in vitro and in vivo liposome characteristics together with improvements in analytical technology will enable generic liposome product."*

In February 2010 the FDA issued a non-binding recommendation which relates to Generic Doxorubicin in Liposomes (http://www.fda.gov).

In the present review I will focus only on few special aspects raised which are related to Doxil physico-chemical properties and their relevancy to Doxil performance. For the detailed FDA Generic Doxil guidelines draft see the FDA website (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM199635.pdf). Citations from the FDA document are in italic type while my comments are in regular (roman) type.

> *"The surface-bound methoxypolyethylene glycol (MPEG) polymer coating protects liposomes from clearance by the mononuclear phagocyte system (MPS) and increases blood circulation time. The PEG layer thickness is known to be thermodynamically limited and estimated to be in the order of several nanometers. The PEG layer thickness should be determined."*

The methoxy PEG (2000 Da) residues of the PEG–DSPE can assume either a mushroom (at low PEG-DSPE mole fraction) or at higher mole fraction, transformation into brush conformation, which involves PEG–PEG interaction [47]. The actual measurement of the PEG layer thickness is not easy to perform as the PEG moiety does not show up in cryo-TEM, and its contrast in SAXS measurements is also poor and requires sophisticated methodology and software (for more details see [118], this issue).

> ***Electrical surface potential or charge***
>
> *Surface charge on liposomes can affect the clearance, tissue distribution, and cellular uptake. Liposome surface charge should be measured.*

Liposome surface potential and zeta potential are not identical as they are measured at different distances from the lipid/water interface (phospholipid headgroup). Both are measured by different methods which are based on two different approaches. The location of the electrical surface potential is fixed at the phospholipid headgroup, while the zeta potential can be at different locations with respect to the phospholipid headgroup. For example, the complete coating of liposomes by PEGylated lipids moves the location of measurement further away from the phospholipid headgroup, thereby reducing the magnitude of the measured liposome zeta potential (a charge-hiding effect). However, the PEG moiety does not affect the electrical surface potential. PEG-DSPE actually introduces a negative surface potential due to its phosphate diester moiety (for more details see [90,95,125]).

> ***In vitro leakage under multiple conditions***
>
> *In vitro drug leakage testing to characterize the physical state of the lipid bilayer and encapsulated doxorubicin should be investigated to support a lack of uncontrolled leakage under a range of physiological conditions and equivalent drug delivery to the tumor cells. Below are some examples of proposed conditions.*

Assaying doxorubicin in vitro release from Doxil and generic Doxil is essential for determining their chemical and physical stability upon storage and during the prolonged plasma circulation time.

In vitro and in vivo drug release profiles are both dependent on the membrane lipid composition and physical state and the integrity of the membrane lipids as well as the stability of the residual transmembrane ammonium sulfate gradient. Reduction in each of the two can be destructive, as it may reduce the therapeutic efficacy and can even be dangerous since it may result in massive drug release to the plasma. While measuring of lipid integrity is simple and requires simple analytical follow-up of lipid stability, mainly using HPLC with suitable ELSD or Corona detectors or LCMS, however, measuring the magnitude and the stability of residual ammonium gradient is less straightforward. Ammonium ions can be determined either by HPLC or specific electrodes (either as ammonium or as ammonia) [48]. Sulfate ions should be determined by HPLC. Determining the change in sulfate to phospholipid ratio is a good measure of integrity of the Doxil liposome membrane, as sulfate permeability coefficient is very low and much lower than that of ammonium ions (10^5-fold lower) or ammonia gas (10^{11}-fold lower) [20]. It is worth noting that ammonia may be released from the Doxil during long-term storage or during its blood circulation at body temperature (37 °C), resulting in reducing the magnitude of ammonium gradient.

- ***Active liposome loading process with an ammonium sulfate gradient***

In order to meet the compositional equivalence and other equivalence tests, an ANDA sponsor would be expected to use an active loading process with an ammonium sulfate gradient. The major steps include 1) formation of liposomes containing ammonium sulfate, 2) liposome size reduction, 3) creation of ammonium sulfate gradient, and 4) active drug loading. An active loading process uses an ammonium gradient.

FDA is requested to deal with all 4 steps of Doxil fabrication. If all these steps go well, it is expected that more than 95% of the doxorubicin will be liposome encapsulated. The active (remote) loading by transmembrane gradient of ammonium sulfate is the heart of the loading matter. It should enable achieving almost complete and stable drug loading, concomitantly having drug release at the tumor. The ammonium gradient is the driving force for the remote loading [19,20,32–34,48,64]. The transmembrane ammonium ion gradient can be tested specifically by ammonium and/or ammonia electrodes [48], and ammonium ion can also be determined by HPLC. The ammonium ion measurements require special precautions. For determination of intraliposome ammonium concentration, there is a need to determine the trapped aqueous volume. To account for losses during preparation, the ammonium amount has to be quantified per amount of total lipid HSPC (measured by HPLC), or total phospholipids (measured by phosphorus determination). For more details on these QC assay see [16,134].

The doxorubicin in DOXIL is largely in the form of a doxorubicin sulfate precipitate inside the liposome. The generic doxorubicin HCl liposome must contain an equivalent doxorubicin precipitate inside the liposome.

The demand for showing equivalent amount and shape of intraliposome doxorubicin-sulfate precipitate/gel is important as this precipitate/gel is highly relevant for maintaining loading stability during storage and during circulation in blood. It can be determined semi-quantitatively by cryo-TEM and SAXS measurements [61,67], and Fig. 2 above.

The relevance of sulfate as the ammonium counterion is well documented in a recent publication by Mamida et al. [126], who compared Doxil with 6 different PEGylated liposomal doxorubicin formulations based on dextran sulfate (DSAS) as ammonium counterion for their performance in a murine breast cancer model, and in tumor-free monkeys. The change in counterion resulted in a very similar doxorubicin plasma PK to that of Doxil. The therapeutic efficacy expressed as decreases in tumor volume was somewhat better for the DSAS-based formulation, but at the cost of a 3.2-fold increase in aspartate transaminase levels (a marker of hepato-toxicity). Also, a 5.0-fold increase in cardiac troponin I levels (a marker of cardiac toxicity), as well as increase in bone marrow hypocellularity (a marker of bone marrow toxicity), and increase in kidney toxicity [126]. This study demonstrates well what we claim above that for Doxil every detail matters!!!

- ***Internal environment (volume, pH, sulfate and ammonium ion concentration)***

This can be measured using acridine orange remote loading [48,63].

The internal environment of the liposome, including its volume, pH, sulfate and ammonium concentration, maintains the precipitated doxorubicin. The measurements of total and free concentrations of components (including sulfate ions) described in liposome composition section allow the inference of the internal concentration inside the liposome.

Total and Doxil-encapsulated doxorubicin should be determined after separation between un-encapsulated and encapsulated drug. Free un-encapsulated doxorubicin can be separated and removed from the liposomes by gel permeation chromatography or by Dowex cation exchanger [48,97,98]. This should be followed by doxorubicin determination using the right HPLC procedure.

Sulfate ion is the counterion which is important to fine tuning of loading stability (see [32,48,64]).

Sulfate ion concentration can be determined by HPLC with an appropriate detector (see above).

Satisfying these requirements requires the following: determination of liposome trapped volume from measurement of sulfate ion by HPLC, or less preferably, ammonium ion by ammonium electrode [48] or HPLC. Level of encapsulated ammonium sulfate should be determined after removal of non-encapsulated ammonium sulfate by exhaustive dialysis, and/or gel permeation chromatography, followed by determination of intraliposome sulfate concentration by HPLC.

Doxil intraliposome pH should be determined by the intra-to-extra-liposome partitioning of radioactive methylamine (Table 2 above and [34]). Similar approaches can also be applied. However in the case of Doxil, the use of chromophores or fluorophores for this determination is not straightforward due to the high background absorbance and fluorescence of doxorubicin [34].

- ***Liposome morphology and number of lamellae***

Liposome morphology and lamellarity should be determined, as drug loading, drug retention, and the rate of drug release from the liposomes are influenced by the degree of lamellarity.

Doxil morphology may be relevant to the level of complement activation [22,23,111], while number of liposome lamellae may be relevant to drug release profile. The determination of Doxil shape, lamellarity, and physical state of the intraliposome drug requires the use of cryo-TEM [67] and Fig. 2 above.

- ***Lipid bilayer phase transitions***

Equivalence in lipid bilayer phase transitions will contribute to demonstrating equivalence in bilayer fluidity and uniformity. The phase transition profiles of the lipid ingredients and liposomes should be comparable to those of Doxil.

HSPC, the main membrane component of Doxil liposomes, has a gel [solid-ordered (SO)] to fluid [liquid-disordered (LD)] phase transition with a Tm ~53 °C [47], and this Tm is not affected much by the ~5 mol% of PEG-DSPE. However, in the presence of the Doxil

high mole% of cholesterol, as expected, there is no SO to LD phase transition [95]. The thermotropic behavior of Doxil can be determined by various methods, the most straightforward and preferred method being differential scanning calorimetry (DSC) [127].

> ❋ ***Liposome size distribution***
>
> *Liposome size distribution is critical to ensuring equivalent passive targeting. The ANDA sponsor should select the most appropriate particle size analysis method to determine the particle size distributions of both test and reference product. The number of liposome product vials to be studied should not be fewer than 30 for each of the test and reference products (i.e., no fewer than 10 from each of three batches).*

Doxil size distribution is a very critical issue, as size may have a large impact on liposome PK and BD, and therefore on therapeutic efficacy and toxicity (see below). Also relevant is the observation that the presence of free drug may induce liposome aggregation and/or formation of drug aggregates. Both types of aggregates may induce complement activation [23,111].

However, size distribution determination is not an easy task and none of the currently available methods gives complete and absolute values. Each of the currently used methods has some pitfalls and/or does not see the full scale [16,128], and references listed therein. The two most commonly used size determination methods for liposomes in the expected size range of Doxil are dynamic light scattering (DLS), and cryo-TEM (the latter being mainly a supportive confirmatory method). Size determination by DLS is based on the determination of diffusion coefficient of nano-particulates, calculated from the exponential decay of the autocorrelation curve of which the radius of the particles are determined. In order to get the best results the instrument that performs the DLS measurement has to get the information about the temperature, refractive index, and viscosity of the solution in which the measurement is performed. Size determination could be a perfect method if the liposome population is very homogeneous in size. But in most situations this is not the case, and the liposome population has a size distribution and therefore there are many autocorrelation curves which have to be deconvoluted to their separate components. This procedure of deconvolution is not simple and requires applying a mathematical model. However there is more than one model available and each of the models may use different mathematics. Even for the same model there is no unique solution. Usually size determination readouts come in 3 different ways: (1) a mean and distribution according to analysis by light intensity (which is much more affected by the larger particles, as intensity is increased by a power of 6 of their size); (2) a mean and distribution of particles by their volume (which again gives more weight to the larger particles); or (3) a mean and distribution by particle number. Only if the three types of readout are very similar can the population be considered homogeneous with respect to size. The higher is the discrepancy between the 3 readouts the more heterogeneous is the liposome population. So when data on size determination are given in order to evaluate them, the evaluator has to know if analysis was corrected for temperature, refractive index, and viscosity and not less important, which of the 3 readouts was used (by intensity, volume, or number). It is preferable to include these 3 readouts in the product description.

Cui et al. evaluated the effect of size distribution on Doxil-like formulations [129]. They prepared smaller than Doxil liposomes (75 nm, 300 mM ammonium sulfate) of lipid composition identical to Doxil. These liposomes showed identical PK parameters in normal mice, however faster drug release for the 75 nm liposomes. Doxil liposomes had higher AUC and Cmax in S-180 sarcoma-bearing mice than the 75 nm Doxil-like liposomes. The 75 nm liposomes were more efficacious therapeutically, but also had a greater toxicity (based on decreases in body weight). However, in this specific publication [129] there is not enough information to evaluate the quality and precision of the size distribution determination. Therefore, it is not recommended to use this information as a go/no go variable.

8. Personal touch

I cannot end this Doxil review without a personal touch. The road to the development of Doxil® covers a major part of my professional career. I have been working on the development of liposomal drugs since 1979. However, I could not perform all my applied multidisciplinary work without the many research years and major efforts I dedicated to basic research in the fields of lipid biochemistry and biophysics. I started to study phospholipid and sphingolipid enzymology when I was a second-year Biology student working, to support my family, in the laboratory of Shimon Gatt, with whom I later performed my graduate studies (M.Sc. and Ph.D. theses). My involvement in lipid biophysics started due to a need to better understand lipid enzymology. Most lipids are not water-soluble and in order to serve as a substrate there is a need to disperse them in an aqueous phase with the aid of detergents (mixed micelles [130], rev. in [131]) or in the form of liposomes [132]. At the time I worked in Gatt's lab, liposomes, which were introduced by Alec Bangham in the mid-1960s, were still in their infancy. Lipid enzymology introduced me to lipid biophysics and therefore I dedicated a large part of my Ph.D. thesis to different aspects of lipid biophysics, under the supervision of Rex Dawson and Peter Quinn of Dawson's lab (lipid monolayers) and in Alec Bangham's lab (liposomes). Both groups were part of the Institute of the Animal Research Council at Babraham, Cambridge, UK. Indeed, my studies at Babraham had an important impact on my Ph.D. thesis and the rest of my scientific career. My Ph.D. thesis was submitted and approved by the Senate of the Hebrew University in 1971. Since then, my independent research was focused on various aspects of lipid biophysics and "liposomology". I was lucky to perform my first and prolonged sabbatical with Tom Thompson at the Department of Biochemistry of the University of Virginia (UVA) Medical School in Charlottesville, VA, (1973–1975) and continued to interact for many years with the UVA team, which included (in addition to Tom Thompson), Chien Huang, Burt Litman, Dov Lichtenberg, Rodney Biltonen, and others. In the 1970s/1980s UVA was one of the world's dominant labs in membrane biophysics. At UVA, liposomes were characterized (like never before) by many physical methods, almost to the level of macromolecules.

In 1979, Alberto Gabizon, (then a young M.D./Ph.D. who arrived to the Oncology Department of Hadassah University Hospital) and I started to work on the development of liposomal doxorubicin formulation for human use (rev. in [1,7]). This direction of my research was intensified in 1984, after a meeting I had with Dimitri Papahadjopoulos from the University of California at San Francisco (UCSF), a long-time friend and colleague "liposomologist". We had met at various scientific conferences since 1973 when I was at UVA. The UVA membrane research group was in tight competition with the UCSF group, although the interests of the two groups were only partially overlapping. The UVA group focused mainly on lipid biophysics and physical chemistry, and the UCSF (Dimitri Papahadjopoulos) group focused more on biologically relevant topics such as fusion, interaction of liposomes with cells, etc. Whenever Dimitri and I met, we talked extensively about science (mainly membrane and liposome research), as well as on culture, art, history, food, and wine. Dimitri kept telling me about Liposome Technology Inc. (LTI), a start-up located at Menlo Park, CA, which focused its R&D in the field of liposome-based diagnostic and medical applications. Dimitri and his previous student, Frank Szoka, were the scientific founders and mentors of LTI. Nick Arvanitidis was convinced by Dimitri

to become LTI CEO, and Frank Martin, another student of Dimitri's, was the first LTI employee. Nick brought with him Sally Davenport, Carl Grove, and Kathy, who had worked in Nick's previous R&D company, to deal with LTI administration. Dimitri asked me if I would be interested to spend a sabbatical at LTI. He told me that it was a great challenge and intellectually very rewarding. He already knew about our efforts in the field of drug delivery, and that we were close to the "first in man" experiment with liposomal doxorubicin, but he was more interested in my knowledge and experience in lipid and liposome biophysics and physical chemistry, as he well understood that this was the heart of the matter of developing liposomal products. I hesitated as, so far, most of my research was academic in nature. Dimitri proposed that I come to his lab at UCSF, give a seminar there, and he would organize my visit at LTI so I would be able to judge for myself whether spending a sabbatical at LTI was of any interest to me. I also got a formal invitation from Nick, LTI CEO, to visit LTI and spend a day there. As things looked serious, I consulted with Hanna, my wife, who supported me and encouraged me to seriously evaluate this interesting proposition. I knew this was not easy for her, as it meant that I would be away from home (in California) for long periods of time, and she would have to take care, alone, of our 4 daughters, the dog, and our home. With her encouragement, I accepted Dimitri's and Nick's offers to visit. My visit at Dimitri's and at LTI was organized for December 1984. My seminar at Dimitri's lab was on glycosphingolipid biophysics, which was followed by discussion with Dimitri's lab people. The next day Dimitri drove me to LTI in Menlo Park, which at that time was a start-up company of ~40 people, where I spent the whole day talking with many company employees. After dinner with Nick and some good wine, Nick and I had a long conversion in which Nick was trying to convince me to spend my sabbatical at LTI. Nick is Greek, and as such he understood well the Mediterranean mentality and way of thinking, so we understood each other very well. Without going into detail and possibly with the aid of the good wine served continuously by Nick, I agreed to seriously consider his proposal. Nick drove me to my San Francisco hotel very late that night. The excitement, together with the 10-h jet lag, made it very difficult for me to sleep.

Returning to Israel, I discussed Nick's proposal with Hanna, and with her support and encouragement, I accepted it. I told Dimitri and Nick that I would not be able to come unless LTI would support our OLV-DOX program in Jerusalem. It took a short time until the LTI board decided to accept my request. Frank Szoka, Dimitri, and Nick called me from the board meeting at 02.00 AM Israel time and woke me up to tell me that LTI had accepted my request. But their condition was that I assured them of my continuous involvement in their relevant research and R&D programs. LTI support meant what was considered a large grant at that time, which would allow us to continue our OLV-DOX research, and especially the "first in man" clinical trial. So, it seemed we had a deal, the small details of which still needed to be finalized. At about that time I was approached by another US company that proposed to license from us the OLV-DOX technology and product. Their proposal was tempting, as it involved what I considered then a large sum of money up front and reasonable royalties. However, this company requested that we would be used only as consultants and not be involved in the research and R&D of the product. I did not like this idea, as we looked upon the product as a "baby" we had to nurture to maturity. I preferred LTI to the other company, as I believed that our day-to-day involvement in the product development was crucial to the program's success. The future would show that I was right. So I convinced Moshe Vigdor, the CEO of Yissum (the Research and Development Company of The Hebrew University of Jerusalem) to accept LTI's proposal. It did not take Nick long to come to Israel and finish the LTI–Yissum first license agreement, which was the basis for a master agreement that continued for 21 years. It started with LTI, with Nick as CEO, and went all the way to Johnson & Johnson. After the approval of Doxil by the FDA there was a change of management at LTI, and Craig Henderson, a top-level oncologist from UCSF (who had been involved in Doxil's clinical development) became LTI CEO. Then the company name was changed to Sequus. Craig and others sold Sequus to ALZA, Mountain View, CA (a major drug delivery company), with Doxil (due to its increasing sales) being one of the main reasons for the deal. It did not take a long time for ALZA to be bought by Johnson & Johnson, which until then was hardly involved in drug delivery systems. Again, Doxil was one of the main reasons for the deal. All the rest is history!!!

During this fantastic very long voyage of 48 wonderful years of active research I met many fascinating people, with whom I interacted and/or collaborated, and many of them remain lifelong friends. The 15 years I worked on liposomal doxorubicin, of which 50% was dedicated to Doxil development, was a unique experience I will never forget. It enabled me to be involved in a very complicated and complex process of drug development and to see its approval worldwide. The reward in terms of satisfaction is unmatched by any of my other achievements. I am trying hard to transfer my experience, part of which is summarized in this review, to many students and others worldwide.

Special acknowledgments

Professionally, I have a very long list of people to thank, in large part described above. Here, however I would like to thank by name only those people with whom I interacted on Doxil-related matters. Alberto Gabizon, for a 32-year partnership in exciting research including Doxil development, and friendship — the many shared papers and patents with him are excellent evidence of our highly productive collaborative interaction; the late Demetrios (Dimitri) Papahadjopoulos, for his friendship and intellectual stimulus; the late Dan Lasic, for his stimulating discussions; and Nick Arvanidis, from whom I learned about priorities in applied research and for the many heated disputes we had.

I also want to thank and acknowledge all the grant agencies that supported my basic research including NIH, ISF, BSF, GIF, MOH, and last but not least, The Hebrew University of Jerusalem.

Most importantly, I want to dedicate this review to my wife, Hanna. Without her encouragement, advice, patience, dedication, and support throughout our 52 years of life together I would not be able to accomplish my part of Doxil development and possibly Doxil would not be developed!

I also want to thank my four daughters, Chagit, Ayelet, Tamar, and Avigail (Abigail), who grew up during the years described in this review; their husbands, Uri, Perri, Ron, and Assaf; and last but not least, our twelve grandchildren, Yael, Yuval, Amit, Omri, Inbar, Mika, Rotem, Guy, Eyal, Gal, Dror, and Kfir, who give us so much joy. Our daughters and grandchildren were my escape during periods of despair.

I also want to thank Sigmund Geller for his 20 years of help in editing my manuscripts including this review, and Amira Weiss, Pablo Kizelsztein, Lila Kart, and Erez Koren for their immense technical help on this review.

References

[1] Y. Barenholz, Doxil®—the first FDA-approved nano-drug: from an idea to a product, in: D. Peer (Ed.), Handbook of Harnessing Biomaterials in Nanomedicine: Preparation, Toxicity, and Applications, Pan Stanford Publishing, Singapore, 2012, pp. 335–398.

[2] A. Gabizon, D. Goren, Z. Fuks, Y. Barenholz, A. Dagan, A. Meshorer, Enhancement of adriamycin delivery to liver metastatic cells with increased tumoricidal effect using liposomes as drug carriers, Cancer Res. 43 (1983) 4730–4735.

[3] A. Gabizon, D. Goren, Z. Fuks, A. Meshorer, Y. Barenholz, Superior therapeutic activity of liposome-associated adriamycin in a murine metastatic tumour model, Br. J. Cancer 51 (1985) 681–689.

[4] A. Gabizon, A. Meshorer, Y. Barenholz, Comparative long-term study of the toxicities of free and liposome-associated doxorubicin in mice after intravenous administration, J. Natl. Cancer Inst. 77 (1986) 459–469.

[5] A. Gabizon, D. Goren, A. Ramu, Y. Barenholz, Design, characterization and antitumor activity of adriamycin-containing phospholipid vesicles, in: G. Gregoriadis, J. Senior, G. Poste (Eds.), Targeting of Drugs with Synthetic Systems, Plenum, London, 1986, pp. 229–238.
[6] A. Gabizon, D. Goren, Y. Barenholz, Investigations on the antitumor efficacy of liposome-associated doxorubicin in murine tumor models, Isr. J. Med. Sci. 24 (1988) 512–517.
[7] A. Gabizon, Y. Barenholz, Adriamycin-containing liposomes in cancer chemotherapy, in: G. Gregoriadis (Ed.), Liposomes as Drug Carriers, Wiley, New York, 1988, pp. 365–379.
[8] A. Gabizon, T. Peretz, A. Sulkes, S. Amselem, R. Ben-Yosef, N. Ben-Baruch, R. Catane, S. Biran, Y. Barenholz, Systemic administration of doxorubicin-containing liposomes in cancer patients: a phase I study, Eur. J. Cancer Clin. Oncol. 25 (1989) 1795–1803.
[9] A. Gabizon, A. Sulkes, T. Peretz, S. Druckmann, D. Goren, S. Amselem, Y. Barenholz, Liposome-associated doxorubicin: preclinical pharmacology and exploratory clinical phase, in: I.J. Fidler, G. Lopez-Berestein (Eds.), Liposomes in the Therapy of Infectious Diseases and Cancer, Alan R. Liss, New York, 1989, pp. 391–402.
[10] D. Goren, A. Gabizon, Y. Barenholz, The influence of physical characteristics of liposomes containing doxorubicin on their pharmacological behavior, Biochim. Biophys. Acta 1029 (1990) 285–294.
[11] D. Goren, Liposomes as doxorubicin carriers: a means to improve the therapeutic index of the drug, in: Ph.D. Thesis, Hebrew University of Jerusalem, Israel, 1990.
[12] S. Amselem, A. Gabizon, Y. Barenholz, A large-scale method for the preparation of sterile and nonpyrogenic liposomal formulations of defined size distributions for clinical use, in: G. Gregoriadis (Ed.), Liposome Technology, 2nd ed., Liposome Preparation and Related Techniques, vol. 1, CRC Press, Boca Raton, FL, 1993, pp. 501–525.
[13] A. Gabizon, D. Papahadjopoulos, Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 6949–6953.
[14] A. Gabizon, R. Chisin, S. Amselem, S. Druckmann, R. Cohen, D. Goren, I. Fromer, T. Peretz, A. Sulkes, Y. Barenholz, Pharmacokinetic and imaging studies in patients receiving a formulation of liposome-associated adriamycin, Br. J. Cancer 64 (1991) 1125–1132.
[15] S. Amselem, R. Cohen, Y. Barenholz, In vitro tests to predict in vivo performance of liposomal dosage forms, Chem. Phys. Lipids 64 (1993) 219–237.
[16] Y. Barenholz, S. Amselem, Quality control assays in the development and clinical use of liposome-based formulations, in: G. Gregoriadis (Ed.), Liposome Technology, 2nd ed., Liposome Preparation and Related Techniques, vol. 1, CRC Press, Boca Raton, FL, 1993, pp. 527–616.
[17] Y. Barenholz, S. Amselem, D. Goren, R. Cohen, D. Gelvan, A. Samuni, E.B. Golden, A. Gabizon, Stability of liposomal doxorubicin formulations: problems and prospects, Med. Res. Rev. 13 (1993) 449–491.
[18] Y. Barenholz, R. Cohen, Rational design of amphiphile-based drug carriers and sterically stabilized carriers, J. Liposome Res. 5 (1995) 905–932.
[19] Y. Barenholz, Relevancy of drug loading to liposomal formulation therapeutic efficacy, J. Liposome Res. 13 (2003) 1–8.
[20] Y. Barenholz, Liposome application: problems and prospects, Curr. Opin. Colloid Interface Sci. 6 (2001) 66–77.
[21] J. Szebeni, C.R. Alving, L. Rosivall, R. Bunger, L. Baranyi, P. Bedocs, M. Toth, Y. Barenholz, Animal models of complement-mediated hypersensitivity reactions to liposomes and other lipid-based nanoparticles, J. Liposome Res. 17 (2007) 107–117.
[22] J. Szebeni, F. Muggia, A. Gabizon, Y. Barenholz, Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention, Adv. Drug Deliv. Rev. 63 (2011) 1020–1030.
[23] J. Szebeni, P. Bedocs, Z. Rozsnyay, Z. Weiszhar, R. Urbanics, L. Rosivall, R. Cohen, O. Garbuzenko, G. Bathori, M. Toth, R. Bunger, Y. Barenholz, Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and Am Bisome, Nanomedicine 8 (2011) 176–184.
[24] K.J. Hwang, Liposome pharmacokinetics, in: M.J. Ostro (Ed.), Liposomes: from Biophysics to Therapeutics, Marcel Dekker Inc., New York, 1987, pp. 109–156.
[25] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.
[26] H. Maeda, G.Y. Bharate, J. Daruwalla, Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect, Eur. J. Pharm. Biopharm. 71 (2009) 409–419.
[27] G. Poste, C. Bucana, A. Raz, P. Bugelski, R. Kirsh, I.J. Fidler, Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery, Cancer Res. 42 (1982) 1412–1422.
[28] G. Poste, Liposome targeting in vivo: problems and opportunities, Biol. Cell 47 (1983) 19–37.
[29] R.H. Blum, S.K. Carter, Adriamycin. A new anticancer drug with significant clinical activity, Ann. Intern. Med. 80 (1974) 249–259.
[30] E.M. McKelvey, J.A. Gottlieb, H.E. Wilson, A. Haut, R.W. Talley, R. Stephens, M. Lane, J.F. Gamble, S.E. Jones, P.N. Grozea, J. Gutterman, C. Colman, T.E. Moon, Hydroxyldaunomycin (adriamycin) combination chemotherapy in malignant lymphoma, Cancer 38 (1976) 1484–1493.
[31] S. Gundersen, S. Kvinnsland, O. Klepp, S. Kvaloy, E. Lund, H. Host, Weekly adriamycin versus VAC in advanced breast cancer. A randomized trial, Eur. J. Cancer Clin. Oncol. 22 (1986) 1431–1434.
[32] D. Zucker, D. Marcus, Y. Barenholz, A. Goldblum, Liposome drugs' loading efficiency: a working model based on loading conditions and drug's physicochemical properties, J. Control. Release 139 (2009) 73–80.
[33] A. Cern, A. Golbraikh, A. Sedykh, A. Tropsha, Y. Barenholz, A. Goldblum, Quantitative Structure–property Relationship Modeling of Remote Liposome Loading of Drugs, J. Control. Release. (2012, this issue).
[34] Y. Barenholz, Amphipathic weak base loading into preformed liposomes having a transmembrane ammonium ion gradient: From the bench to approved DOXIL, 3rd ed., in: G. Gregoriadis (Ed.), Liposome Technology: Entrapment of Drugs and Other Materials into Liposomes, vol. 2, Informa Healthcare, New York, 2007, pp. 1–25.
[35] A. Rahman, A. Kessler, N. More, B. Sikic, G. Rowden, P. Woolley, F.S. Schein, Liposomal protection of adriamycin-induced cardiotoxicity in mice, Cancer Res. 40 (1980) 1532–1537.
[36] A. Rahman, G. White, N. More, F.S. Schein, Pharmacological, toxicological, and therapeutic evaluation in mice of doxorubicin entrapped in cardiolipin liposomes, Cancer Res. 45 (1985) 796–803.
[37] A. Rahman, A. Fumagalli, B. Barbieri, P.S. Schein, A.M. Casazza, Antitumor and toxicity evaluation of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes, Cancer Chemother. Pharmacol. 16 (1986) 22–27.
[38] A. Rahman, D. Carmichael, M. Harris, J.K. Roh, Comparative pharmacokinetics of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes, Cancer Res. 46 (1986) 2295–2299.
[39] R.B. Weiss, The anthracyclines: will we ever find a better doxorubicin? Semin. Oncol. 19 (1992) 670–686.
[40] R.T. Skeel, Handbook of Cancer Chemotherapy, Lippincott Williams & Wilkins, Philadelphia, 1999.
[41] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, L. Gianni, Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity, Pharmacol. Rev. 56 (2004) 185–229.
[42] J. Kenyon, Chemotherapy and cardiac toxicity – the lesser of two evils, Doctors Lounge Website, 2010.
[43] X. Peng, B. Chen, C.C. Lim, D.B. Sawyer, The cardiotoxicology of anthracycline chemotherapeutics: translating molecular mechanism into preventative medicine, Mol. Interv. 5 (2005) 163–171.
[44] C.H. Takimoto, E. Calvo, Principles of oncologic pharmacotherapy, in: R. Pazdur, L.D. Wagman, K.A. Camphausen, W.J. Hoskins (Eds.), Cancer Management: a Multidisciplinary Approach11th ed., , 2008.
[45] J.H. Beijnen, H. Rosing, P.A. de Vries, W.J. Underberg, Stability of anthracycline antitumour agents in infusion fluids, J. Parenter. Sci. Technol. 39 (1985) 220–222.
[46] P.A. Andrews, D.E. Brenner, F.T. Chou, H. Kubo, N.R. Bachur, Facile and definitive determination of human adriamycin and daunorubicin metabolites by high-pressure liquid chromatography, Drug Metab. Dispos. 8 (1980) 152–156.
[47] O. Garbuzenko, Y. Barenholz, A. Priev, Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer, Chem. Phys. Lipids 135 (2005) 117–129.
[48] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, Biochim. Biophys. Acta 1151 (1993) 201–215.
[49] S. Bandak, A. Ramu, Y. Barenholz, A. Gabizon, Reduced UV-induced degradation of doxorubicin encapsulated in polyethyleneglycol-coated liposomes, Pharm. Res. 16 (1999) 841–846.
[50] A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, R. Cohen, F. Martin, A. Huang, Y. Barenholz, Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes, Cancer Res. 54 (1994) 987–992.
[51] A. Gabizon, H. Shmeeda, Y. Barenholz, Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies, Clin. Pharmacokinet. 42 (2003) 419–436.
[52] H. Hashizume, P. Baluk, S. Morikawa, J.W. McLean, G. Thurston, S. Roberge, R.K. Jain, D.M. McDonald, Openings between defective endothelial cells explain tumor vessel leakiness, Am. J. Pathol. 156 (2000) 1363–1380.
[53] R. Bassermann, Changes of vascular pattern of tumors and surrounding tissue during different phases of metastatic growth, Recent Results Cancer Res. 100 (1986) 256–267.
[54] R.K. Jain, Transport of molecules across tumor vasculature, Cancer Metastasis Rev. 6 (1987) 559–593.
[55] Y. Barenholz, G. Haran, Efficient Loading and Controlled Release of Amphipathic Molecules, in, United States Patent 5,244,544, 1994.
[56] Y. Barenholz, G. Haran, Method of Amphipathic Drug Loading in Liposomes by pH Gradient, in, United States Patent 5,192,549, 1993.
[57] D.W. Deamer, R.C. Prince, A.R. Crofts, The response of fluorescent amines to pH gradients across liposome membranes, Biochim. Biophys. Acta 274 (1972) 323–335.
[58] J.W. Nichols, D.W. Deamer, Catecholamine uptake and concentration by liposomes maintaining pH gradients, Biochim. Biophys. Acta 455 (1976) 269–271.
[59] T.D. Madden, A.S. Janoff, P.R. Cullis, Incorporation of amphotericin B into large unilamellar vesicles composed of phosphatidylcholine and phosphatidylglycerol, Chem. Phys. Lipids 52 (1990) 189–198.
[60] E.M. Bolotin, R. Cohen, L.K. Bar, N. Emanuel, S. Ninio, D.D. Lasic, Y. Barenholz, Ammonium sulfate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes, J. Liposome Res. 4 (1994) 455–479.
[61] D.D. Lasic, B. Ceh, M.C. Stuart, L. Guo, P.M. Frederik, Y. Barenholz, Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery, Biochim. Biophys. Acta 1239 (1995) 145–156.

[62] R. Ulmansky, K. Turjeman, M. Baru, G. Katzavian, M. Harel, A. Sigal, Y. Naparstak, Y. Barenholz, Glucocorticoids in nano-liposomes administered intravenously and subcutaneously to adjuvant arthritis rats are superior to the free drugs in suppressing arthritis and inflammatory cytokines, J. Control. Release. (2012, this issue).
[63] S. Clerc, Y. Barenholz, A quantitative model for using acridine orange as a transmembrane pH gradient probe, Anal. Biochem. 259 (1998) 104–111.
[64] V. Wasserman, P. Kizelsztein, G. Garbuzenko, R. Kohen, H. Ovadia, R. Tabakman, Y. Barenholz, The antioxidant tempamine: in vitro antitumor and neuroprotective effects and optimization of liposomal encapsulation and release, Langmuir 23 (2007) 1937–1947.
[65] W.D. Stein, Transport and Diffusion Across Cell Membranes, Academic Press, Orlando, FL, 1986.
[66] G.J. Grant, Y. Barenholz, B. Piskoun, M. Bansinath, H. Turndorf, E.M. Bolotin, DRV liposomal bupivacaine: preparation, characterization, and in vivo evaluation in mice, Pharm. Res. 18 (2001) 336–343.
[67] D.D. Lasic, P.M. Frederik, M.C. Stuart, Y. Barenholz, T.J. McIntosh, Gelation of liposome interior. A novel method for drug encapsulation, FEBS Lett. 312 (1992) 255–258.
[68] R.M. Abra, R.B. Bankert, F. Chen, N.K. Egilmez, K. Huang, R. Saville, J.L. Slater, M. Sugano, S.J. Yokota, The next generation of liposome delivery systems: recent experience with tumor-targeted, sterically-stabilized immunoliposomes and active-loading gradients, J. Liposome Res. 12 (2002) 1–3.
[69] A.T. Horowitz, Y. Barenholz, A.A. Gabizon, In vitro cytotoxicity of liposome-encapsulated doxorubicin: dependence on liposome composition and drug release, Biochim. Biophys. Acta 1109 (1992) 203–209.
[70] A.A. Gabizon, Y. Barenholz, Method for Drug Loading in Liposomes, in, International Patent Appl., WO/2005/046643, 2005.
[71] G.J. Grant, Y. Barenholz, E.M. Bolotin, M. Bansinath, H. Turndorf, B. Piskoun, E.M. Davidson, A novel liposomal bupivacaine formulation to produce ultralong-acting analgesia, Anesthesiology 101 (2004) 133–137.
[72] R. Cohen, H. Kanaan, G.J. Grant, Y. Barenholz, Prolonged analgesia from Bupisome and Bupigel formulations: from design and fabrication to improved stability, J. Control. Release. (2012, this issue).
[73] D.D. Lasic, J.J. Vallner, P.K. Working, Sterically stabilized liposomes in cancer therapy and gene delivery, Curr. Opin. Mol. Ther. 1 (1999) 177–185.
[74] T. Peleg-Shulman, D. Gibson, R. Cohen, R. Abra, Y. Barenholz, Characterization of sterically stabilized cisplatin liposomes by nuclear magnetic resonance, Biochim. Biophys. Acta 1510 (2001) 278–291.
[75] A. Schroeder, R. Honen, K. Turjeman, A. Gabizon, J. Kost, Y. Barenholz, Ultrasound triggered release of cisplatin from liposomes in murine tumors, J. Control. Release 137 (2009) 63–68.
[76] T.M. Allen, A. Chonn, Large unilamellar liposomes with low uptake into the reticuloendothelial system, FEBS Lett. 223 (1987) 42–46.
[77] T.M. Allen, Liposomes with Enhanced Circulation Time, in, United States Patent 4,837,028, 1989.
[78] M.C. Woodle, F.J. Martin, A. Yau-Yang, C.T. Redmann, Liposomes with Enhanced Circulation Time, in, United States Patent 5,013,556, 1991.
[79] M.C. Woodle, Improved long circulating (Stealth®) liposomes using synthetic lipids, Proc. 17th Int Symp Control Rel Bioact Mater, Controlled Release Society, Illinois, 1990, pp. 77–78.
[80] M.C. Woodle, D.D. Lasic, Sterically stabilized liposomes, Biochim. Biophys. Acta 1113 (1992) 171–199.
[81] M.C. Woodle, Surface-modified liposomes: assessment and characterization for increased stability and prolonged blood circulation, Chem. Phys. Lipids 64 (1993) 249–262.
[82] C.A. Hunt, D.P. Papahadjopoulous, Method for Producing Liposomes in Selected Size Range, in, United States Patent 4,529,561, 1985.
[83] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes, FEBS Lett. 268 (1990) 235–237.
[84] G. Blume, G. Cevc, Liposomes for the sustained drug release in vivo, Biochim. Biophys. Acta 1029 (1990) 91–97.
[85] D.D. Lasic, F. Martin (Eds.), Stealth Liposomes, CRC Press, Boca Raton, FL, 1995.
[86] A. Abuchowski, J.R. McCoy, N.C. Palczuk, T. van Es, F.F. Davis, Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase, J. Biol. Chem. 252 (1977) 3582–3586.
[87] F.M. Veronese, J.M. Harris, Introduction and overview of peptide and protein pegylation, Adv. Drug Deliv. Rev. 54 (2002) 453–456.
[88] F.M. Veronese, G. Pasut, PEGylation, successful approach to drug delivery, Drug Discov. Today 10 (2005) 1451–1458.
[89] O. Tirosh, R. Kohen, J. Katzhendler, A. Alon, Y. Barenholz, Oxidative stress effect on the integrity of lipid bilayers is modulated by cholesterol level of bilayers, Chem. Phys. Lipids 87 (1997) 17–22.
[90] O. Tirosh, Y. Barenholz, J. Katzhendler, A. Priev, Hydration of polyethylene glycol-grafted liposomes, Biophys. J. 74 (1998) 1371–1379.
[91] T.M. Allen, From fusion to magic bullets: the influence of Dimitri Papahdjopoulos, J. Liposome Res. 5 (1995) 657–667.
[92] A.A. Gabizon, Y. Barenholz, M. Bialer, Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmacokinetic studies in rodents and dogs, Pharm. Res. 10 (1993) 703–708.
[93] N. Emanuel, E. Kedar, E.M. Bolotin, N.I. Smorodinsky, Y. Barenholz, Preparation and characterization of doxorubicin-loaded sterically stabilized immunoliposomes, Pharm. Res. 13 (1996) 352–359.
[94] N. Emanuel, E. Kedar, E.M. Bolotin, N.I. Smorodinsky, Y. Barenholz, Targeted delivery of doxorubicin via sterically stabilized immunoliposomes: pharmacokinetics and biodistribution in tumor-bearing mice, Pharm. Res. 13 (1996) 861–868.
[95] O. Garbuzenko, S. Zalipsky, M. Qazen, Y. Barenholz, Electrostatics of PEGylated micelles and liposomes containing charged and neutral lipopolymers, Langmuir 21 (2005) 2560–2568.
[96] Z. Symon, A. Peyser, D. Tzemach, O. Lyass, E. Sucher, E. Shezen, A. Gabizon, Selective delivery of doxorubicin to patients with breast carcinoma metastases by stealth liposomes, Cancer 86 (1999) 72–78.
[97] S. Druckmann, A. Gabizon, Y. Barenholz, Separation of liposome-associated doxorubicin from non-liposome-associated doxorubicin in human plasma: implications for pharmacokinetic studies, Biochim. Biophys. Acta 980 (1989) 381–384.
[98] S. Amselem, A. Gabizon, Y. Barenholz, Optimization and upscaling of doxorubicin-containing liposomes for clinical use, J. Pharm. Sci. 79 (1990) 1045–1052.
[99] R. Solomon, A. Gabizon, Clinical pharmacology of liposomal antinacyclines: focus on pegylated liposomal Doxorubicin, Clin. Lymphoma Myeloma 8 (2008) 21–32.
[100] A. Gabizon, L. Huang, F. Martin, Y. Barenholz, Doxorubicin encapsulated in polyethylene-glycol coated liposomes: initial clinical-pharmacokinetic studies in solid tumors, in: D.D. Lasic, F. Martin (Eds.), Stealth Liposomes, CRC Press, Boca Raton, FL, 1995, pp. 245–255.
[101] R.K. Jain, R.T. Tong, L.L. Munn, Effect of vascular normalization by antiangiogenic therapy on interstitial hypertension, peritumor edema, and lymphatic metastasis: insights from a mathematical model, Cancer Res. 67 (2007) 2729–2735.
[102] R.K. Jain, Transport of molecules in the tumor interstitium: a review, Cancer Res. 47 (1987) 3039–3051.
[103] A. Schroeder, J. Kost, Y. Barenholz, Ultrasound, liposomes, and drug delivery: principles for using ultrasound to control the release of drugs from liposomes, Chem. Phys. Lipids 162 (2009) 1–16.
[104] W.C. Zamboni, A.C. Gervais, M.J. Egorin, J.H. Schellens, E.G. Zuhowski, D. Pluim, E. Joseph, D.R. Hamburger, P.K. Working, G. Colbern, M.E. Tonda, D.M. Potter, J.L. Eiseman, Systemic and tumor disposition of platinum after administration of cisplatin or STEALTH liposomal-cisplatin formulations (SPI-077 and SPI-077 B103) in a preclinical tumor model of melanoma, Cancer Chemother. Pharmacol. 53 (2004) 329–336.
[105] J.M. Meerum Terwogt, G. Groenewegen, D. Pluim, M. Maliepaard, M.M. Tibben, A. Huisman, W.W. ten Bokkel Huinink, M. Schot, H. Welbank, E.E. Voest, J.H. Beijnen, J.M. Schellens, Phase I and pharmacokinetic study of SPI-77, a liposomal encapsulated dosage form of cisplatin, Cancer Chemother. Pharmacol. 49 (2002) 201–210.
[106] G.J. Veal, M.J. Griffin, E. Price, A. Parry, G.S. Dick, M.A. Little, S.M. Yule, B. Morland, E.J. Estlin, J.P. Hale, A.D. Pearson, H. Welbank, A.V. Boddy, A phase I study in paediatric patients to evaluate the safety and pharmacokinetics of SPI-77, a liposome encapsulated formulation of cisplatin, Br. J. Cancer 84 (2001) 1029–1035.
[107] J.H. Beijnen, J. den Hartigh, W.J. Underberg, Qualitative aspects of the degradation of mitomycins in alkaline solution, J. Pharm. Biomed. Anal. 3 (1985) 71–79.
[108] O.G. Mouritsen, K. Jorgensen, A new look at lipid–membrane structure in relation to drug research, Pharm. Res. 15 (1998) 1507–1519.
[109] D.S. Alberts, P.Y. Liu, S.P. Wilczynski, M.C. Clouser, A.M. Lopez, D.P. Michelin, V.J. Lanzotti, M. Markman, Randomized trial of pegylated liposomal doxorubicin (PLD) plus carboplatin versus carboplatin in platinum-sensitive (PS) patients with recurrent epithelial ovarian or peritoneal carcinoma after failure of initial platinum-based chemotherapy (Southwest Oncology Group Protocol S0200), Gynecol. Oncol. 108 (2008) 90–94.
[110] F. Joly, I. Ray-Coquard, M. Fabbro, M. Donoghoe, K. Boman, A. Sugimoto, M. Vaughan, A. Reinthaller, I. Vergote, G. Ferrandina, T. Dell'Anna, J. Huober, E. Pujade-Lauraine, Decreased hypersensitivity reactions with carboplatin-pegylated liposomal doxorubicin compared to carboplatin–paclitaxel combination: analysis from the GCIG CALYPSO relapsing ovarian cancer trial, Gynecol. Oncol. 122 (2011) 226–232.
[111] J. Szebeni, Y. Barenholz, Complement activation, immunogenicity and immune suppression as potential side effects of liposomes, in: D. Peer (Ed.), Handbook of Harnessing Biomaterials for Nanomedicine: Preparation, Toxicity, and Applications, Pan Stanford Publishing, Singapore, 2012, pp. 309–334.
[112] A.N. Gordon, J.T. Fleagle, D. Guthrie, D.E. Parkin, M.E. Gore, A.J. Lacave, Recurrent epithelial ovarian carcinoma: a randomized phase III study of pegylated liposomal doxorubicin versus topotecan, J. Clin. Oncol. 19 (2001) 3312–3322.
[113] Y.P. Zhang, R. Ceh, D.D. Lasic, Liposomes in drug delivery, in: S. Damiziu (Ed.), Polymeric Biomaterials, Marcel Dekker Inc., New York, 2001, pp. 783–821.
[114] D. Needham, G. Anyarambhatla, G. Kong, M.W. Dewhirst, A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model, Cancer Res. 60 (2000) 1197–1201.
[115] D. Needham, M.W. Dewhirst, The development and testing of a new temperature-sensitive drug delivery system for the treatment of solid tumors, Adv. Drug Deliv. Rev. 53 (2001) 285–305.
[116] A. Schroeder, Y. Avnir, S. Weisman, Y. Najajreh, A. Gabizon, Y. Talmon, J. Kost, Y. Barenholz, Controlling liposomal drug release with low frequency ultrasound: mechanism and feasibility, Langmuir 23 (2007) 4019–4025.
[117] D. Zucker, Y. Barenholz, Optimization of vincristine–topotecan combination – paving the way for improved chemotherapy regimens by nanoliposomes, J. Control. Release 146 (2010) 326–333.
[118] D. Zucker, A.V. Andriyanov, A. Steiner, U. Raviv, Y. Barenholz, Characterization of PEGylated nanoliposomes co-remotely loaded with topotecan and vincristine: relating structure and pharmacokinetics to therapeutic efficacy, J. Control. Release. (2012, this issue).

[119] A. Cabanes, S. Even-Chen, J. Zimberoff, Y. Barenholz, E. Kedar, A. Gabizon, Enhancement of antitumor activity of polyethylene glycol-coated liposomal doxorubicin with soluble and liposomal interleukin 2, Clin. Cancer Res. 5 (1999) 687–693.
[120] E. Kedar, E. Braun, Y. Rutkowski, N. Emanuel, Y. Barenholz, Delivery of cytokines by liposomes. II. Interleukin-2 encapsulated in long-circulating sterically stabilized liposomes: immunomodulatory and anti-tumor activity in mice, J. Immunother. Emphasis Tumor Immunol. 16 (1994) 115–124.
[121] E. Kedar, Y. Rutkowski, E. Braun, N. Emanuel, Y. Barenholz, Delivery of cytokines by liposomes. I. Preparation and characterization of interleukin-2 encapsulated in long-circulating sterically stabilized liposomes, J. Immunother. Emphasis Tumor Immunol. 16 (1994) 47–59.
[122] B. Diop-Frimpong, V.P. Chauhan, S. Krane, Y. Boucher, R.K. Jain, Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 2909–2914.
[123] A. Gabizon, Y. Barenholz, Liposomal anthracyclines: from basics to clinical approval of PEGylated liposomal doxorubicin, in: A.S. Janoff (Ed.), Liposomes: Rational Design, Marcel Dekker Inc., New York, 1999, pp. 343–362.
[124] W. Jiang, R. Lionberger, L.X. Yu, In vitro and in vivo characterizations of PEGylated liposomal doxorubicin, Bioanalysis 3 (2011) 333–344.
[125] O. Tirosh, R. Kohen, J. Katzhendler, R. Gorodetsky, Y. Barenholz, Novel synthetic phospholipid protects lipid bilayers against oxidative damage: role of hydration layer and bound water, J. Chem. Soc. Perkin 1 2 (1997) 383–389.
[126] R.N.V.S. Mamidi, S. Weng, S. Stellar, C. Wang, N. Yu, T. Huang, A.P. Tonelli, M.F. Kelley, A. Angiuoli, M.C. Fung, Pharmacokinetics, efficacy and toxicity of different pegylated liposomal doxorubicin formulations in preclinical models: is a conventional bioequivalence approach sufficient to ensure therapeutic equivalence of pegylated liposomal doxorubicin products? Cancer Chemother. Pharmacol. (2010) 1–12.
[127] R.L. Biltonen, D. Lichtenberg, The use of differential scanning calorimetry as a tool to characterize liposome preparations, Chem. Phys. Lipids 64 (1993) 129–142.
[128] D. Lichtenberg, Y. Barenholz, Liposomes: preparation, characterization, and preservation, Methods Biochem. Anal. 33 (1988) 337–462.
[129] J.X. Cui, C.L. Li, W.M. Guo, Y.H. Li, C.X. Wang, L. Zhang, Y.L. Hao, Y.L. Wang, Direct comparison of two pegylated liposomal doxorubicin formulations: Is AUC predictive for toxicity and efficacy? J. Control. Release 118 (2007) 204–215.
[130] Y. Barenholz, A. Roitman, S. Gatt, Enzymatic hydrolysis of sphingolipids. II. Hydrolysis of sphingomyelin by an enzyme from rat brain, J. Biol. Chem. 241 (1966) 3731–3737.
[131] S. Gatt, Y. Barenholz, Enzymes of complex lipid metabolism, Annu. Rev. Biochem. 42 (1973) 61–90.
[132] S. Gatt, A. Herzl, Y. Barenholz, Hydrolysis of sphingomyelin liposomes by sphingomyelinase, FEBS Lett. 30 (1973) 281–285.
[133] P.K. Working, A.D. Dayan, Pharmacological-toxicological expert report. CAELYX. (Stealth liposomal doxorubicin HCl), Hum. Exp. Toxicol. 15 (1996) 751–785.
[134] H. Shmeeda, S. Even-Chen, R. Honen, R. Cohen, C. Weintraub, Y. Barenholz, Enzymatic assays for quality control and pharmacokinetics of liposome formulations: comparison with nonenzymatic conventional methodologies, Methods Enzymol. 367 (2003) 272–292.

British Journal of Cancer (2007) 96, 21 – 28

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clinical studies

Schedule-dependent activity of 5-fluorouracil and irinotecan combination in the treatment of human colorectal cancer: *in vitro* evidence and a phase I dose-escalating clinical trial

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Several schedules of 5-fluorouracil (FU) and irinotecan (IRI) have been shown to improve overall survival in advanced colorectal cancer (CRC). Preclinical evidence suggests that the sequential administration of IRI and FU produces synergistic activity, although their clinical use has not been fully optimised. We investigated the interaction between short-term exposure to SN-38, the active metabolite of IRI, and prolonged exposure to FU in human CRC HT-29 cells and observed that the synergism of action between the two agents can be increased by extending the time of cell exposure to FU and reducing the interval between administration of the two agents. Based on these findings, we performed a phase I trial in 25 advanced CRC patients using a modified IRI/FU regimen as first-line therapy and evaluated three dose levels of IRI (150–300 mg/m^2) and two of continuous infusion of FU (800–1000 mg/m^2) in a 3-weekly schedule. The most severe grade III–IV toxicities were neutropoenia in four cycles and diarrhoea in three. One patient achieved complete response (4%), 12 a partial response (48%), the overall response rate was 52% (±20, 95% CI); seven of 25 patients had stable disease (28%), the overall disease control was 80% (±16, 95% CI). This modified IRI/FU schedule is feasible and exhibits potentially interesting clinical activity.

British Journal of Cancer (2007) **96,** 21 – 28. doi:10.1038/sj.bjc.6603496 www.bjcancer.com
Published online 12 December 2006

Keywords: 5-fluorouracil; irinotecan; SN-38; colon carcinoma cells; phase I trial

Combination therapy with FU, IRI and oxaliplatin (l-OHP) administered in two- or three-drug regimens is the mainstay of treatment for advanced CRC (Venook, 2005; O'Neil and Goldberg, 2005). Indeed, regimens combining FU with IRI or l-OHP are equally effective in terms of response rate and overall survival (Tournigand *et al*, 2004) and represent the standard first-line treatment in advanced CRC (O'Neil and Goldberg, 2005). Although three combinations of IRI and FU (IFL, FOLFIRI and AIO + IRI) have been evaluated in phase III studies (Saltz *et al*, 2000; Douillard *et al*, 2000; Köhne *et al*, 2005), several other schedules have been proposed (Venook, 2005; Atalay *et al*, 2003). However, no randomised clinical trials have compared these different FU/IRI schedules, so that we do not know whether one regimen is better than others.

The most effective regimens combining FU and IRI were designed on the basis of preclinical evidence suggesting that the antiproliferative activity of the two agents is schedule-dependent (Guichard *et al*, 1997; Guichard *et al*, 1998; Mullany *et al*, 1998). Indeed, the administration of IRI before FU produced additive or synergistic effects in all colon carcinoma cell lines tested (Guichard *et al*, 1998; Mans *et al*, 1999), whereas both the exposure of cells to FU before IRI and the simultaneous administration of both drugs produced antagonistic or only additive activity, depending on the colon tumour cell model (Mans *et al*, 1999). Similar findings were also reported *in vivo*, in athymic mice xenografts of colon carcinoma cells (Guichard *et al*, 1997).

Several combination regimens with FU and IRI have been evaluated as first-line therapy for advanced CRC, achieving a response rate of 30–50% and an overall survival of 14–20 months (Venook, 2005; O'Neil and Goldberg, 2005). Interestingly, the most commonly used schedules – that is the FOLFIRI and IFL regimens – consist in the sequential administration of IRI followed by FU bolus and/or continuous infusion (c.i.). However, considering that the half-life of IRI is about 10 h (Robert and Rivory, 1998), both regimens also combine the two drugs simultaneously in a weekly (IFL regimen) or bi-weekly (FOLFIRI regimen) schedule (Douillard *et al*, 2000; Saltz *et al*, 2000). While there is no clinical evidence to prove that the drug interactions observed in preclinical models also occur in humans and may affect the effectiveness of FU- and IRI-based chemotherapy, the results obtained *in vitro* clearly indicate a schedule-dependency of the interaction between the two agents and suggest the possibility of improving the efficacy of the combination. We therefore looked more closely at the

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Received 15 August 2006; revised 26 October 2006; accepted 29 October 2006; published online 12 December 2006

dependency of the synergism between FU and SN-38, the active metabolite of IRI, on the extent of cell exposure to FU and the interval between the two drugs in human colon carcinoma HT-29 cells and translated the findings obtained *in vitro* into clinical experience, evaluating a modified IRI/FU regimen in a phase I trial.

PATIENTS AND METHODS

Cell Cultures and Chemicals. HT-29 human colon carcinoma cells were cultured in DMEM containing 10% foetal bovine serum, glutamine and Penicillin/Streptomycin (Sigma-Aldrich, Italy) (Khatib *et al*, 2001). SN-38 was kindly provided by Aventis Inc., Paris, France. Stock solution of SN-38 was prepared in DMSO at 10 mM. FU was purchased from Sigma-Aldrich and diluted in phosphate buffer saline. Either drugs or the same DMSO volume were added to the cultures at the concentrations specified in the Results; incubation was carried out continuously and fresh drug-containing medium was changed at 24-h intervals.

The cell-cycle phase distribution and the rate of necrosis and apoptosis were evaluated as previously reported (Landriscina *et al*, 2000; Sciamanna *et al*, 2005).

In order to select HT-29 colon carcinoma cells resistant to FU (HT29 FUR) or to SN-38 (HT29 SN-38R), cells were continuously incubated in the presence of increasing concentrations of FU and SN-38 starting from 0.1 nM for both drugs (Lesuffleur *et al*, 1991). Cells were finally stabilised in the presence of 9 μM FU and 100 nM SN-38. The resistance to each drug was assessed by MTT dye assay (see below) and by measuring apoptosis in the presence of increasing concentrations of FU or SN-38.

Immunoblot analysis and cytotoxicity assay

Immunoblot analysis was performed as previously reported (Landriscina *et al*, 2000). Specific bands were revealed using a mouse monoclonal anti-thymidylate synthase (TS) antibody (Histoline Laboratories, Italy).

Growth inhibition by cytotoxic agents was measured using the MTT (Sigma-Aldrich, Italy) dye assay as previously described (Zeghari-Squalli *et al*, 1999). Briefly, 10^4 cells were seeded into 24-well plates and incubated, 24 h later, in the presence of increasing concentrations of FU (10^{-4}–100 μM) or SN-38 (10^{-6}–10 μM) as specified in the Results. Three independent cytotoxicity assays were used to calculate EC_{50} SN-38 and EC_{50} FU. Combination assays were performed using EC_{50} SN-38 (0.11 μM) or FU (5.2 μM) with increasing concentrations of FU or SN-38, respectively. SN-38 was always administered for 6 h, whereas FU was administered for 24–96 h, as specified in the Results. In the sequential schedule, the second drug was administered immediately after the first drug or, in some experiments, after an incubation of cells in a drug-free medium for 24–96 h. After the removal of both drugs, cells were incubated in a drug-free medium for 72 h and 50 μl of a 125 μM MTT solution were then added to each well. The plates were incubated for additional 3 h at 37°C to allow MTT metabolism into formazan crystals. The formazan crystals were finally solubilised by adding 200 μl of 0.04 N HCl in isopropanol to each microplate well. Adsorbance at 540 nm was measured using a Bio-Tek microplate reader (model EL-340; BioMetallics, Priceston, NJ, USA). Wells containing only DMEM, 10% FBS and MTT were used as controls. Each experiment was performed at least three times, using four replicates for each drug concentration.

Analysis of combination effects

Combination analysis was performed using the method described by Chou *et al*. (Chou and Talalay, 1984; Chou *et al*, 1994). The influence of the two drugs on the combination was evaluated by comparing the sequential assay with assays involving the two drugs simultaneously or alone. The combination effect was evaluated from isoeffect analysis CIs, calculated as follows:

$$CI = C_{FU}/Cx_{FU} + C_{SN-38}/Cx_{SN-38}$$

where Cx_{FU} and $Cx_{SN\,38}$ are respectively the concentrations of FU and SN-38 alone needed to achieve a given effect (x%) and C_{FU} and C_{SN-38} are the concentrations of the two drugs needed to obtain the same effect when FU and SN-38 are combined. The CIs were calculated under the assumption of a mutually exclusive drug interaction. The combination was considered as positive (synergistic) when the combination index was <1 and negative (antagonistic) when it was >1.

Patients

Patients with locally advanced or metastatic CRC, histologically or cytologically proven, were eligible for this study. Minimum age for enrolment was 18 years. Other requisites of eligibility were disease measurability according to RECIST criteria (Therasse *et al*, 2000); PS (ECOG) 0–2; adequate organ function: white blood cell count $>3000/\mu$l, platelet count $>100\,000/\mu$l, Hgb >12 g/dl, creatinine <1.5 mg/dl, total bilirubin <2 mg/dl and transaminase levels <3 times upper normal limits. FU- and folinic acid (LFA)-based adjuvant chemotherapy and prior surgery for primary tumour were allowed. All patients were informed of the investigational nature of the study and expressed written informed consent. The study was approved by the local Ethics Committee.

Clinical study design

To translate the results of the preclinical part of this study, an open-label, dose-escalating phase I trial was designed in which groups of four to six patients were to receive increasing doses of IRI and c.i. FU until dose-limiting toxicity (DLT) was demonstrated in at least two of six patients. Four dose levels of IRI and two of FU were evaluated in this study. We obtained five cohorts of patients: (i) IRI 150 mg/m^2 and FU 800 mg/m^2 as a 4-day c.i., (ii) IRI 200 mg/m^2 and FU 800 mg/m^2 as a 4-day c.i., (iii) IRI 250 mg/m^2 and FU 800 mg/m^2 as a 4-day c.i., (iv) IRI 250 mg/m^2 and FU 1000 mg/m^2 as a 5-day c.i. and (v) IRI 300 mg/m^2 and FU 1000 mg/m^2 as a 5-day c.i. IRI was administered intravenously as 1 h infusion, while FU was given starting 24 h after IRI administration by means of portable infusion pumps in an outpatient setting. Cycles were repeated every 3 weeks until the maximum number of 12 was reached.

Toxicity was evaluated according to NCI-CTC version 2. DLT was defined as the occurrence of grade III nonhaematological toxicity, except alopecia, asthaenia, nausea and vomiting, or grade IV neutropoenia complicated by fever or lasting for more than 5 days, or grade IV thrombocytopoenia, or a delay of more than 2 weeks in treatment due to toxicity. A minimum of four patients was observed for at least one complete cycle of combination therapy before escalating to the next dose level. In the event that fewer than two patients experienced DLT at the same dose level, a minimum of four patients were entered at the next higher dose level. All patients in the prior cohort were required to have completed one cycle of therapy before enrolment in the next cohort began. If two instances of DLT were observed at any dose level, the maximum tolerated dose (MTD) was considered to have been exceeded and a total of six patients were to be treated at the previous dose level to confirm its tolerability.

Response was evaluated after four cycles according to RECIST criteria and confirmed within 6 weeks. Patients received up to 12 cycles of chemotherapy provided they had stable disease, partial or complete response. Treatment was interrupted in the event of

unacceptable toxicity, disease progression, patient refusal or physician's decision.

RESULTS

The synergism between SN-38 and FU *in vitro* depends on the extent of cell exposure to FU and the interval between administration of the two drugs

In preliminary experiments, we confirmed in human colon carcinoma HT-29 cells the well known evidence that the synergism between FU and SN-38 is schedule-dependent, with maximal supra-additive effect when SN-38 is administered first (Guichard *et al*, 1997; Guichard *et al*, 1998; Mullany *et al*, 1998). Cells were therefore exposed to (i) SN-38 alone for 6 h, FU alone for 24 h, (ii) the simultaneous combination of the two drugs (SN-38 and FU for 6 h followed by FU for 18 h) or (iii) both sequential combinations of the two drugs (SN-38 for 6 h and FU for 24 h and vice versa). The CI isobologram equation was used for the analysis of the interaction between SN-38 and FU (Chou and Talalay, 1984; Chou *et al*, 1994).

Figure 1A illustrates the results obtained after exposure of HT-29 cells (i) to increasing concentrations of FU alone (10^{-4}–100 μM) or (ii) to the combination of EC_{30} SN-38 (0.11 μM) and increasing concentrations of FU using both sequences or after concomitant exposure to EC_{30} SN-38 and increasing concentrations of FU.

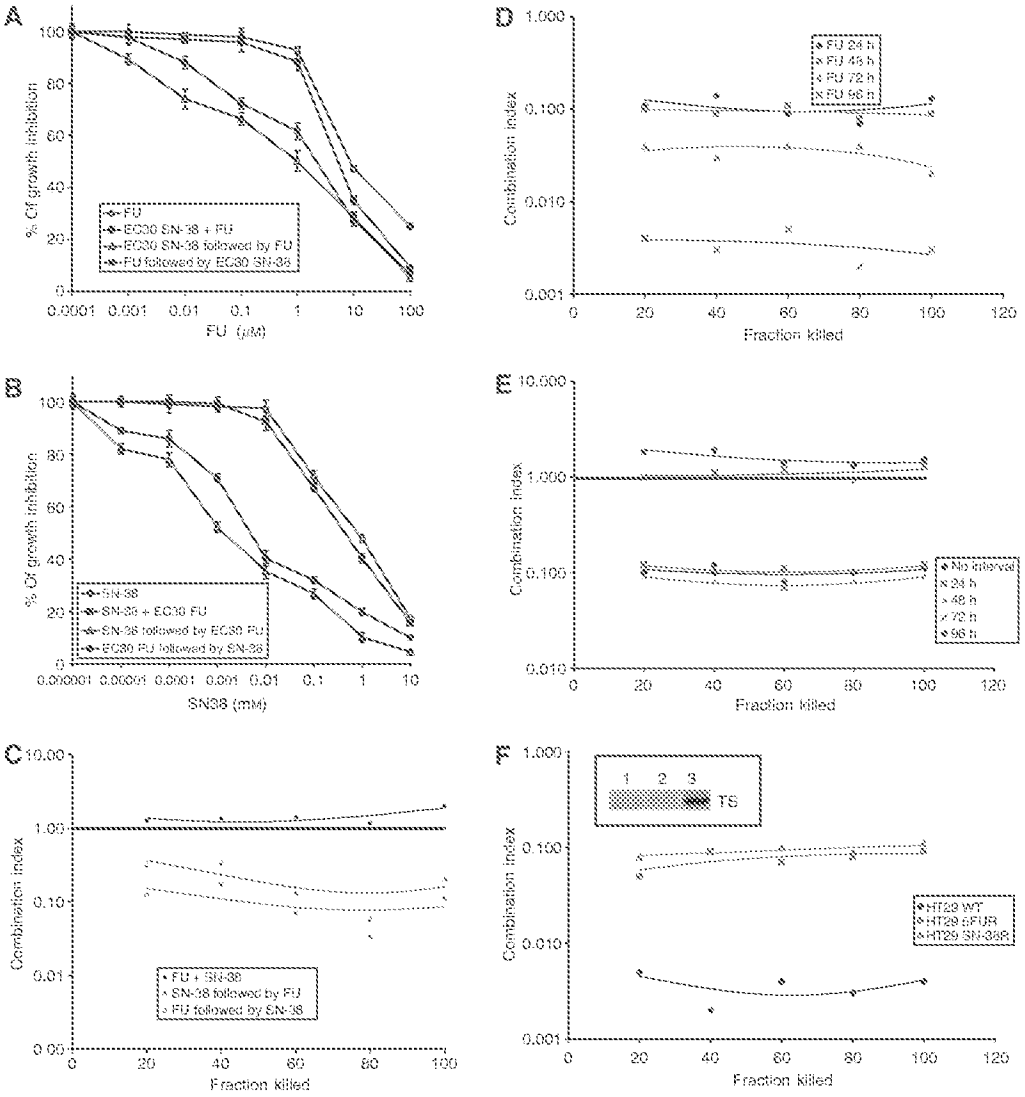

Figure 1 The schedule-dependent synergism between SN-38 and FU. (**A**) Cytotoxicity evaluated by MTT assay after exposure of human colon carcinoma HT-29 cells to increasing concentrations of FU (10^{-4}–100 μM) for 24 h, the sequential combination of EC_{30} SN-38 (0.11 μM) for 6 h and increasing concentrations of FU for 24 h using both sequences and the concomitant treatment with EC_{30} SN-38 for 6 h and increasing concentrations of FU for 24 h. (**B**) Cytotoxicity evaluated by MTT assay after exposure of HT-29 to increasing concentrations of SN-38 (10^{-6}–10 μM) for 6 h, the sequential combination of EC_{30} FU (5.2 μM) for 24 h and increasing concentrations of SN-38 for 6 h using both sequences and the concomitant treatment with EC_{30} FU for 24 h and increasing concentrations of SN-38 for 6 h. (**C**) Plot of the CIs *vs* the cytotoxicity, calculated from data reported in (**A**,**B**), using the methods described by Chou *et al* (20–21) and under the assumption of a mutually exclusive drug interaction. CI > 1, antagonism; CI = 1, additive effect; CI < 1, synergism. (**D**) Plot of the CIs *vs* the cytotoxicity calculated from MTT assay data of HT-29 cells exposed sequentially to SN-38 for 6 h followed by FU for 24, 48, 72 and 96 h. (**E**) Plot of the CIs *vs* the cytotoxicity calculated from MTT assay data of HT-29 cells exposed to SN-38 for 6 h followed by FU for 24 h after an interval between the two drugs of 0, 24, 48, 72 and 96 h during which cells were incubated in a drug-free medium. (**F**) Plot of the CIs *vs* the cytotoxicity obtained from MTT assay data of HT-29, HT-29 SN-38R and HT-29 FUR cells exposed to the sequence of SN-38 followed by FU for 96 h. Insert: Thymidylate synthase (TS) protein expression in wild-type HT-29 (line 1), HT-29 SN-38R (line 2) and HT-29 FUR (line 3) cells.

Figure 1B shows HT-29 cells grown in the presence of increasing concentrations of SN-38 alone (10^{-6}–10 μM), EC_{50} FU (5.2 μM) and increasing concentrations of SN-38 in both sequences or concomitantly with EC_{50} FU and increasing concentrations of SN-38. As reported in Figure 1C, the maximal synergism between FU and SN-38 was achieved when SN-38 was administered before FU (green line). However, a synergism of action was also observed with the reverse sequence (red line), whereas only an additive effect was found with the concomitant exposure of HT-29 cells to both drugs (black line).

As in clinical experience IRI is always administered as a short infusion, while FU is administered either by bolus or as c.i. (Venook, 2005; O'Neil and Goldberg, 2005), we designed specific experiments to evaluate the possibility of improving the synergism between SN-38 and FU by prolonging the extent of cell exposure to FU. We therefore evaluated the synergism between a brief exposure (6 h) to SN-38 followed by a longer exposure to FU (24–96 h). As reported in Figure 1D, the synergism between the two drugs was increased by prolonging the exposure of cells to FU, reaching the maximal activity when cells were exposed to FU for 96 h (blue line).

In a third set of experiments (Figure 1E), HT-29 cells were exposed to the sequence of SN-38 for 6 h followed by FU for 24 h, but the second drug was added to the cell culture immediately after (black line) or after an interval of 24 (red line), 48 (green line), 72 (blue line) or 96 h (brown line) during which cells were incubated in a drug-free medium. Interestingly, the synergism between SN-38 and FU was unchanged whether the drug-free interval between SN-38 and FU was 24 or 48 h. In contrast, when cells were exposed to the sequence of SN-38 and FU with a drug-free interval of 72 or 96 h in between, the combination of the two cytotoxic agents obtained only additive effects.

The sequential combination of SN-38 and FU results in an increase in apoptosis and S-phase of the cell cycle

HT-29 cells were treated with the two drugs alone or with the combination of SN-38 and FU and evaluated for the rate of apoptosis and necrosis and the cell cycle distribution. Cells treated with increasing concentrations of SN-38 (0.001–1 μM) and FU (0.1–100 μM) revealed a dose-dependent increase in apoptosis which is maximal at 100 nM SN-38 and 10 μM FU (data not shown). Cells were exposed to 10 nM SN-38 or 1 μM FU for 24 h, the combination of both drugs for 24 h or sequentially exposed to both drugs for 24 h. The sequential exposure of cells to SN-38 before FU elicited the maximal increase in apoptosis, while the reverse sequence produced intermediate levels of apoptosis compared with the simultaneous exposure (Table 1). Interestingly, when the cells were sequentially exposed to the two agents with an incubation of 96 h in a drug-free medium after SN-38, the rate of apoptosis was significantly lower and was comparable to that observed with the simultaneous exposure (Table 1).

The cell cycle distribution in HT-29 cells exposed to (i) 1 μM FU for 24 h, (ii) 10 nM SN-38 for 24 h, (iii) the combination of both drugs for 24 h and (iv) the sequence of both drugs for 24 h was further evaluated. FU produced an arrest of cells in S-phase of the cell cycle, whereas SN-38 produced an arrest in the G2-M phase (Table 2). Interestingly, HT-29 colon carcinoma cells sequentially exposed to SN-38 followed by FU exhibited a significantly higher increase in the S-phase fraction with no arrest in the G2–M phase, while HT-29 cells exposed sequentially to FU followed by SN-38 exhibited an arrest of the cell cycle in both S- and G2–M phases with a magnitude similar to that produced by the single agents. In contrast, cells simultaneously exposed to SN-38 and FU exhibited an arrest of the cell cycle in the S-phase similar to that induced by FU alone, but not in the G2–M phase (Table 2).

The synergism between SN-38 and FU is partially conserved in colon carcinoma HT-29 cells resistant to FU or SN-38

In order to evaluate whether the synergism between SN-38 and FU is able to overcome resistance to the individual agents, we obtained HT-29 cells resistant to SN-38 (HT-29 SN-38R) or FU (HT-29 FUR) and evaluated the interaction between the two drugs in both cell lines. HT-29 SN-38R and HT-29 FUR cells required concentrations of SN-38 or FU about 10 times higher than wild-type HT-29 cells to exhibit similar rates of cytotoxicity or apoptosis (data not shown). Moreover, HT-29 FUR cells exhibited increased protein levels of TS (Figure 1D, inset), the molecular target of FU (van Triest *et al*, 1999), which is in agreement with the well known observation that the upregulation of TS is responsible for resistance to FU (Wong *et al*, 2001). The cytotoxicity of the sequence of SN-38 followed by FU for 96 h was evaluated in HT-29, HT-29 SN-38R and HT-29 FUR cells. In single agent-resistant cells the synergism between SN-38 and FU was still observed, albeit with a magnitude lower than that obtained in wild-type HT29 cells (Figure 1F).

Patient population, toxicity and clinical activity

Between January 2003 and December 2004 25 patients were enrolled in the clinical study. Patients' characteristics are listed in Table 3, while toxicities are reported in Table 4. A total of 203 cycles was administered without observing treatment-related deaths. We reported three DLTs, all of which were grade III diarrhoea, in separate patients. The first patient at step 3 resumed treatment at a dose of 75% after recovering from grade II nausea/vomiting, while grade III diarrhoea occurred during the first cycle and was not repeated after dose reduction. The second and the third patients experienced grade III diarrhoea at the second and fourth cycle, respectively, and resumed treatment at full doses with no further grade III toxicities. As we did not observe a minimum of two DLTs at the same dose level, the MTD was not reached. Grade

Table 2 Cell cycle distribution of human colon carcinoma HT-29 cells exposed to different combinations of SN-38 and FU

	G0–G1	S	G2–M
Control	62.8±0.8	27.0±0.9	10.2±1.0
10 nM SN-38	42.8±1.1	31.5±1.4	25.7±0.9
1 μM FU	45.9±1.4	44.1±1.6	10.0±0.7
10 nM SN-38+1 μM FU	40.9±1.6	46.0±0.7	13.1±1.3
10 nM SN-38→1 μM FU	37.2±1.4	60.8±1.9	2.0±0.2
1 μM FU→10 nM SN-38	34.5±1.1	45.4±2.3	20.1±1.2

Table 1 Analysis of cell viability in human colon carcinoma HT-29 cells exposed to different combinations of SN-38 and FU

	Control	10 nM SN-38	1 μM FU	SN-38+FU	SN-38→FU	FU→SN-38	SN-38→96 h→FU
Viable cells	92.0±1.7	87.3±1.3	84.6±1.9	71.7±1.3	59.7±1.9	66.3±2.3	74.6±1.7
Apoptosis	6.8±0.8	11.9±0.7	13.1±0.5	26.5±0.9	38.9±0.5	31.9±0.7	23.9±1.5
Necrosis	1.2±0.1	0.8±0.4	2.3±0.3	1.8±1.1	1.4±0.3	1.8±0.5	1.5±0.7

III neutropoenia was recorded in four patients and was managed with G-CSF administration. Mucositis and gastrointestinal toxicity (i.e. diarrhoea and nausea/vomiting) were the most relevant and frequently observed grade I/II toxicities; they were generally mild and rapidly reversible. The treatment was never interrupted due to toxicity.

Efficacy data are summarised in Table 5. The most remarkable result is 1 complete response, which lasted over 10 months. Twelve patients achieved a partial response (48%). Stable disease was observed in seven patients (28%), while progressive disease was recorded in five patients (20%). Overall response and disease control rates were 52% (±20, 95% CI) and 80% (±16, 95% CI), respectively. Median time to progression was 7 months (range 2–11 months).

Three patients who had received adjuvant chemotherapy were enrolled in this trial. The first had relapsed 2 months after the end of adjuvant treatment, while the other two relapsed after 24 and 48 months, respectively. The first two patients were enrolled at step 3 and the third at step 5 and, interestingly, all of them achieved a partial response. The first two patients recorded a time to further progression of 9 months, while the third, who also

Table 3 Baseline characteristics of the patients

Patients		**25**	
Age			
Range		35–79	
Median		60	
Sex			
Female		7	
Male		18	
PS ECOG	N		%
0	19		76
1	5		20
2	1		4
Previous surgery			
Resection/colectomy		18	
Bypass		3	
None		4	
Adjuvant CT		5	
Metastatic site	N		%
Liver	21		84.0
Lung	3		13.0
Nodes	3		13.0
Peritoneum	4		16.0
Other	2		8.7

Table 5 Overall and partial response rates in patients who received different dose levels of the combination of FU and IRI

	CR	**PR**	**S**	**PD**
Step 1	0	0	2	2
Step 2	0	2	1	1
Step 3	0	4	0	1
Step 4	0	3	3	0
Step 5	1	3	1	1
Total	1	12	7	5
%	4	48	28	20
Response rate (%)		52		
Disease control rate (%)		80		

Table 4 Adverse events in patients who received different dose levels of the combination of FU and IRI

	Level 1 (27 cycles)	**Level 2 (29 cycles)**	**Level 3 (45 cycles)**	**Level 4 (55 cycles)**	**Level 5 (47 cycles)**	**Total (203 cycles)**
Hematological						
Anaemia						
Grade I–II	0	0	0	1 (1.8%)	0	1 (0.5%)
Grade III–IV	0	0	0	0	0	0
Neutropoenia						
Grade I–II	1 (3.7%)	0	0	0	0	1 (0.5%)
Grade III–IV	0	0	3 (6.6%)	1 (1.8%)	0	4 (2.0%)
Nonhaematological						
Nausea/Vomiting						
Grade I–II	3 (11.1%)	4 (13.8%)	10 (22.2%)	10 (18.2%)	9 (19.1%)	36 (17.7%)
Grade III–IV	0	0	0	1 (1.8%)	0	1 (0.5%)
Diarrhoea						
Grade I–II	8 (29.6%)	6 (20.7%)	11 (24.4%)	7 (12.7%)	9 (19.1%)	41 (20.2%)
Grade III–IV	0	0	1 (2.2%)	1 (1.8%)	1 (2.1%)	3 (1.5%)
Stipsis						
Grade I–II	0	0	0	4 (7.3%)	0	4 (2.0%)
Grade III–IV	0	0	0	0	0	0
Stomatitis						
Grade I–II	1 (3.7%)	1 (3.4%)	9 (20.0%)	7 (12.7%)	2 (3.6%)	20 (9.8%)
Grade III–IV	0	0	0	0	0	0
Fatigue						
Grade I–II	4 (14.8%)	1 (3.4%)	1 (2.2%)	2 (3.6%)	2 (3.6%)	10 (4.9%)
Grade III–IV	0	0	0	0	0	0
Flu-like syndrome						
Grade I–II	1 (3.7%)	0	1 (2.2%)	1 (1.8%)	0	3 (1.5%)

received radiation therapy on a juxta-vertebral lymph-node metastasis, was progression-free at the time of this statistical analysis (11 months after the end of chemotherapy).

Most patients had a good PS at the end of treatment and all of them received second-line treatment.

DISCUSSION

Over the past 10 years the treatment of advanced CRC has progressed dramatically, with a shift from monotherapy to combination therapy and, more recently, to sequential combination therapy (Venook, 2005; O'Neil and Goldberg, 2005; Kelly and Goldberg, 2005). The introduction of IRI and l-OHP in the first- and second-line setting has increased the complexity of delivery of care to patients. Moreover, the recent development of molecular-targeted agents that are tumour-specific and have different toxicity profiles from chemotherapeutic agents has further widened the range of therapies for this disease (Venook, 2005; O'Neil and Goldberg, 2005). As these more efficacious agents allow patients to survive longer and to receive more lines of therapy, issues have arisen concerning the choice of the best schedule and the best sequence of treatments. However, while most of the regimens combining l-OHP and FU differ only marginally, the combinations of IRI and FU are characterised by major differences in terms of doses and schedules (Venook, 2005; O'Neil and Goldberg, 2005; Tournigand *et al*, 2004; Saltz *et al*, 2000; Douillard *et al*, 2000).

In the present study we investigated, at preclinical level, the interaction between FU and SN-38, the active metabolite of IRI, in order to obtain *in vitro* evidence for optimising chemotherapeutic schedules. We observed that (i) the sequential exposure of colon carcinoma cells to the two agents produces a supra-additive effect with maximal cytotoxic activity when cells are pre-exposed to SN-38 before FU, (ii) this synergism of action is partially conserved in colon carcinoma cells resistant to SN-38 or FU, and, interestingly, (iii) it is possible to strengthen this synergism of action further by prolonging the exposure of tumour cells to FU and by administering the two agents sequentially with minimal interval in between.

Other preclinical studies previously suggested that preincubation of colon carcinoma cells with IRI before FU enhances the incorporation of FU derivatives into the DNA and DNA–protein complexes with a parallel and more persistent decrease in TS activity (Guichard *et al*, 1998). Furthermore, increased DNA damage was also observed in SW620 and HT-29 colon carcinoma cells when cells were pre-exposed to IRI before FU (Mans *et al*, 1999). These results are in agreement with our findings that the sequential exposure of colon carcinoma cells to SN-38 before FU produces a significant increase either in apoptosis or in the S-phase arrest. Indeed, while FU produced an arrest of cells in S-phase of the cell cycle and SN-38 produced an arrest in the phase G2–M, as previously reported (Mullany *et al*, 1998; Yoshikawa *et al*, 2001; McDonald and Brown, 1998), tumour cells sequentially exposed to SN-38 followed by FU exhibited a significantly higher increase in the S-phase fraction with no arrest in the G2–M phase. Thus, it is likely that preincubation of colon carcinoma cells with SN-38 facilitates in turn a more prolonged inhibition of TS by FU, an increase in the incorporation of FU derivatives into DNA, an enhanced and persistent S-phase arrest and apoptotic cell death. This hypothetical mechanism of action provides a molecular rationale to our results showing that the synergistic activity of the SN-38 and FU sequence is partially conserved in colon carcinoma cells resistant to FU and characterised by increased levels of TS. It is also in agreement with the clinical observation that the FU- and IRI-based combination therapy is effective in patients pretreated with FU (Andre *et al*, 1999) and whose tumours are generally characterised by increased levels of TS (Wong *et al*, 2001) as well as with our results obtained in three patients previously treated with FU-based adjuvant chemotherapy who achieved partial response with this modified FU/IRI regimen. Furthermore, our results suggest that IRI-resistant CRC cells may be more sensitive to schedules with c.i. FU, although the molecular mechanism of this synergism is still unclear.

The evidence that pre-incubation of HT-29 colon carcinoma cells with FU before SN-38 achieves synergism of action is partially in contrast with results reported by other authors, which suggest that the sequential exposure of cells to FU before IRI produces only additive activity (Mans *et al*, 1999). These differences may depend on the specific colon tumour cell model used.

Based on these preclinical findings and considering the low toxicity profile of infusional FU (Poplin *et al*, 2005), we designed a modified IRI/FU schedule with IRI administered on day 1 followed by a 4- or 5-day infusion of FU. We tested this alternative FU/IRI-based regimen in a phase I trial, evaluating three dose levels of IRI and two of FU in a 3-weekly schedule. Compared with the commonly used two-drug regimens (Venook, 2005), our schedule proved feasible and did not increase either haematological toxicity or the rate of high-grade diarrhoea and stomatitis. This is even more relevant in view of the toxicity profile of some traditional combination regimens of IRI and FU (i.e. IFL) in which a large proportion of patients experienced grade III–IV haematological and nonhaematological toxicity (Saltz *et al*, 2000). Although the MTD was not reached it is unlikely that this depends on the dose levels of IRI and FU. Indeed, in our modified IRI/FU schedule the theoretical weekly dose intensities of IRI and FU at the highest dose levels are 90 and 1660 mg/m^2, respectively, very similar to the dose intensities of IRI and FU in the FOLFIRI and IFL regimens (Saltz *et al*, 2000; Douillard *et al*, 2000). Thus, taking into account that the rate of treatment delays or dose reductions reported in our study is low, it is reasonable to speculate that, at least at the highest dose levels, IRI and FU are not under-dosed.

Our regimen, tested as first-line treatment in 25 patients with advanced CRC, obtained a response rate >50%, a disease control rate of 80% and a time to progression of 7 months. These results are promising even though they were achieved in a dose-escalating phase I trial whose major aim was not to evaluate the antitumour activity. Taking into account that at the first dose level, we did not observe any response, probably because IRI was under-dosed, these findings are even more significant. Indeed, if only patients enrolled between the second and fifth dose levels are considered, the overall response and disease control rates reached 61.9 and 85.7%, respectively. Similar results were recently achieved in a phase I dose-escalating trial of IRI and c.i. FU as first-line treatment of metastatic colorectal cancer. Interestingly, the combination was well tolerated and demonstrated a significant clinical activity, obtaining an overall response rate of 55%, a clinical response benefit of 82% and a time to progression of 8 months (Saunders *et al*, 2004). Thus, the results of our study clearly suggest that the schedule of administration of the two drugs is critical to achieve the maximal supra-additive cytotoxic activity and that the lack of full synergism in some traditional schedules of IRI and FU may depend on the use of bolus FU (i.e. IFL) (Saltz *et al*, 2000) and/or the need to optimise the sequence of administration of the two agents (i.e. FOLFIRI) (Douillard *et al*, 2000).

Several options have been proposed to improve the efficacy of standard two-drug regimens for advanced CRC (Venook, 2005). Some studies have evaluated the combination of IRI, l-OHP and FU concurrently in a single regimen, the rationale being that nonspecific resistance to therapy may develop after first-line therapy. Indeed, these studies demonstrated that three-drug regimens achieve very high response rates (50–70%), but also DLTs such as neutropoenia and diarrhoea (Souglakos *et al*, 2002; Goetz *et al*, 2003). Other studies have evaluated the combination of FU with l-OHP and/or IRI administered as chronomodulated infusion. These trials also reported interesting response rates and

optimal toxicity profiles, but raised questions about the feasibility of chronomodulated chemotherapy (Garufi *et al*, 2003). However, the analysis of seven phase III trials in advanced CRC suggested that exposure to all three drugs, regardless of their sequence, is a key element able to extend the overall survival of patients to 18–21 months (Grothey *et al*, 2004). Such a prospect strongly reinforces the need to optimise doses and schedules of doublet chemotherapy, in order to deliver the three drugs sequentially and obtain maximal cytotoxic activity and minimal toxicity. Moreover, these efforts seem even more relevant in the light of the introduction in the clinical management of advanced CRC of new molecular-targeted agents with a cytostatic mechanism of action requiring precise timing when combined with traditional chemotherapeutic drugs to maximise their efficacy (Venook, 2005). The combination of IRI and cetuximab represents a salvage chemotherapy in IRI-resistant patients (Venook, 2005), while the inclusion of bevacizumab in IRI/FU combination has increased both response rates and overall survival (Kelly and Goldberg, 2005). Optimising the efficacy of a combination of IRI and FU could help to enhance the overall efficacy of chemotherapy in CRC as first- or second-line therapy. Thus, this modified IRI/FU regimen may represent a precious alternative schedule with very low toxicity profile, promising clinical activity and, therefore, worth being tested in a larger phase II trial.

clinical studies

REFERENCES

Andre T, Louvet C, Maindrault-Goebel F, Couteau C, Mabro M, Lotz JP, Gilles-Amar V, Krulik M, Carola E, Izrael V, de Gramont A (1999) CPT-11 (irinotecan) addition to bimonthly, high-dose leucovorin and bolus and continuous-infusion 5-fluorouracil (FOLFIRI) for pretreated metastatic colorectal cancer. GERCOR. *Eur J Cancer* **35:** 1343–1347

Atalay G, Cardoso F, Paesmans M, Goldberg RM, Bleiberg H (2003) Second-line treatment in advanced colon cancer: are multiple phase II trials informative enough to guide clinical practice? *Anticancer Drugs* **14:** 703–713

Chou TC, Motzer RJ, Tong Y, Bosl GJ (1994) Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rationale approach to clinical protocol design. *J Natl Cancer Inst* **86:** 1517–1524

Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationship: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22:** 27–55

Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, Rougier P (2000) Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomized trial. *Lancet* **355:** 1041–1047

Garufi C, Bria E, Vanni B, Zappala AM, Sperduti I, Terzoli E (2003) A phase II study of irinotecan plus chronomodulated oxaliplatin, 5-fluorouracil and folinic acid in advanced colorectal cancer patients. *Br J Cancer* **89:** 1870–1875

Goetz MP, Erlichman C, Windebank AJ, Reid JM, Sloan JA, Atherton P, Adjei AA, Rubin J, Pitot H, Galanis E, Ames MM, Goldberg RM (2003) Phase I and pharmacokinetic study of two different schedules of oxaliplatin, irinotecan, fluorouracil, and leucovorin in patients with solid tumors. *J Clin Oncol* **21:** 3761–3769

Grothey A, Sargent D, Goldberg RM, Schmoll HJ (2004) Survival of patients with advanced colorectal cancer improves with the availability of fluorouracil-leucovorin, irinotecan, and oxaliplatin in the course of treatment. *J Clin Oncol* **22:** 1209–1214

Guichard S, Cussac D, Hennebelle I, Bugat R, Canal P (1997) Sequence-dependent activity of the irinotecan-5FU combination in human colon-cancer model HT-29 *in vitro* and *in vivo*. *Int J Cancer* **73:** 729–734

Guichard S, Hennebelle I, Bugat R, Canal P (1998) Cellular interaction of 5-fluorouracil and the camptothecin analogue CPT-11 (irinotecan) in human colorectal carcinoma cell line. *Biochem Pharmacol* **55:** 667–676

Kelly H, Goldberg RM (2005) Systemic therapy for metastatic colorectal cancer: current options, current evidence. *J Clin Oncol* **23:** 4553–4560

Khatib AM, Siegfried G, Prat A, Luis J, Chretien M, Metrakos P, Seidah NG (2001) Inhibition of proprotein convertases is associated with loss of growth and tumorigenicity of HT-29 human colon carcinoma cells: importance of insulin-like growth factor-1 (IGF-1) receptor processing in IGF-1-mediated functions. *J Biol Chem* **276:** 30686–30693

Köhne CH, van Cutsem E, Wils J, Bokemeyer C, El-Serafi M, Lutz MP, Lorenz M, Reichardt P, Ruckle-Lanz H, Frickhofen N, Fuchs R, Mergenthaler HG, Langenbuch T, Vanhoefer U, Rougier P, Voigtmann R, Muller L, Genicot B, Anak O, Nordlinger B, European Organisation for Research and Treatment of Cancer Gastrointestinal Group (2005) Phase III study of weekly high-dose infusional fluorouracil plus folinic acid with or without irinotecan in patients with metastatic colorectal cancer: European Organisation for Research and Treatment of Cancer Gastrointestinal Group Study 40986. *J Clin Oncol* **23:** 4856–4865

Landriscina M, Prudovsky I, Mouta Carriera C, Soldi R, Tarantini F, Maciag T (2000) Amlexanox reversibly inhibits cell migration and proliferation and induces the Src-dependent disassembly of actin stress fiber *in vitro*. *J Biol Chem* **275:** 32753–32762

Lesuffleur T, Kornowski A, Luccioni C, Muleris M, Barbat A, Beaumatin J, Dussaulx E, Dutrillaux B, Zweibaum A (1991) Adaptation to 5-fluorouracil of the heterogeneous human colon tumor cell line HT-29 results in the selection of cells committed to differentiation. *Int J Cancer* **49:** 721–730

Mans DRA, Grivicich I, Peters GJ, Schwartsmann G (1999) Sequence-dependent growth inhibition and DNA damage formation by the irinotecan-5-fluorouracil combination in human colon carcinoma cell lines. *Eur J Cancer* **13:** 1851–1861

McDonald AC, Brown R (1998) Induction of p53-dependent and p53-independent cellular responses by topoisomerase I inhibitors. *Br J Cancer* **78:** 745–751

Mullany S, Svingen PA, Kaufmann SH, Erlichman C (1998) Effect of adding the topoisomerase I poison 7-ethyl-10-hydroxycamptothecin (SN-38) to 5-fluorouracil and folinic acid in HCT-8 cells: elevated dTTP pools and enhanced cytotoxicity. *Cancer Chemother Pharmacol* **42:** 391–399

O'Neil BH, Goldberg RM (2005) Chemotherapy for advanced colorectal cancer: let's not forget how we got here (until we really can). *Semin Oncol* **32:** 35–42

Poplin EA, Benedetti JK, Estes NC, Haller DG, Mayer RJ, Goldberg RM, Weiss GR, Rivkin SE, Macdonald JS (2005) Phase III Southwest Oncology Group 9415/Intergroup 0153 randomized trial of fluorouracil, leucovorin, and levamisole *vs* fluorouracil continuous infusion and levamisole for adjuvant treatment of stage III and high-risk stage II colon cancer. *J Clin Oncol* **23:** 1819–1825

Robert J, Rivory L (1998) Pharmacology of irinotecan. *Drugs Today* **34**(9): 777–803

Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N, Elfring GL, Miller LL (2000) Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. *N Engl J Med* **343:** 905–914

Saunders MP, Hogg M, Carrington B, Sjursen AM, Allen J, Beech J, Swindell R, Valle JW (2004) Phase I dose-escalating trial of irinotecan with continuous infusion 5-FU first line, in metastatic colorectal cancer. *Br J Cancer* **91:** 1447–1452

Sciamanna I, Landriscina M, Pittoggi C, Quirino M, Mearelli C, Beraldi R, Mattei E, Serafino A, Cassano A, Sinibaldi-Vallebona P, Garaci E, Barone C, Spadafora C (2005) Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. *Oncogene* **24:** 3923–3931

Souglakos J, Mavroudis D, Kakolyris S, Kouroussis CH, Vardakis N, Androulakis N, Agelaki S, Kalbakis K, Tsetis D, Athanasiadis N, Samonis G, Georgoulias V (2002) Triplet combination with irinotecan plus oxaliplatin plus continuous-infusion fluorouracil and leucovorin as first-line treatment in metastatic colorectal cancer: a multicenter phase II trial. *J Clin Oncol* **20:** 2651–2657

Therasse P, Arbuck SG, Eisenhauer E, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumours. *J Natl Cancer Inst* **92:** 205–216

Tournigand C, Andre T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, de Gramont A (2004) FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* **22:** 229–237

Van Triest B, Pinedo HM, van Hensbergen Y, Smid K, Telleman F, Schoenmakers PS, van der Wilt CL, van Laar JA, Noordhuis P, Jansen G, Peters GJ (1999) Thymidylate synthase level as the main predictive parameter for sensitivity to 5-fluorouracil, but not for folate-based thymidylate synthase inhibitors, in 13 nonselected colon cancer cell lines. *Clin Cancer Res* **5:** 643–654

Venook A (2005) Critical evaluation of current treatments in metastatic colorectal cancer. *The Oncol* **10:** 250–261

Weng NA, Brett L, Stewart M, Leitch A, Longley DB, Dunlop MG, Johnston PG, Lessells AM, Jodrell DI (2001) Nuclear thymidylate synthase expression, p53 expression and 5FU response in colorectal carcinoma. *Br J Cancer* **85:** 1937–1943

Yoshikawa R, Kusunoki M, Yanagi H, Noda M, Furuyama J, Yamamura T, Hashimoto-Tamaoki T (2001) Dual antitumor effects of 5-fluorouracil on the cell cycle in colorectal carcinoma cells: a novel target mechanism concept for pharmacokinetic modulating chemotherapy. *Cancer Res* **61:** 1029–1037

Zeghari-Squalli N, Raymond E, Cvitkovic E, Goldwasser F (1999) Cellular pharmacology of the combination of the DNA topoisomerase I inhibitor SN-38 and the diaminocyclohexane platinum derivative oxaliplatin. *Clin Cancer Res* **5:** 1189–1196

Journal of Chromatography B, 1015–1016 (2016) 34–43

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of an UPLC–MS/MS method for the quantification of irinotecan, SN-38 and SN-38 glucuronide in plasma, urine, feces, liver and kidney: Application to a pharmacokinetic study of irinotecan in rats

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ARTICLE INFO

Article history:
Received 7 December 2015
Received in revised form 4 February 2016
Accepted 6 February 2016
Available online 9 February 2016

Keywords:
Irinotecan
SN-38
Pharmacokinetics
UPLC–MS/MS
SN-38 glucuronide

ABSTRACT

The objective of this research is to develop and validate a sensitive and reproducible UPLC–MS/MS method to quantify irinotecan, its active metabolite SN-38 and SN-38 glucuronide (phase II metabolite of SN-38) simultaneously in different bio-matrices (plasma, urine, feces), tissues (liver and kidney) and to use the method to investigate its pharmacokinetic behavior in rats. Irinotecan, SN-38 and SN-38 glucuronide has been resolved and separated by C18 column using acetonitrile and 0.1% formic acid in water used as the mobile phases. Triple quadruple mass spectrometer using multiple reaction monitoring (MRM) with positive scan mode were employed to perform mass analysis. The results showed that the linear response range of irinotecan and SN-38 in plasma, feces, liver and kidney is 4.88–10000 nM, 39–5000 nM, 48.8–6250 nM and 48.8–6250 nM, respectively ($R^2 > 0.99$). In case of SN-38 glucuronide, the standard curves were linear in the concentration range of 6.25–2000 nM, 4.88–1250 nM, 9.8–1250 nM and 9.8–1250 nM in plasma, feces, liver and kidney homogenates, respectively. The lower limit of detection (LLOD) of irinotecan, SN-38 and SN-38 glucuronide was determined to be less than 25 nM in all bio-matrices as well as tissue homogenates. Recoveries of irinotecan, SN-38 and SN-38 glucuronide at three different concentrations (low, medium and high) were not less than 85% at three different concentrations in plasma and feces. The percentage matrix factors in different bio-matrices and tissues were within 20%. The UPLC–MS/MS method was validated with intra-day and inter-day precision of less than 15% in plasma, feces, liver and kidney. Owing to the high sensitivity of this method, only 20 μl of plasma, urine and homogenates of liver, kidney and feces is needed. The validated method has been successfully employed for pharmacokinetic evaluation of irinotecan in male wistar rats to quantify irinotecan, SN-38 and SN-38 glucuronide in plasma, feces, and urine samples.

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1. Introduction

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecine, CPT-11, Fig. 1B) is an important component of FOLFIRI and FOLFIRINOX chemotherapy regimen which are used as first line treatment against colorectal and advanced pancreatic cancer [1,2]. It has also shown clinical activity against other types of cancers such as lung, ovarian, cervical, gastric, refractory lymphoma and leukemia etc. [3–5]. Irinotecan is a pentacyclic semisynthetic derivative of camptothecin which is isolated from the bark of a tree named *Camptotheca acuminate* in 1966 [6,7]. Irinotecan, through the action of liver carboxylesterase gets converted into its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin, Fig. 1C) which is 100–1000 times as potent as irinotecan [8,9]. Anticancer activity of irinotecan is attributed to the inhibitory effect of SN-38 on DNA topoisomerase I which plays an important role in DNA replication and transcription [10,11]. Formation of an irreversible complex of SN-38, DNA topoisomerase I enzyme and the ligated DNA strand leads to the breakage of double stranded DNA which ultimately causes cell death. Owing to the poor solubility of SN-38, irinotecan is currently used as a prodrug in clinical applications.

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http://dx.doi.org/10.1016/j.jchromb.2016.02.012
1570-0232/Published by Elsevier B.V.

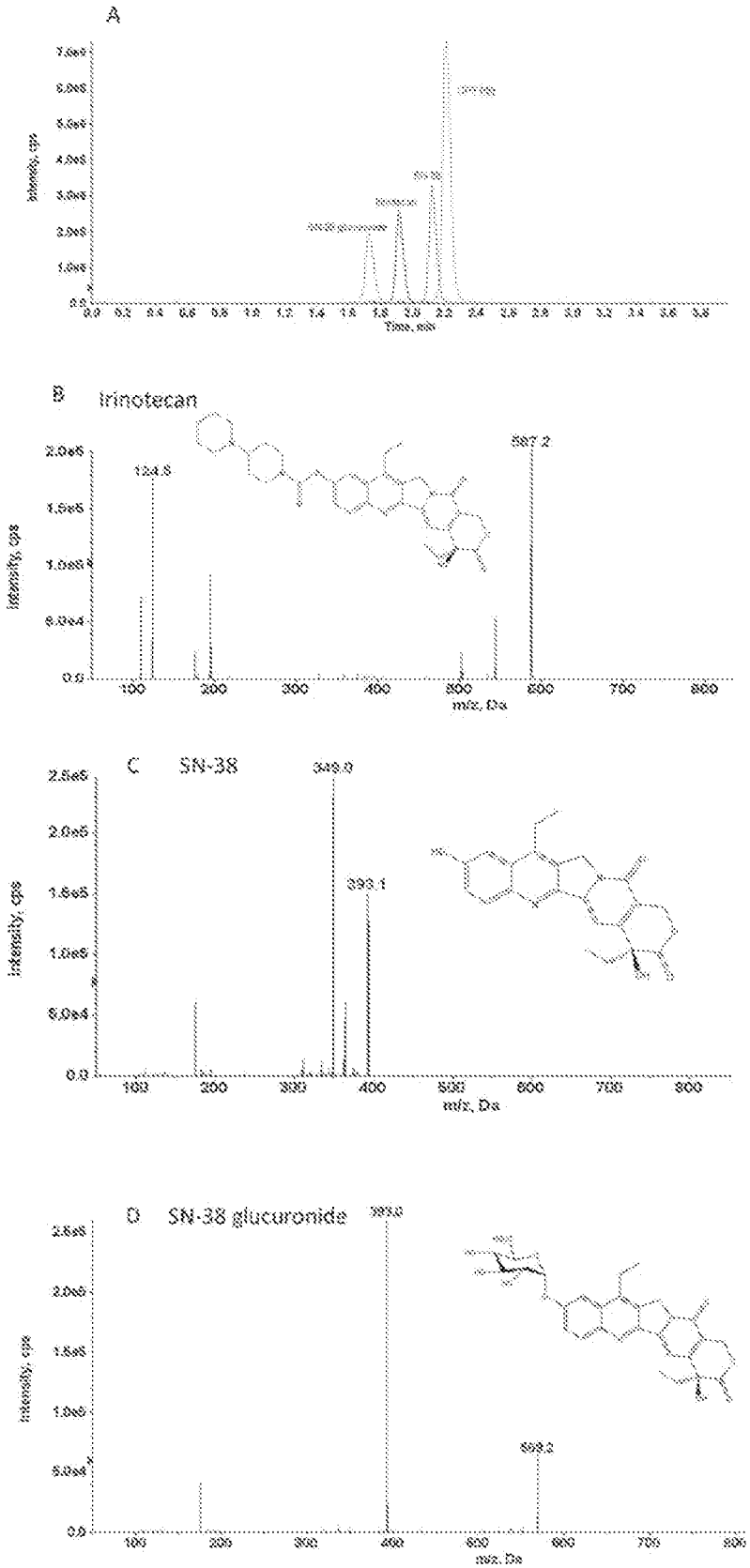

Fig. 1. (A) Representative UPLC chromatogram of irinotecan, SN-38, SN-38 glucuronide and CPT (IS). (B) Chemical structure and representative MS/MS Chromatogram of irinotecan. (C) Chemical structure and representative MS/MS Chromatogram of SN-38. (D) Chemical structure and representative MS/MS Chromatogram of SN-38 glucuronide. (E) Chemical structure and representative MS/MS Chromatogram of CPT (IS).

Irinotecan exhibits a complicated metabolic profile by interacting with various drug metabolizing enzymes as well as efflux transporters. Studies indicated that both biliary as well as intestinal excretion of irinotecan and its metabolites are regarded as significant contributing factors to elevate the colonic SN-38 content which causes late onset diarrhea [12–16]. In spite of knowing the fact that predominant efflux transporters (P-gp and MRP2) mediate the excretion of irinotecan and its metabolites, the role of efflux transporters on the disposition of irinotecan and its metabolites at different metabolic organs (liver, intestine and kidney) has not been clearly defined till date. Quantification of irinotecan and its metabolites in different bio-matrices (bile, plasma, intestinal perfusate and urine) as well as in different metabolic organs (liver and kidney) will allow us to elucidate the role of predominant efflux transporters on the disposition and the intestinal toxicity of irinotecan.

Generally, inactivation of SN-38 to its phase II metabolite SN-38 glucuronide (Fig. 1D) occurs through an enzymatic reaction mediated by the UDP-glucuronosyltransferase 1A1 isoform (UGT1A1) [17,18]. UGT1A1 plays an important role in the disposition as well as in the intestinal toxicity of irinotecan. In the colon, SN-38 glucuronide, after interacting with the bacterial β glucuronidase enzyme secreted from commensal microbiota, can regenerate toxic SN-38 which has the potential to kill the normal cells in the intestine resulting in late onset diarrhea [19]. At present, several compounds are being synthesized which can predominantly inhibit the activity of the bacterial β-glucuronidase resulting in the reduction of the generation of SN-38 from inactive SN-38 glucuronide [20]. In addition, preclinical studies indicated that around 2–22% of SN-38 glucuronide was excreted into the bile over 24 h which was comparatively higher than biliary excretion of SN-38 (7–9%) [15,21]. Similarly clinical study of irinotecan also indicated that around 3% of dose is excreted through urine [12]. So in this regard, direct quantification of SN-38 glucuronide in different biomatrices as well as in major metabolic organs will enable us to get the holistic idea of the disposition of irinotecan.

It has been shown that the intact lactone ring of camptothecin (CPT) derivative is an important factor to retain its anticancer activity [10,11,22]. However, the lactone ring of CPT derivatives undergoes a pH sensitive, reversible hydrolysis and converts into carboxylate form at physiologic pH [23]. Boyd et al. showed that pH played an important role in the inter conversion of lactone and carboxylate forms of CPT in the solution. Experiments showed that at pH 3–5, lactone is the most stable form of CPT, whereas at pH 9, it rapidly converts into carboxylate form [24]. Till date, HPLC methods coupled with fluorescence detectors have been the most predominant method for detection of CPT derivatives as they can detect both lactone and carboxylate forms in different biological matrices.

Different bioanalytical techniques, mostly based on reverse phase liquid chromatography coupled with fluorescence detection, have been proposed to determine irinotecan and SN-38 in various biological matrices. However, these fluorescence methods suffered from several drawbacks such as lengthy preparation time, limited sensitivity and high sample volumes [25–27]. Recently, liquid chromatography coupled with mass spectrometry (LC–MS and LC–MS/MS) has become one of the preferred analytical tools for the rapid and efficient quantification of small and large molecules in different biological matrices due to the unique combination of high specificity, sensitivity and high sample throughput possibilities. Although several LC–MS/MS methods have been developed and implemented for the quantification of irinotecan and SN-38 in rabbit, mouse and human plasma, very few of them developed a UPLC–MS/MS method of SN-38 glucuronide [28–31]. Similarly, though quantification of irinotecan and SN-38 was done in mouse tissues (brain, liver, kidney etc.), there was no report of the quantification of SN-38 glucuronide in these tissues [32,33]. Also, to the best of our knowledge there is no LC–MS/MS method available to quantify irinotecan, SN-38 and SN-38 glucuronide simultaneously in urine and feces. In this study, we developed and validated a simple, rapid and sensitive LC–MS/MS method for simultaneous quantification of total concentration of irinotecan, SN-38 and SN-38 glucuronide in rat plasma, feces, urine, liver and kidney homogenate to support pharmacokinetic studies in rats.

2. Experimental

2.1. Chemicals and reagents

Irinotecan, SN-38, CPT, uridine-5′-diphosphate-β, D-glucuronic acid ester (UDPGA), D-saccharic-1,4-lactone monohydrate, magnesium chloride, Hanks' balanced salt solution (powder form) and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Expressed human UGT isoforms (UGT1A1) was purchased from BD Biosciences (Woburn, MA, USA). Solid phase extraction (C18) columns were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile, methanol and water (LC–MS grade) were purchased from EMD (Gibbstown, NJ, USA). Water was deionized by a Milli-Q water purification system of Millipore (Bedford, MA, USA). Intravenous irinotecan hydrochloride injection (20 mg/ml) was purchased from Teva Pharmaceuticals (Pearl River, NY, USA).

2.1.1. Bio synthesis of SN-38 glucuronide

SN-38 glucuronide was biosynthesized using human expressed UGTs (UGT1A1 isoform). In this study, SN-38 was incubated with UGT1A1 for 24 h which resulted in the formation of 95.8% SN-38 glucuronide. Then, the mixture containing both SN-38 and SN-38 glucuronide was extracted with a liquid–liquid extraction method using dichloromethane (DCM) as the organic solvent to remove SN-38. The resulting aqueous layer of SN-38 glucuronide was collected, and each 5 ml of the collected layer were applied to a C18 solid phase extraction column. After washing out the salt, 1 ml of methanol was used to elute SN-38 glucuronide. The eluted methanol fractions from multiple extractions were pooled and air dried, and the residue was reconstituted with 100 μl of 50% methanol-water to concentrate SN-38 glucuronide. In each step, the respective peak area of SN-38 and SN-38 glucuronide were measured in UPLC using PDA detector at wavelength of 375.2 and 380 nm respectively, to calculate the purity of SN-38 glucuronide in the mixture. Apart from UPLC quantification, MS/MS spectrum of SN-38 glucuronide was also analyzed to confirm its presence in the solution.

2.2. Instruments and conditions

2.2.1. UPLC

The chromatographic separation of irinotecan, SN-38 and SN-38 glucuronide was achieved by a Waters Acquity™ UPLC H-Class system with a diode array detector (DAD) and a flow-through-needle sample manager. Analysis was carried out using Acquity UPLC BEH C18 Column (2.1 mm × 50 mm, 300°A, 1.7 μm, Waters, Milford, MA, USA). Mobile phase A (0.1% formic acid in water [v/v]) and mobile phase B (100% acetonitrile) were operated with a gradient elution at a flow rate of 0.4 ml/min as follows: 10% B → 25% B (0–0.5 min), 25% B → 40% B (0.5–1 min), 40% B (1–2.5 min), 40% B → 10% B (2.5–4.5 min). The column temperature and sample temperature was 60 °C and 20 °C. The injection volume was 10 μl. 100 nM camptothecin (CPT; Fig. 1 E) in 50% methanol-water was used as internal standard (IS).

2.2.2. Mass spectrometry

LC–MS/MS analysis for irinotecan, SN-38 and SN-38 glucuronide was performed with an API 5500 Qtrap triple quadruple mass spec-

trometer coupled with a TurbolonSpray™ (Applied Biosystem-MDS SCIEX, Framingham, MA, USA). The system was operated in positive electrospray ionization (ESI) and multiple reactions monitoring (MRM) scan mode. All data were acquired and processed using Analyst® 1.5.2 software with hotfixes (AB SCIEX).

2.3. Method validation

2.3.1. Calibration curve and LLOD

Calibration standards were prepared in 50% aqueous methanolic solution by diluting a stock solution of irinotecan, SN-38 and SN-38 glucuronide to final concentrations of 50, 50 and 10 μM respectively and were kept at −80 °C. Working solutions were prepared by serial dilutions of stock solutions with 50% methanol-water at the following concentrations: 6.25, 12.5, 25, 50, 100, 250, 500, 1000 nM of irinotecan; 6.25, 12.5, 25, 50, 100, 250, 500, 1000 nM of SN-38 and 3.12, 6.25, 12.5, 25, 50, 100, 250, 500 nM of SN-38 glucuronide. The standard and quality control solutions (QCs) were prepared by mixing 20 μl of blank plasma, liver, kidney and fecal homogenates with each 20 μl working solutions of CPT-11, SN-38 and SN-38 glucuronide in 1.5 ml mini-eppendorf tubes. Quality control samples (QCs) were prepared at the following concentrations: 40, 200 and 1000 nM for CPT-11, 40, 200 and 1000 nM for SN-38 and 8, 40 and 200 nM for SN-38 glucuronide. All sample solutions of rat plasma, feces, liver and kidney homogenates were also added with 20 μl working solutions of CPT (100 nM). Then the solution is extracted with 360 μl methanol-acetonitrile (1:1, [v/v]) solution, vortex-mixed for 1 min and centrifuged at 15,500 rpm for 15 min. The supernatants were transferred to another tube and evaporated to dryness under a steady stream of air at room temperature. The residue was reconstituted with 80 μl of 50% aqueous methanolic solution and centrifuged again at 15,500 rpm for 15 min. After centrifugation, 10 μl of the supernatant was injected to UPLC–MS/MS system for analysis. In case of feces, we followed the same protocol except we added 360 μl acetonitrile to extract irinotecan, SN-38 and SN-38 glucuronide. The linearity of each calibration curve was determined by plotting the ratio of the peak areas of analyte (irinotecan/SN-38/SN-38 glucuronide) to internal standard (I.S.; CPT in 50% methanol) in rat plasma, feces and homogenized liver and kidney. A least-square linear regression method ($1/x^2$ weight) was used to determine the slope, intercept and correlation coefficient of linear regression method. The lower limit of quantification (LLOQ) was determined based on the signal-to-noise ratio of at least 10:1.

2.3.2. Accuracy and precision

The "intra-day" and "inter-day" precision and accuracy of the method were determined with quality control (QC) samples at three different concentrations (six injections for each concentration) on the same day or on three different days [34].

2.3.3. Extraction recovery and matrix effect

Extraction recovery of irinotecan, SN-38 and SN-38 glucuronide in different bio-matrices (plasma, feces, liver and kidney homogenate) was calculated by plotting the ratio of the peak areas of analyte (irinotecan/SN-38/SN-38 glucuronide) to internal standard (CPT) in blank rat plasma/feces/liver or kidney homogenate spiked before extraction procedure divided by the ratio of the peak areas of analyte to internal standard for the same quantity of the respective compound spiked into extracted blank matrix. Similarly, matrix effects were calculated by dividing the ratio of the peak area of the compound and internal standard spiked into extracted blank matrix by the ratio of the peak area of same compound and internal standard in neat solution at the same concentration. All these experiments and evaluations were performed according to the recommended validation procedures reported by Matuszewski [34].

2.3.4. Stability

Short-term (25 °C for 4 h), post-processing (20 °C for 8 h), long-term (−80 °C for 1 month) and three freeze-thaw cycle stabilities of irinotecan, SN-38 and SN-38 glucuronide were determined by analyzing three replicates of QC samples at three different concentrations.

2.4. In vivo rat pharmacokinetic study

2.4.1. Animals

Male Wistar rats (6–10 weeks, Body weight between 250 and 280 g, n = 6) were purchased from Harlan Laboratory (Indianapolis, IN). Rats were kept in an environmentally controlled room (temperature: 25 ± 2 °C, humidity: 50 ± 5%, 12 h dark-light cycle) for at least 1 week before the experiments. The rats were fasted overnight before the day of the experiment.

2.4.2. Animal experiment design

Irinotecan was administered at a dose of 5 mg/kg via intravenous injection through the tail vein. Blood samples (about 20–50 μl) were collected in heparinized tubes at 0, 15, 30, 60, 120, 240, 360, 480, and 1440 min post-injection, via tail snip with isoflurane as anesthetic. Urine samples were collected at 4, 8 and 24 h; whereas feces were collected at 24 h. Plasma samples were prepared and stored at −80 °C until analysis. The procedures were approved by the University of Houston's Institutional Animal Care and Uses Committee (IACUC).

2.4.3. Tissue homogenization

Liver and kidney were excised from the sacrificed rats and stored frozen at −80 °C in polypropylene tubes until homogenization. The frozen tissues were thawed, chopped and weighed at 4 °C. Accurately 50 mg of chopped tissue was homogenized in 2 ml of ice-cold homogenizing solution (pH 7.4) containing 10 mM potassium phosphate, 250 mM sucrose and 1 mM EDTA dehydrate with a polytron tissue homogenizer. Homogenization was paused 20 s after every 30 s of homogenization at a medium speed. The homogenization was repeated 3–4 times until a uniform homogenate was obtained. Final tissue extract was stored at approximately −80 °C prior to analysis. The homogenizer probe was washed sequentially with water, methanol and water after every homogenization.

2.4.4. Sample preparation

Plasma, liver and kidney homogenate samples (20 μl) were spiked with 20 μl of I.S. (CPT in 50% methanol, 100 nM) and vortexed for 1 min and then extracted with 360 μl of 50% methanol in acetonitrile (1:1). All solutions were vortexed and centrifuged at 15,500 rpm for 15 min. The supernatants were transferred to another tube and evaporated to dryness under a steady stream of air at room temperature. The residue was reconstituted with 80 μl of 50% methanol in water (1:1) and centrifuged again at 15,500 rpm for 15 min. After centrifugation, 10 μl of the supernatant was injected to UPLC–MS/MS system for analysis. In case of fecal samples, they were first lyophilized to evaporate the moisture and then 1 g of dried feces was accurately weighed and homogenized with 10 ml of homogenizing solution. The homogenized samples were centrifuged for 5 min to remove heavy particles and the supernatant was collected. Then, 20 μl fecal homogenate solution was spiked with 20 μl of I.S. (CPT in 50% methanol, 100 nM) and vortexed for 1 min and extracted with 18 volume of acetonitrile for every volume of fecal homogenate. In case of urine, all the standard and samples were diluted 100 times with 50% methanol-water solution, centrifuged at 15,500 rpm for 15 min and 10 μl of

the supernatant was injected to UPLC–MS/MS system for quantification. The density of the blood is treated as 1 g/mL in the tissue distribution study.

2.4.5. Preparation of standards, quality controls and sample solutions

Calibration standards and quality control (QC) samples were prepared as described in Section 2.3.1.

2.4.6. Pharmacokinetic parameter calculation

The pharmacokinetic parameters of irinotecan, SN-38 and SN-38 glucuronide were calculated by the non-compartmental method, using Phoenix WinNonLin (Pharsight Corporation, Mountain View, California) program.

3. Results and discussion

3.1. Optimization of the UPLC–MS/MS condition

Different combinations of mobile and stationary phases were employed to enhance the sensitivity of detection of irinotecan, SN-38 and SN-38 glucuronide. Acetonitrile, methanol, 0.1–5% formic acid in acetonitrile, and 0.1–5% formic acid in methanol as organic phase and 1–2.5 mM ammonium acetate in water, 0.05–1% formic acid in water as aqueous phase were tested as potential mobile phases. We tested both C8 and C18 column as stationary phases to resolve irinotecan, SN-38 and SN-38 glucuronide. Based on the intensity of the signal, signal-noise ratio and shape of the peak, 0.1% formic acid in water, 100% acetonitrile and C18 column were found to be the optimal aqueous mobile phase, organic mobile phase and stationary phase, respectively. Apart from that, it has been found that column temperature at 60 °C and the flow rate of 0.45 ml/min produced sharp and symmetrical peaks (Fig. 1A). For MS/MS analysis, both positive and negative scan mode were employed to analyze irinotecan, SN-38 and SN-38 glucuronide. A representative MS/MS spectrum of irinotecan, SN-38 and SN-38 glucuronide is shown in Fig. 1B, C and D, respectively. Based on the intensity of the analytes, positive scan mode was found to be more sensitive compared to the negative scan mode. To improve the specificity, multiple reactions monitoring (MRM) scan type was used. The MRM transitions from precursor ions to product ions were optimized as *m/z* 587.6 → 124.04 for irinotecan, 393.1 → 349.06 for SN-38, 569.05 → 393 for SN-38 glucuronide and 349 → 305 for CPT (IS), based on their most abundant precursor ions and corresponding product ions (Fig. 1B, C, D and E). Additional compound-dependent parameters in MRM mode for irinotecan, SN-38, SN-38 glucuronide and CPT were summarized in Table 1. The main working parameters for mass spectrum were used in the QTRAP 5500 system as follows: ion-spray voltage, 5.5 kV; temperature, 500 °C; curtain gas, 20 psig; gas 1, 20 psig; gas 2; 20 psig, collision gas, medium.

3.2. Method validation

3.2.1. Linearity and sensitivity

Method validation was conducted using blank rat plasma, untreated feces, liver and kidney tissue samples. In plasma, the standard curves of irinotecan, SN-38 and SN-38 glucuronide were linear in the concentration range of 4.88–10000 nM, 4.88–10000 nM, and 6.25–2000 nM, respectively ($R^2 > 0.99$). In case of feces samples, the standard curves of irinotecan, SN-38, SN-38 glucuronide were linear in the concentration range of 39–5000 nM, 39–5000 nM, and 4.88–1250 nM SN-38 glucuronide, respectively ($R^2 > 0.99$). The assay also exhibited excellent linear response over selected concentration range of 48.8–6250 nM, 48.8–6250 nM and 9.8–1250 nM for irinotecan, SN-38 and SN-38 glucuronide, respectively in liver and kidney homogenates ($R^2 > 0.99$). The lower limit of detection (LLOD) of irinotecan and SN-38 was determined to be 2.44 nM, 19.5 nM and 24.44 nM in plasma, feces, liver and kidney homogenate, respectively. In case of SN-38 glucuronide, LLOD is 6.25 nM, 2.44 nM and 4.9 nM in plasma, feces, liver and kidney homogenate, respectively. However, the LLOD values of irinotecan, SN-38 and SN-38 glucuronide in rat plasma weren't comparable with the values (116 pg/ml for irinotecan, 58 pg/ml for SN-38 and 105 pg/ml for SN-38 glucuronide) reported by Marangon et al. [31], which might be explained by the differences of nature of sample analyzed based on species (rat vs human).

3.2.2. Accuracy and precision

Accuracy, intra-day and inter-day precision were determined by running six replicates of QC samples at three different concentration levels (low, medium, high) of irinotecan, SN-38 and SN-38 glucuronide in blank rat plasma, feces, liver and kidney tissues. The precision and accuracy of these measurements were shown in Table 2. These results demonstrated that the precision and accuracy values were in the acceptance range (<15%) according to FDA guidance. Unlike LLOD values, the precision and accuracy values were quite comparable with the values reported by Marangon et al. [31].

3.2.3. Recovery, matrix effect and stability

The mean extraction recoveries determined using three replicates of QC samples at three concentration levels (the same concentrations as QC sample) in rat plasma, feces, liver and kidney tissues were shown in Table 2, Table 3, Table 4 and Table 5, respectively. 50% methanol–acetonitrile and 100% acetonitrile were used as protein precipitating solvent to extract irinotecan, SN-38 and SN-38 glucuronide from blank rat plasma, liver, kidney and feces. The result showed that the recoveries were not less than 85% for all the analytes at three different concentrations in plasma and feces. In comparison to human plasma (44–84%), the recoveries of irinotecan and its metabolites in rat plasma were quite higher (95–114%) [31].

Similarly, to test if the matrix effects have any impact on the UPLC–MS analysis of irinotecan, SN-38 and SN-38 glucuronide, the relative peak areas of the analyte spiking in the evaporated blank plasma, feces, liver and kidney tissues at three different concentration levels (low, medium and high) were compared to similarly prepared standard solutions. As shown in Table 2, Table 3, Table 4 and Table 5, no measurable matrix effect was observed.

Stabilities of irinotecan, SN-38 and SN-38 glucuronide in plasma were evaluated by analyzing triplicates of QC samples at three different concentrations following storage at 25 °C for 4 h, at −80 °C for 3 days, and after going through three freeze–thaw cycles (−80 °C and 25 °C). All the samples showed 85–115% recoveries after various stability tests.

3.3. Application to the method to determine the plasma, urine and feces concentration of irinotecan in rats after i.v. administration

The validated method was utilized to determine irinotecan, SN-38 and SN-38 glucuronide content in plasma, feces and urine after intravenous administration of 5 mg/kg irinotecan in male wistar rats. Mean plasma concentration of irinotecan, SN-38 and SN-38 glucuronide as a function of time after i.v. administration of irinotecan is shown in Fig. 2. The C_{max} of irinotecan, SN-38 and SN-38 glucuronide are 6.02 ± 0.19 μM, 2.13 ± 0.70 μM and 0.08 ± 0.01 μM, respectively; whereas the T_{max} and AUC of irinotecan, SN-38 and SN-38 glucuronide are 33.75 min, 26.25 min, 100 min and 15.72 h μM, 18.53 h μM and 0.70 h μM, respectively. The plasma concentration-time profile of irinotecan continued to exhibit multi exponential phases denoting rapid distribution and slower elimination. In case of urinary excretion it was found that in comparison to SN-38 (16.50 μM) and its glucuronidated

Table 1
Compound dependent parameters.

Analyte	Q1(*m/z*)	Q3(*m/z*)	Time (msec)	DP (Volts)	EP (Volts)	CE(Volts)	CXP(Volts)
Irinotecan	587	124	100	161	10	43	8
SN-38	393	349	100	196	10	35	20
SN-38G	569	393	100	201	10	37	24
CPT	349	305	100	201	10	31	14

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

Table 2
Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in plasma using MRM method (n = 6).

Compound	Conc. (nM)	Extraction recovery Average ± SD (%)	Intra-day		Inter-day		Matrix effect Average ± SD (%)
			Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	
CPT-11	40	106.9 ± 4.45	104.34	13.19	103.48	6.04	85.67 ± 3.78
	200	100.44 ± 0.43	108.93	9.28	107.03	8.45	86.56 ± 3.81
	1000	109.06 ± 3.82	107.74	3.90	106.8	7.14	88.61 ± 0.50
SN-38	40	114.31 ± 13.28	110.35	5.44	85.98	1.80	92.79 ± 3.15
	200	108.5 ± 16.96	86.01	4.21	84.85	4.84	90.16 ± 2.01
	1000	102.34 ± 5.21	92.94	3.38	82.51	4.15	94.59 ± 1.03
SN-38G	8	99.12 ± 2.50	103.78	12.25	98.9	7.94	91.03 ± 1.66
	40	95.08 ± 0.44	105.90	3.11	93.73	5.21	90.42 ± 2.44
	200	99.52 ± 1.49	94.36	2.48	90.88	3.15	103.7 ± 0.81

Table 3
Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in liver homogenate using MRM method (n = 6).

Compound	Conc. (nM)	Extraction recovery Average ± SD (%)	Intra-day		Inter-day		Matrix effect Average ± SD (%)
			Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	
CPT-11	40	92.09 ± 8.11	93.87	2.89	91.01	0.22	101.01 ± 12
	200	83.94 ± 5.65	95.04	5.97	103.50	0.13	95.97 ± 10.9
	1000	82.26 ± 4.38	91.55	2.66	107.77	0.94	96.3 ± 3
SN-38	40	80.28 ± 0.92	92.21	6.58	97.49	0.23	96.16 ± 5.4
	200	80.58 ± 3.64	95.39	3.46	97.94	0.34	84.23 ± 3.13
	1000	83.88 ± 5.98	96.75	3.74	97.53	2.19	95 ± 9.18
SN-38G	8	111.56 ± 4.75	96.04	2.75	108.4	0.01	91.96 ± 10.55
	40	95.51 ± 1.05	87.46	4.18	98.32	0.08	84.85 ± 3.95
	200	84.9 ± 3.62	95.16	3.26	98.43	0.42	97.41 ± 0.98

Table 4
Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in kidney homogenate using MRM method (n = 6).

Compound	Conc. (nM)	Extraction recovery Average ± SD (%)	Intra-day		Inter-day		Matrix effect Average ± SD (%)
			Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	
CPT-11	40	75.38 ± 4.94	91.79	8.62	98	0.06	111.25 ± 6.10
	200	79.15 ± 3.24	93.84	5.01	105.84	0.38	111.21 ± 5.50
	1000	77.82 ± 5.37	97.16	2.02	100.88	1.21	112.38 ± 3.90
SN-38	40	74.25 ± 6.23	95.32	5.79	100.07	0.06	97.17 ± 3.39
	200	77.67 ± 7.32	95.55	2.15	108.42	4.84	86.24 ± 3.55
	1000	78.96 ± 4.07	93.15	2.84	102.04	1.45	88.61 ± 0.50
SN-38G	8	86.83 ± 1.82	90.13	3.59	98.82	0.02	86.63 ± 2.95
	40	81.88 ± 6.09	98.95	0.98	109	0.07	90.88 ± 2.90
	200	83.77 ± 9.95	94.67	2.15	97.41	0.60	84.66 ± 6.21

Table 5
Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in feces using MRM method.

Compound	Conc. (nM)	Extraction recovery Average ± SD (%)	Intra-day		Inter-day		Matrix effect Average ± SD (%)
			Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	
CPT-11	40	107.40 ± 8.95	90.84	2.9	81.60	1.13	94.75 ± 5.05
	200	109.14 ± 8.45	97.22	2.33	95.86	3.53	91.86 ± 4.07
	1000	107.07 ± 1.77	97.63	0.15	81.70	2.95	85.8 ± 6.39
SN-38	40	107.02 ± 2.68	96.66	1.69	80.29	3.61	88.26 ± 2.79
	200	106.9 ± 4.51	98.97	0.94	99.43	8.88	92.31 ± 2.84
	1000	102.49 ± 2.64	93.65	2.98	89.46	4.78	115.4 ± 8.43
SN-38G	8	83.8 ± 13.56	103.78	12.25	84.09	7.17	81.17 ± 4.31
	40	93.87 ± 16.84	105.9	3.11	100.45	0.39	81.92 ± 6.08
	200	86.63 ± 16.83	94.36	2.48	106.97	9.10	97.84 ± 1.4

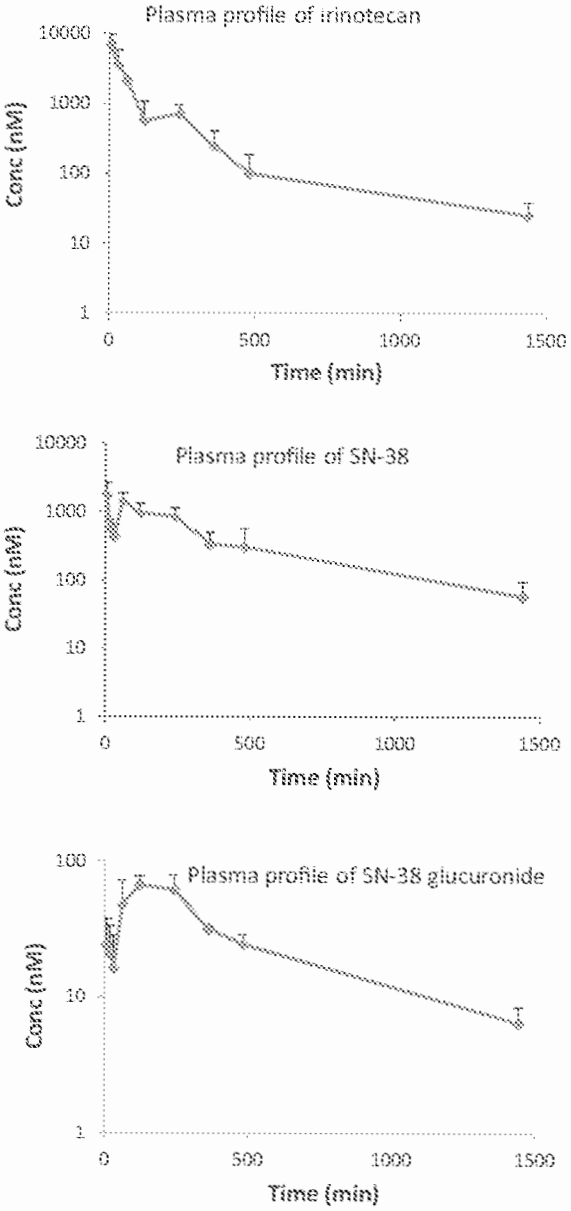

Fig. 2. Plasma profile of irinotecan, SN-38 and SN-38 glucuronide after intravenous administration of 5 mg/kg irinotecan.

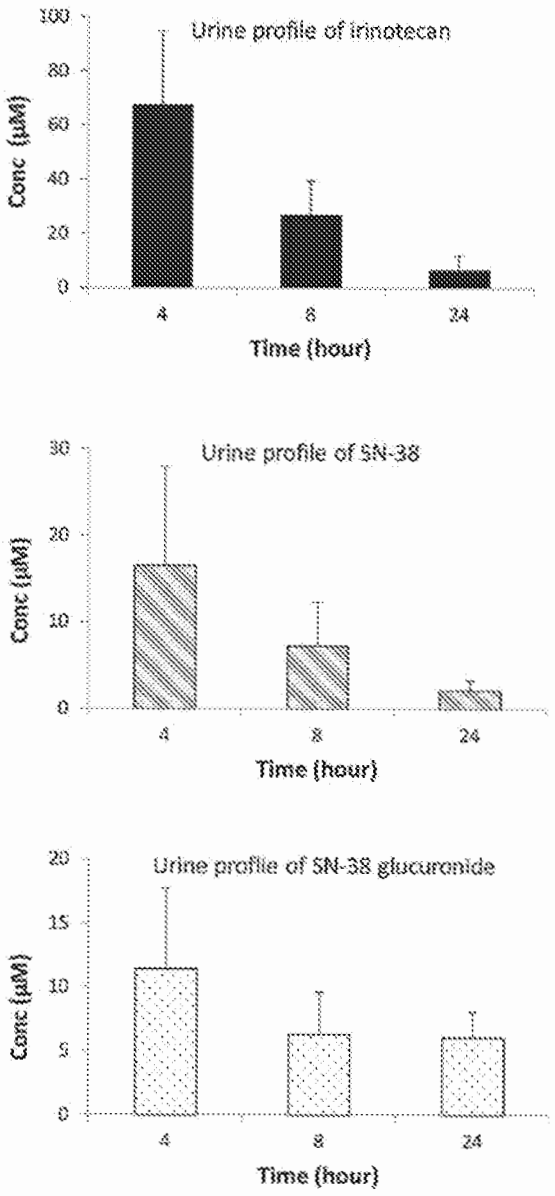

Fig. 3. Urine profile of irinotecan, SN-38 and SN-38 glucuronide after intravenous administration of 5 mg/kg irinotecan.

form (11.47 μM), irinotecan (67.49 μM) predominantly excreted in unchanged form after 4 h (Fig. 3). However, after 24 h, the concentration of irinotecan (6.43 μM) and SN-38 glucuronide (6.07 μM) in urine is quite comparable. After analyzing the feces we found that, after 24 h (Fig. 4), fecal content of irinotecan (818.35 μg/g) and SN-38 (423.95 μg/g) is much higher than the fecal content of SN-38 glucuronide (9.60 μg/g). Low fecal excretion of SN-38 glucuronide might be ascribed to the hydrolysis of SN-38 glucuronide by bacterial β-glucuronidase in feces which was supported by different reports [35,36].

4. Conclusion

In this study, we developed and validated a rapid, sensitive and specific UPLC–MS/MS method for quantifying irinotecan, SN-38 and SN-38 glucuronide simultaneously in rat plasma, feces, urine, liver, kidney, and the validated method was successfully applied to the in vivo pharmacokinetic studies of irinotecan in rats. This is the

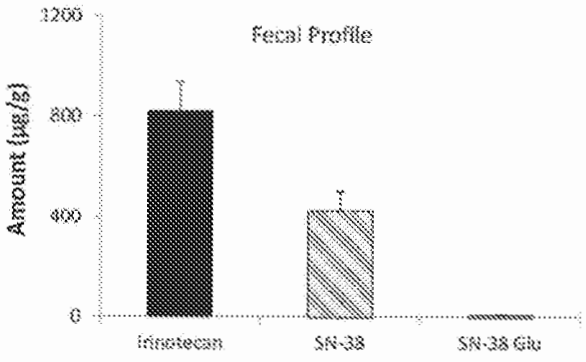

Fig. 4. Fecal profile of irinotecan, SN-38 and SN-38 glucuronide in feces collected for 24 h, after intravenous administration of 5 mg/kg irinotecan.

first UPLC–MS/MS method that allows us to simultaneously quantify irinotecan, its active metabolite SN-38 and SN-38-glucuronide in different bio-matrices (plasma, urine and feces) as well as in different tissues (liver and kidney). Apart from that, this method has certain advantages such as high sensitivity (nM), short analysis time (4.5 min), good recovery with negligible matrix effect. This method can also be extrapolated to clinical studies owing to its high sensitivity and small sample volume requirement; although full validation of the method in human matrices is required prior to support any kind of clinical studies.

References

[1] C. Tournigand, et al., FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study, J. Clin. Oncol. 22 (2) (2004) 229–237.

[2] K. Akhtar, et al., Current status of pharmacological treatment of colorectal cancer, World J. Gastrointest. Oncol. 6 (6) (2014) 177–183.

[3] R. Ohno, et al., An early phase II study of CPT-11: a new derivative of camptothecin, for the treatment of leukemia and lymphoma, J. Clin. Oncol. 8 (11) (1990) 1907–1912.

[4] M. Fukuoka, et al., A phase II study of CPT-11: a new derivative of camptothecin, for previously untreated non-small-cell lung cancer, J. Clin. Oncol. 10 (1) (1992) 16–20.

[5] Y. Shimada, et al., Phase II study of CPT-11: a new camptothecin derivative, in metastatic colorectal cancer. CPT-11 Gastrointestinal Cancer Study Group, J. Clin. Oncol. 11 (5) (1993) 909–913.

[6] M.E. Wall, M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPhail, G.A. Sim, Plant antitumor agents. I. The isolation and structure of camptothecine, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata, J. Am. Chem. Soc. 88 (16) (1966) 3888–3890.

[7] S. Sawada, et al., Synthesis and antitumor activity of 20(S)-camptothecin derivatives: carbamate-linked, water-soluble derivatives of 7-ethyl-10-hydroxycamptothecin, Chem. Pharm. Bull. (Tokyo) 39 (6) (1991) 1446–1450.

[8] M.E. Rothenberg, et al., Phase I and pharmacokinetic trial of weekly CPT-11, J. Clin. Oncol. 11 (11) (1993) 2194–2204.

[9] R.R. Mathijssen, et al., Clinical pharmacokinetics and metabolism of irinotecan (CPT-11), Clin. Cancer Res. 7 (8) (2001) 2182–2194.

[10] M.R. Redinbo, et al., Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA, Science 279 (5356) (1998) 1504–1513.

[11] L. Stewart, et al., A model for the mechanism of human topoisomerase I, Science 279 (5356) (1998) 1534–1541.

[12] E. Gupta, et al., Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea, Cancer Res. 54 (14) (1994) 3723–3725.

[13] J.R. Rechl, Gastrointestinal toxicity of irinotecan, Oncology (Williston Park) 12 (8 Suppl. 8) (1998) 72–78.

[14] Y. Sugiyama, Y. Kato, and X. Chu, Multiplicity of biliary excretion mechanisms for the camptothecin derivative irinotecan (CPT-11), its metabolite SN-38, and its glucuronide: role of canalicular multispecific organic anion transporter and P-glycoprotein. Cancer Chemother Pharmacol, 1998. 42 Suppl: p. S44-9.

[15] T. Itoh, et al., Biliary excretion of irinotecan and its metabolites, J. Pharm. Pharm. Sci. 7 (1) (2004) 13–18.

[16] E. Gupta, et al., Pharmacokinetic modulation of irinotecan and metabolites by cyclosporin A, Cancer Res. 56 (6) (1996) 1309–1314.

[17] M. Ciotti, et al., Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus, Biochem. Biophys. Res. Commun. 280 (1) (2001) 199–202.

[18] J.F. Gagne, et al., Common human UGT1A polymorphisms and the altered metabolism of irinotecan active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), Mol. Pharmacol. 62 (3) (2002) 608–617.

[19] G. Catimel, et al., Phase I and pharmacokinetic study of irinotecan (CPT-11) administered daily for three consecutive days every three weeks in patients with advanced solid tumors, Ann. Oncol. 6 (2) (1995) 133–140.

[20] B.D. Wallace, et al., Alleviating cancer drug toxicity by inhibiting a bacterial enzyme, Science 330 (6005) (2010) 831–835.

[21] N. Yamada, T. Yokokura, Nonlinear pharmacokinetics of CPT-11 in rats, Cancer Res. 50 (6) (1990) 1721–1725.

[22] W.J. Slichenmyer, et al., The current status of camptothecin analogues as antitumor agents, J. Natl. Cancer Inst. 85 (4) (1993) 271–291.

[23] K. Sano, et al., Simple non-ion-paired high-performance liquid chromatographic method for simultaneous quantitation of carboxylate and lactone forms of 14 new camptothecin derivatives, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 795 (1) (2003) 25–34.

[24] C. Boyd, et al., High-performance liquid chromatographic technique for the simultaneous determination of lactone and hydroxy acid forms of camptothecin and SN-38 in tissue culture media and cancer cells, Anal. Biochem. 297 (1) (2001) 15–24.

[25] Z.P. Hu, et al., Simultaneous determination of irinotecan (CPT-11) and SN-38 in tissue culture media and cancer cells by high performance liquid chromatography: application to cellular metabolism and accumulation studies, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 850 (1-2) (2007) 575–580.

[26] E. Gravel, et al., Fluorescence detection combined with either HPLC or MEKC for pharmaceutical quality control in a hospital chemotherapy production unit: application to camptothecin derivatives, J. Pharm. Biomed. Anal. 39 (3-4) (2005) 581–586.

[27] N. Yang, et al., Simultaneous determination of the lactone and carboxylate forms of irinotecan (CPT-11) and its active metabolite SN-38 by high-performance liquid chromatography: application to plasma pharmacokinetic studies in the rat, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 821 (2) (2005) 221–228.

[28] G. Corona, et al., Fast liquid chromatography-tandem mass spectrometry method for routine assessment of irinotecan metabolic phenotype, Ther. Drug Monit. 32 (5) (2010) 636–646.

[29] C. Ahn, et al., A rapid, simple and reliable HPLC-triple quadrupole tandem mass spectrometer method for a simultaneous quantification of irinotecan and its active metabolite 7-ethyl-10-hydroxycamptothecin (SN38) in mouse plasma, Biomed. Chromatogr. 28 (7) (2014) 919–922.

[30] D.J. Park, et al., Determination of irinotecan and its metabolite SN-38 in rabbit plasma and tumors using a validated method of tandem mass spectrometry coupled with liquid chromatography, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 962 (2014) 147–152.

[31] E. et al. Marangon, Development and validation of a high-performance liquid chromatography-tandem mass spectrometry method for the simultaneous determination of irinotecan and its main metabolites in human plasma and its application in a clinical pharmacokinetic study, PLoS One 10 (2) (2015) e0118194.

[32] S. Bardin, et al., Liquid chromatographic-tandem mass spectrometric assay for the simultaneous quantification of Camptosar and its metabolite SN-38 in mouse plasma and tissues, J. Chromatogr. A 1073 (1-2) (2005) 249–255.

[33] L. Goldwirt, et al., A new UPLC-MS/MS method for the determination of irinotecan and 7-ethyl-10-hydroxycamptothecin (SN-38) in mice: application to plasma and brain pharmacokinetics, J. Pharm. Biomed. Anal. 66 (2012) 325–333.

[34] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (13) (2003) 3019–3030.

[35] A. Sparreboom, et al., Irinotecan (CPT-11) metabolism and disposition in cancer patients, Clin. Cancer Res. 4 (11) (1998) 2747–2754.

[36] J.G. Slatter, et al., Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(14)C]CPT-11 in cancer patients, Drug Metab. Dispos. 28 (4) (2000) 423–433.

Safety, Pharmacokinetics, and Efficacy of CPX-1 Liposome Injection in Patients with Advanced Solid Tumors

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Abstract

Purpose: CPX-1 is a novel, liposome-encapsulated formulation of irinotecan and floxuridine designed to prolong *in vitro* optimized synergistic molar ratios of both drugs postinfusion. This open-label, single-arm, dose-escalating phase I study was designed to determine the maximum tolerated dose and pharmacokinetics of CPX-1 in patients with advanced solid tumors.

Experimental Design: Patients received CPX-1 at 30, 60, 100, 150, 210, or 270 units/m^2 (1 unit = 1 mg irinotecan + 0.36 mg floxuridine) infused over 90 minutes every 14 days in 28-day cycles. Pharmacokinetic samples were collected on days 1 and 15 of cycle 1.

Results: Thirty-three patients were enrolled, treated, and evaluated for safety; 30 patients were evaluated for response. A 1:1 plasma irinotecan to floxuridine molar ratio was maintained for 8 to 12 hours. Grade 3/4 toxicities included diarrhea (24.2%), neutropenia (12.1%), and hypokalemia (12.1%); 1 patient (270 units/m^2) died of persistent diarrhea, which led to dehydration and renal failure (grade 5). Partial response occurred in 3 (12%) of the 25 subjects evaluated through Response Evaluation Criteria in Solid Tumors. Progression-free survival lasting >6 months occurred in 9 patients, 6 with colorectal cancer. Among 15 colorectal cancer patients (10 with prior irinotecan), the calculated median progression-free survival was 5.4 months; 11 patients (72.7%) achieved disease control and 2 patients (13%) had partial response.

Conclusions: Outpatient CPX-1 was well tolerated and antitumor activity was shown in patients with advanced solid tumors. The recommended dose for future studies is 210 units/m^2. This is the first clinical evaluation of fixed drug ratio dosing designed to maintain synergistic molar ratios for enhanced therapeutic benefit.

The backbone of systemic therapy for cancer is combination chemotherapy. Historically, oncologists have assumed that greater antitumor activity can be achieved by administering combinations of active cytotoxic agents at maximally tolerated doses rather than using single-agent strategies (1, 2). However, *in vitro* studies of chemotherapy doublets with established clinical activity have suggested that efficacy may depend on the molar ratio of the two agents (3–7). Certain ratios of drug combinations can be synergistic, whereas other ratios of the same agents can be additive or antagonistic. This observation has important implications for maximizing the effectiveness of combination chemotherapy and suggests that improved efficacy may be achieved if the molar ratios that tumor cells are exposed to can be controlled *in vivo*. Because different anticancer drugs are pharmacokinetically dissimilar, conventional aqueous drug formulations will not allow either the maintenance or the delivery of optimal molar ratios to the tumor, resulting in exposure to suboptimal or antagonistic ratios (8). This problem can be overcome by encapsulating drug combinations within nanoscale carriers capable of maintaining synergistic drug ratios systemically through pharmacokinetic control. This approach has been shown to markedly increase the therapeutic index of several cancer drug combinations in preclinical studies (4, 5, 7).

Since the approval of irinotecan in 1996, the combination of i.v. irinotecan plus 5-fluorouracil (5-FU) and leucovorin has become a standard chemotherapy for first- and second-line treatments of metastatic colorectal cancer either alone or in combination with targeted biological agents such as bevacizumab and epidermal growth factor receptor inhibitors (9–15). In these treatment regimens, irinotecan and 5-FU are both administered i.v. with irinotecan injected as a short (30-120 minutes) infusion and 5-FU most frequently given as a bolus injection plus a 22-hour infusion the same day as irinotecan. In view of our *in vitro* observations

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Received 2/25/08; revised 7/9/08; accepted 8/25/08.

Grant support: Celator Pharmaceuticals, Inc.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Presented in part at the 42nd Annual Meeting of the American Society of Clinical Oncology, June 2-6, 2006, in Atlanta, GA, and the 43rd Annual Meeting of the American Society of Clinical Oncology, June 1-5, 2007, in Chicago, IL.

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doi:10.1158/1078-0432.CCR-08-0515

Translational Relevance

This study represents the first step in the clinical testing of a novel concept in combination chemotherapy for cancer. Ratiometric dosing is an innovation that could enhance many combinations of drugs currently used to treat various cancers and may affect the development of future combinations. Based on sound theory and experimental data, this phase I study confirms the safety and efficacy of the first ratiometrically designed drug combination tested.

of drug ratio–dependent synergy for irinotecan and fluorinated pyrimidine, we developed a liposome-based formulation coencapsulating irinotecan and floxuridine to assess whether controlling the ratio of these two drugs *in vivo* could improve therapeutic activity (5, 7). Floxuridine was used rather than 5-FU due to its compatibility with drug delivery technology as well as the fact that i.v. 5-FU and floxuridine have been shown to exhibit equivalent clinical activity (16, 17).

In preclinical studies, irinotecan and floxuridine exhibited the highest degree of *in vitro* synergy when molar ratios were between 1:1 and 1:5 (5). Consequently, a 1:1 molar ratio was chosen as the optimal ratio on the basis of demonstrated synergy across the highest proportion of cell lines studied. CPX-1 (Celator Pharmaceuticals, Inc.) was developed by fixing the molar ratio of irinotecan and floxuridine at 1:1 within a liposomal carrier that was able to control the plasma concentrations of both drugs such that the 1:1 molar ratio was maintained for up to 24 hours. *In vivo* tests of CPX-1 in a variety of human xenograft and allogeneic solid tumor models showed consistently superior antitumor efficacy when compared with both conventional combinations of irinotecan and floxuridine at the maximum tolerated dose, as well as liposomal formulations of the individual drugs administered at the maximum tolerated dose (5, 7). Furthermore, the degree of antitumor activity observed for CPX-1 was consistent with *in vivo* synergy when compared with the individual liposomal agents. A striking observation was that liposome delivery of an irinotecan to floxuridine molar ratio shown to be antagonistic *in vitro* provided less antitumor activity than liposomal irinotecan alone, despite administration of the same dose of irinotecan and the addition of fluoropyrimidine, an agent known to be active in that *in vivo* model (5).

CPX-1 is being developed with the hypothesis that it will be more efficacious than conventional irinotecan and fluoropyrimidine in the treatment of sensitive malignancies. Administration of chemotherapy combinations within drug carriers to achieve synergistic molar ratios at the site of drug action has not, to date, been studied in the clinic as a means of improving the efficacy of antitumor treatment. The objectives of this phase I study were to determine the maximum tolerated dose of CPX-1 for use in future studies and to assess the toxicity profile at the maximum tolerated dose. Data were collected to determine the duration of maintenance of the intended 1:1 molar ratio and the bioavailability of irinotecan and floxuridine. Early observations of CPX-1 efficacy are also presented.

Materials and Methods

The study was conducted between January 2005 and June 2007. Both clinical sites obtained approval from their respective Research Ethics Boards and all patients provided written informed consent.

Patients. Eligible patients provided written informed consent; were ≥18 years of age; and had confirmed histologic or cytologic advanced solid tumor not curable by conventional surgery, radiotherapy, or chemotherapy. Enrollment criteria included Eastern Cooperative Oncology Group performance status of ≤2, absolute neutrophil count $>1.5 \times 10^9$/L, platelet count $>100 \times 10^9$/L, serum creatine <1.5 times the upper limit of normal, aspartate transaminase and alanine transaminase <3 times the upper limit of normal (<5 times the upper limit of normal if caused by liver metastasis), serum total bilirubin <1.25 times the upper limit of normal, and an international normalized ratio <1.5. Exclusion criteria included any serious medical condition, laboratory abnormality, or psychiatric illness that prevented patients from providing informed consent; treatment with chemotherapy, radiation therapy, or other investigational anticancer therapeutic drugs within 4 wk before study entry (6 wk for prior nitrosoureas or mitomycin chemotherapy); the presence of primary brain tumors, lymphoma, or other hematologic malignancies; the presence of brain metastases that were not stable for ≥6 mo; pregnant or lactating females; the presence of clinically significant cardiac disease (New York Heart Association Class III or IV); severe, debilitating pulmonary disease; the presence of an infection that required continuing i.v. antibiotic treatment; severe or active enteropathy, or recent onset of diarrhea (defined as an excess of two to three stools above the normal daily rate within 4 wk before study entry); a history of Wilson's disease or other copper-related disorder (due to the copper used in the formulation of CPX-1); and hypersensitivity to irinotecan, floxuridine, or liposomal products.

All laboratory variables were evaluated before treatment and 4 wk posttreatment. Hematology exams were done twice weekly during cycle 1 and once weekly thereafter; biochemistry analyses were done weekly; coagulation studies were done on day 1 of each cycle. Because CPX-1 contains copper, serum copper levels were measured weekly during cycle 1 and on day 1 of each cycle thereafter.

Premedication. Antiemetics and antidiarrheal agents (loperamide) were administered at onset of nausea and vomiting or diarrhea during cycle 1 and could be used prophylactically for subsequent treatment cycles. For hypersensitivity or infusion-related reactions, treatment was stopped and potentially reinitiated (depending on the severity of the reaction), with or without i.v. diphenhydramine and dexamethasone.

Drug formulation and dosing regimen. CPX-1 is a liposomal formulation of irinotecan HCl trihydrate and floxuridine held in a fixed 1:1 molar ratio. The CPX-1 liposome membrane is composed of distearylphosphatidylcholine, distearylphosphatidylglycerol, and cholesterol in a 7:2:1 molar ratio.

In dogs given four weekly i.v. doses, the toxic low dose of CPX-1 was irinotecan 5 mg/kg plus floxuridine 1.8 mg/kg; twice this CPX-1 dose was not lethal (data on file). Based on this toxicology information, the starting dose of CPX-1 was irinotecan 30 mg/m^2 plus floxuridine 10.8 mg/m^2, which corresponded to 30 units/m^2. Although many dosing regimens have been evaluated for irinotecan, the most common are once every 3 wk or once weekly dosing (10–12). It is commonly assumed that exposure time rather than concentration is more important for response to cell cycle–specific drugs; thus, a weekly schedule might be better than an every 3 weeks schedule. However, the half-lives of irinotecan and SN-38 in dogs receiving CPX-1 were 3 to 6 times longer than historical data for irinotecan in dogs (18); thus, a decision was made to use an every 2 weeks schedule.

Treatment cycles were repeated once every 28 d in the absence of unacceptable toxicity or evidence of disease progression. Patients with stable disease or partial response continued therapy until disease

Table 1. Patient characteristics

Characteristic	CPX-1 dose (units/m^2)						
	30 (*n* = 4)	60 (*n* = 4)	100 (*n* = 4)	150 (*n* = 4)	210 (*n* = 13)	270 (*n* = 4)	Total (*N* = 33)
Gender, *n*							
Male	1	4	2	1	9	3	20
Female	3	0	2	3	4	1	13
Age, median (range), y	54 (45-72)	55.5 (37-68)	58.5 (43-62)	51 (51-64)	58 (21-79)	51.5 (34-70)	57 (21-79)
Race, *n*							
White	3	4	3	4	12	3	29
Black	0	0	0	0	0	0	0
Asian	1	0	1	0	1	0	3
Other	0	0	0	0	0	1	1
ECOG status							
0	0	0	1	0	2	1	4
1	2	3	3	4	10	2	24
2	2	1	0	0	1	1	5
Cancer type, *n*							
Colorectal		1	2	1	10	1	15
Pancreatic			1		2		3
Ovarian			1	1		1	3
Esophageal		1				1	2
Breast	2						2
Sarcomas				1	1		2
Gastric	1	1					2
Renal cell	1						1
Prostate		1					1
NSCLC				1			1
Sphenoid sinus						1	1
Cycles of treatment completed, *n*							
0-1	0	2	0	0	2	2	6
2-3	2	0	0	1	2	1	6
4-5	2	1	2	1	4	0	10
6-10	0	1	0	2	5	1	9
11-15	0	0	1	0	0	0	1
16-20	0	0	1	0	0	0	1
Median	3	2.5	7.5	5	3.5	1.5	4

Abbreviations: ECOG, Eastern Cooperative Oncology Group; NSCLC, non–small cell lung cancer.

progression or until two cycles of treatment were completed after the maximum response was reached.

Dose escalation was based on a modified Fibonacci sequence (19); successive groups of patients received CPX-1 30, 60, 100, 150, 210, and 270 units/m^2 (cohorts 1-6, respectively) infused i.v. over 90 min once every 14 d (one cycle = 28 d). Dose-limiting toxicity was defined as any grade 3 or 4 nonhematologic toxicity that occurred during cycle 1, with the exception of nausea or vomiting in patients without prophylactic antiemetic treatment. Hematologic dose-limiting toxicity was defined as absolute neutrophil count $<0.5 \times 10^9$/L for ≥7 d, febrile neutropenia (defined as absolute neutrophil count $<0.5 \times 10^9$/L and either fever ≥38.5°C or hospitalization for febrile neutropenia), platelet count $<25 \times 10^9$/L (with or without bleeding), or grade 3 thrombocytopenia (platelet count $<50 \times 10^9$/L and $\geq 25 \times 10^9$/L) associated with bleeding. Grade 3 alanine transaminase or aspartate transaminase toxicity did not constitute dose-limiting toxicity in subjects with liver metastases starting treatment with alanine transaminase or aspartate transaminase 3 to 5 times the upper limit of normal. Treatment was delayed or reduced for hematologic and other toxicities that were possibly, probably, or definitely related to protocol therapy. Toxicities were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

Subsequent cohorts began the next dose level when dose-limiting toxicities were confirmed absent after 28 d of follow-up. If one patient at a given dose level experienced dose-limiting toxicities, additional patients were treated at the same dose level up to a total of six patients. Dose escalation continued only if no more than one of the six patients experienced dose-limiting toxicities. If two or more patients experienced dose-limiting toxicities at a given dose level, further dose escalation was discontinued. Additional patients were then added to the next lower dose level until a total of six patients were treated. If no more than one of the six patients experienced dose-limiting toxicities at this dose level, it was declared the maximum tolerated dose. Additional patients were treated at the maximum tolerated dose to confirm safety and to explore preliminary evidence of antitumor activity (extension cohort). Subjects with prior irinotecan treatment were allowed entry if there was no evidence of primary irinotecan resistance and if their last irinotecan dose was administered >12 mo before the initiation of CPX-1 treatment.

Patients who received at least one cycle of CPX-1 treatment and had at least one measurable lesion were considered evaluable for response; patients who did not complete one cycle of CPX-1 treatment because they had early disease progression (i.e., the progression of target or nonmeasurable lesions or the appearance of new lesions) were considered evaluable for progression-free survival. Disease status was assessed using Response Evaluation Criteria in Solid Tumors guidelines (20). Response duration was measured as the time between the first documentation of complete remission or partial remission and the onset of progressive disease. Stable disease duration was measured from the start of therapy until the onset of progressive disease.

Pharmacokinetics. Blood samples for pharmacokinetic analyses were collected before CPX-1 infusion and at 45 and 85 min and at 2, 4, 6, and 8 h after the initiation of CPX-1 infusion on days 1 and 15; additional samples were collected at 24, 48, 72, 96, and 168 h after the

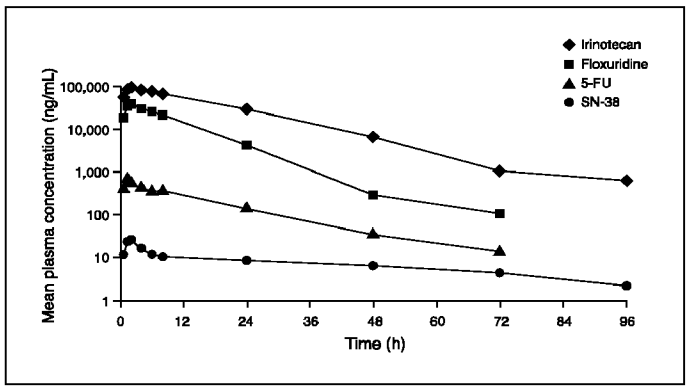

Fig. 1. Mean plasma concentrations on days 1 to 4 following CPX-1 210 units/m^2 infusion (n = 13).

initiation of infusion on day 1. Plasma was analyzed for total irinotecan (we did not distinguish free from encapsulated nor the lactone form from the carboxylic acid), 7-ethyl-10-hydroxy-camptothecin (SN-38), total floxuridine (free plus encapsulated), and 5-FU using validated, specific high-performance liquid chromatographic mass spectrometric methods with lower limits of quantitation of 240, 1, 50, and 10 ng/mL, respectively. Both intraday and interday coefficients of variation for low-, medium-, and high-quality control samples were ≤15% and accuracy was ≤±15% for all analytes (data on file).

Pharmacokinetic variables were determined from the plasma concentration-time data of all evaluable patients. Scheduled time was used to present mean concentration-time profiles and to calculate the area under the curve (AUC) from time 0 to 8 h ($AUC_{0\text{-}8}$). Actual time (defined as the time elapsed between the initiation of infusion and blood collection) was used for standard noncompartmental pharmacokinetic analysis using WinNonLin v 5.1 (Pharsight Corp.).

Calculated pharmacokinetic variables included maximum observed plasma concentration (C_{max}); the time to C_{max} (T_{max}); the elimination rate constant obtained from a linear regression of the natural log (ln) transformed concentration versus time data in the terminal phase (λz); the terminal elimination half-life ($t_{1/2}$), calculated as $\ln(2)/\lambda z$; the area under the plasma concentration-time curve extrapolated to time infinity ($AUC_{0\text{-}\infty}$), estimated using linear trapezoidal summation from time zero; $AUC_{0\text{-}8}$; the systemic clearance (CL), calculated as dose/$AUC_{0\text{-}\infty}$; the volume of distribution (V_d) calculated as dose/($AUC_{0\text{-}\infty} \times \lambda z$); the AUC accumulation ratio (AC[AUC]), calculated as $AUC_{0\text{-}8}$ day 15/$AUC_{0\text{-}8}$ day 1; and the C_{max} accumulation ratio (AC[C_{max}]) calculated as C_{max} day 15/C_{max} day 1. Dose proportionality was evaluated by linear regression of mean $AUC_{0\text{-last}}$ and C_{max} values estimated on day 1 versus dose.

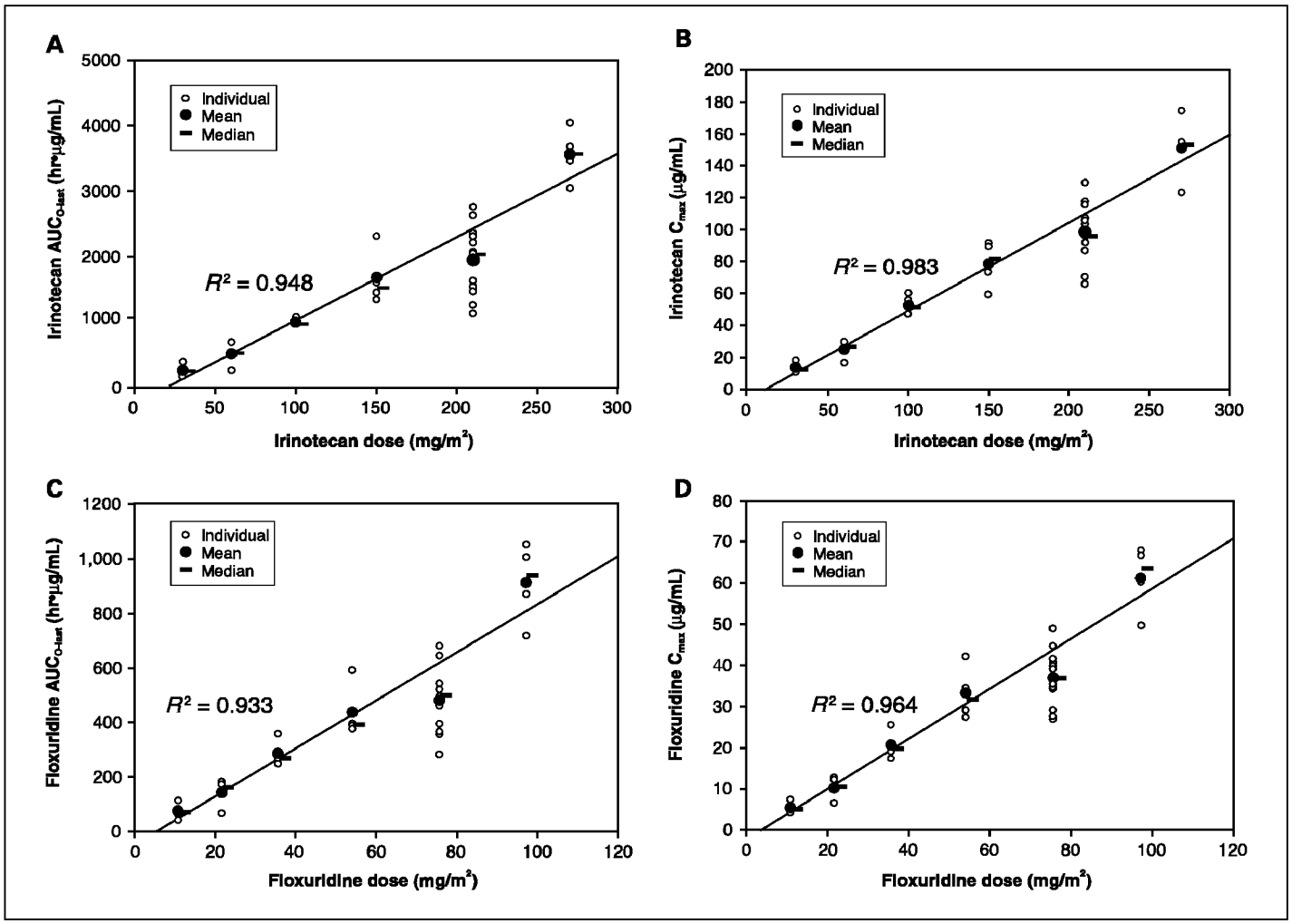

Fig. 2. Mean, individual, and median irinotecan (A) $AUC_{0\text{-last}}$ and (B) C_{max} values versus dose on day 1 and mean, individual, and median floxuridine (C) $AUC_{0\text{-last}}$ and (D) C_{max} values versus dose on day 1.

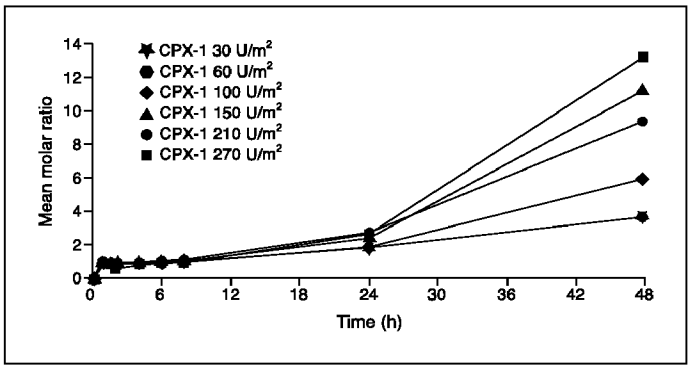

Fig. 3. Mean molar ratios of irinotecan and floxuridine on days 1 and 2.

To determine if there were relationships between treatment-related toxicities and pharmacokinetic variables, scatterplots and correlational statistics were evaluated.

Results

Patient characteristics. Thirty-three patients were enrolled and treated on cohorts 1 to 6 and the extension cohort (Table 1). Patients had a median age of 57 years and were predominantly male. All but four patients were white, and the majority of patients had an Eastern Cooperative Oncology Group performance status of 1. Eleven different tumor types were represented in the patient population; colorectal cancer was most common (15 patients, 45.5%). The average number of prior chemotherapy regimens was 2.5 among all patients. Details for prior therapies among the 15 colorectal patients are presented in Table 5.

Dose escalation. The starting dose for this study was 30 units/m^2 (cohort 1). Because dose-limiting toxicity occurred in four patients in cycle 1 of cohort 6 (270 units/m^2), the maximum tolerated dose was considered to have been exceeded. Thus, an additional two patients were enrolled at the next lowest dose level (cohort 5, 210 units/m^2). None of the six patients at the 210 units/m^2 dose level had dose-limiting toxicity; this dose level was declared the maximum tolerated dose. Subsequently, seven patients with colorectal cancer were enrolled at 210 units/m^2 (extension cohort) to obtain additional safety and efficacy data. A total of 153 cycles of treatment were administered to 33 patients. The median number of cycles of treatment by cohort is shown in Table 1 and for the study as a whole was four cycles per patient.

Pharmacokinetics. No patients were excluded from safety or pharmacokinetic analyses. Irinotecan, SN-38, floxuridine, and 5-FU were detected in the plasma of all patients following CPX-1 treatment. Mean (arithmetic) plasma concentrations after the first infusion in patients who received 210 units/m^2 (n = 13) on day 1 are depicted in Fig. 1. All analytes disappeared from the plasma in an apparently monophasic manner. Mean (arithmetic) pharmacokinetic variables for each dose group are shown in Supplementary Table. Pharmacokinetic variables of irinotecan and floxuridine (C_{max} and $AUC_{0\text{-last}}$) increased proportionately with CPX-1 doses (Supplementary Table; Fig. 2).

For all doses, accumulation ratios (AC[C_{max}] and AC[AUC]) of irinotecan, SN-38, floxuridine, and 5-FU were close to 1 (data not shown). The target 1:1 molar ratio of irinotecan to floxuridine was maintained in the plasma of all patients for 8 to 12 hours. The mean molar ratios are presented in Fig. 3.

There were no clear relationships between absolute or dose-normalized C_{max} or AUC for any of the analytes and screening bilirubin, day 8 absolute neutrophil count or National Cancer Institute-Common Toxicity Criteria toxicity grades for diarrhea, nausea or vomiting.

Safety. One patient in cohort 6 died after the first dose of CPX-1 from dose-limiting drug–related diarrhea and vomiting, which led to dehydration and renal failure. Another patient died of progressive metastatic sarcoma unrelated to study treatment. A list of adverse events (all grades) is presented in Table 2. Discontinuations due to adverse events occurred at 100 units/m^2 (one patient) after 20 cycles of treatment, 210 units/m^2 (one patient) after one cycle of treatment, and 270 units/m^2 (three patients) after one dose of treatment (two patients) and after three cycles (one patient). Severe adverse events that were dose related included grade 3 or 4 diarrhea (24.2%), neutropenia (12.1%), and hypokalemia (12.1%; Table 3). Gastrointestinal toxicities were present at all dose levels, but severity and duration increased with higher doses. Selected grade 3/4 events (diarrhea, neutropenia, nausea/vomitting, or fatigue) occurred in 9 of 24 patients with no prior irinotecan therapy and 5 of 9 patients with prior irinotecan therapy.

Serum copper levels became elevated above baseline in a dose-dependent manner. Elevations were observed in cohorts 3 (25% of patients), 4 (75%), 5 (100%), and 6 (100%), and in

Table 2. Adverse events

Adverse event type, *n* (%)	CPX-1 dose (units/m^2)						
	30 (*n* = 4)	60 (*n* = 4)	100 (*n* = 4)	150 (*n* = 4)	210 (*n* = 13)	270 (*n* = 4)	Total (*N* = 33)
Blood and lymphatic	0	0	1	0	4	2	7 (21.2)
Gastrointestinal	4	4	4	4	13	4	33 (100.0)
General and administration site	3	3	4	4	12	2	28 (84.8)
Hepatobiliary	0	0	1	0	3	0	4 (12.1)
Metabolism and nutrition	0	1	1	2	9	2	15 (45.5)
Musculoskeletal and connective tissue	3	3	2	3	8	2	21 (63.6)
Nervous system	1	2	1	4	9	2	19 (57.6)
Psychiatric	1	0	2	1	5	1	10 (30.3)
Respiratory, thoracic, and mediastinal	1	1	3	1	8	1	15 (45.5)
Skin and subcutaneous	3	1	2	2	9	2	19 (57.6)
Vascular	1	0	1	2	2	1	7 (21.2)

Table 3. All grade 3-5 adverse events (events related and unrelated to CPX-1)

CPX-1 dose, units/m^2	Sample size, *n*	Grade 3 adverse events	Grade 4 adverse events	Grade 5 adverse event
		Adverse event (cycle, day)	Adverse event (cycle, day)	Adverse event (cycle, day)
30	4	Arthralgia (C4, D6)		
		Spinal compression fracture (C4, D2)		
60	4	Diarrhea (C1, D4)		
100	4	Diarrhea (C8, D12)	Neutropenia* (C3, D22)	
		Hypokalemia (C10, D18)		
		Neutropenia* (C1, D11)		
		Sepsis (C20, D23)		
150	4	Ascites (C6, D67)		
		Vomiting (C3, D2)		
210	13	Hypokalemia (C2, D28)	Large intestine obstruction (C1, D28)	
		Hypokalemia (C5, D13)	Osteosarcoma metastatic (C3, D37)	
		Hypokalemia (C4, D14)	Pulmonary embolism (C3, D1)	
		Diarrhea (C2, D22)		
		Diarrhea (C1, D24)		
		Hyperbillirubinemia (C4, D51)		
		Hyperbillirubinemia (C2, D15)		
		Neutropenia (C1, D16)		
		Neutropenia (C1, D33)		
		Anemia (C1, D8)		
		Anemia (C5, D8)		
		Constipation (C1, D29)		
		Fatigue (C2, D17)		
		Hyponatremia (C3, D14)		
		Increased blood alkaline phosphate (C4, D36)		
		Edema peripheral (C3, D33)		
		Pain in extremity (C3, D12)		
		Pyrexia (C1, D32)		
		Small intestine obstruction (C1, D47)		
		Urinary tract infection (C5, D13)		
270	4	Diarrhea (C1, D2)	Diarrhea (C1, D8)	Renal failure (C1, D13)
		Diarrhea (C1,D9)		
		Cellulitis staph (C1, D15)	Neutropenia (C1, D10)	
		Dehydration (C2, D16)		
		Flatulence (C1, D9)		
		Groin pain (C1, D15)		
		Nausea (C1, D3)		
		Thrombocytopenia (C1, D15)		
		Vomiting (C2, D17)		
Total	33			

*Grade 3 and grade 4 neutropenia occurred in one patient.

the Extension Cohort (100%) during cycle 1; however, there was no evidence of copper accumulation. No instances of elevated copper levels were defined as severe adverse events. One patient with biliary obstruction unrelated to CPX-1 treatment had increasing serum copper levels above normal limits (on day 1 of cycles 2, 3, and 4, respectively) that returned to normal 5 days after the implantation of a biliary stent.

Response evaluation. Complete response, partial response, stable disease, and progressive disease were evaluated in 30 of 33 patients. Three patients were not evaluated because adverse events led to early removal from the study before scheduled tumor assessment could be completed. Three patients achieved a partial response, 21 patients achieved stable disease, and 6 patients had progressive disease (Table 4). Partial response occurred in 3 of 25 (12%) subjects evaluated by Response Evaluation Criteria in Solid Tumors criteria. Disease control (complete remission, partial remission, or stable disease) was observed in 11 of 15 (73.3%) patients with colorectal cancer. Among the 18 subjects with other tumor types, 1 partial response (non–small cell lung carcinoma) and 11 stable diseases were observed. Progression-free survival lasting >6 months was observed in six patients with colorectal cancer and one patient each with pancreatic, ovarian, and non–small cell lung carcinoma (Table 4). The median progressive-free survival among colorectal cancer patients was 5.4 months. Colorectal cancer patients with prior exposure to irinotecan had no reduction in progressive-free survival compared with patients who were irinotecan-naive (Table 5).

Discussion

In vitro findings that particular molar ratios of chemotherapy doublets may enhance antitumor efficacy, whereas other ratios may be antagonistic (4, 5, 8), provide the basis for the concept of fixed drug ratio dosing. This novel concept has important implications for the design of combination chemotherapy

Table 4. Best response by cancer type

Cancer type	n	CR, *n*	PR, *n*	SD, *n*	PD, *n*	NE, *n*	PFS lasting >6 mo, *n*
Colorectal	15	0	2	9	2	2	6
Pancreatic	3	0	0	2	1	0	1
Ovarian	3	0	0	3	0	0	1
Esophageal	2	0	0	0	1	1	0
Breast	2	0	0	2	0	0	0
Sarcomas	2	0	0	2	0	0	0
Gastric	2	0	0	1	1	0	0
Renal cell	1	0	0	0	1	0	0
Prostate	1	0	0	1	0	0	0
NSCLC	1	0	1	0	0	0	1
Sphenoid sinus	1	0	0	1	0	0	0

NOTE: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; PFS, progression-free survival; NSCLC, non–small cell lung cancer.

regimens and suggests that control of molar ratios using drug delivery technology may be used to enhance antitumor efficacy. CPX-1 was designed according to fixed drug ratio dosing principles to present and maintain a 1:1 molar ratio of irinotecan and floxuridine, which was found to be consistently synergistic in a variety of *in vitro* tumor models and more active than standard irinotecan and floxuridine combination therapy in preclinical tumor models (7). This is the first report of results from a CPX-1 clinical trial.

The intended 1:1 molar ratio of irinotecan and floxuridine was maintained in patient plasma for up to 12 hours, indicating that extended exposure at the predetermined, synergistic ratio was accomplished. The peak plasma levels and AUC for total irinotecan and total floxuridine were higher than what would be expected following their administration in a conventional combination treatment regimen. For irinotecan, Pitot et al. (21) found a mean C_{max} and AUC of 2,810 ng/mL and 18,091 ng/mL-h, respectively, in patients receiving a 90-minute infusion of 240 mg/m^2 conventional drug. After a 90-minute infusion of 210 units/m^2 of CPX-1, we found the mean C_{max} and AUC of irinotecan to be 35- and 108-fold higher. Similarly, after a single, i.v. bolus dose of 1,650 mg/m^2 of floxuridine, Creaven et al. (22) reported a mean AUC of 15,744 ng/mL-h. After a single, 90-minute, i.v. infusion of CPX-1 at 210 units/m^2 (containing 75.6 mgm^2 of floxuridine), the AUC of floxuridine was 486,905 ng/mL-h. These data suggest that most of the drug in plasma after CPX-1 administration is in the encapsulated form and that the pharmacokinetics reflects the disposition of the liposome-encapsulated drugs rather than the "free" drugs. The low V_d for irinotecan and floxuridine was close to the physiologic volume of the plasma, suggesting that, like other liposomal drugs, CPX-1 did not escape the vascular compartment until it encountered "leaky" capillaries in tumors or the sinusoidal capillaries of the reticuloendothelial system.

Table 5. CPX-1 activity in patients with colorectal cancer

Patient number	CPX-1 dose, units/m^2	Previous treatment history					PFS (mo)	Best response
		Adjuvant/neoadjuvant	First line	Second line	Third line	Fourth line		
02-010	270	5-FU ± LV	CAPOX				0.8+	N/A
01-004	60		FOLFOX	CAPIRI			0.8	PD
02-012	210	FOLFIRI	FOLFOX	Capecitabine	Cetuximab	Paclitaxel/carbo/mAb	1.9	PD
01-019	210		FOLFIRI	FOLFOX/Avastin			2.0+	N/A
02-004	100		Oxaliplatin	CAPOX	5-FU ± LV		3.8	SD
01-011	210	5-FU ± LV	FOLFIRI	FOLFOX	Cetuximab		3.8	SD
02-015	210	Capecitabine					4.6	SD
02-014	210	5-FU ± LV	FOLFOX	Vatalinib			5.4	SD
01-017	210	5-FU ± LV	FOLFOX	FOLFIRI	Cetuximab	Erlotinib	5.6	SD
02-007	150		IROX	Capecitabine			6.7	PR
02-013	210	Capecitabine					7.4+	SD
02-016	210	5-FU ± LV	FOLFIRI	FOLFOX	Capecitabine		7.7	SD
01-012	210	5-FU ± LV	FOLFOX	FOLFIRI			7.7	SD
01-018	210	5-FU ± LV	FOLFOX	FOLFIRI			9.3	PR
02-005	100	5-FU ± LV	FOLFIRI	CAPOX			12.0	SD

Abbreviations: LV, leucovorin; CAPOX, capecitabine and oxaliplatin; FOLFOX, oxaliplatin, fluorouracil, and folinic acid; CAPIRI, capecitabine and irinotecan; FOLFIRI, 5-FU, leucovorin, and irinotecan; carbo, carboplatin; IROX, irinotecan and oxaliplatin; +, patient censored at last assessment; N/A, not assessed.

The fact that the metabolites SN-38 and 5-FU were detected in all patients suggests that both encapsulated drugs are bioavailable. For SN-38, the mean C_{max} for patients receiving 210 mg/m^2 of CPX-1 was less than that for patients receiving 240 mg/m^2 of conventional irinotecan (26.6 versus 41 ng/mL), but the AUCs were comparable (769 ng/mL-h for CPX-1–derived irinotecan versus 638 ng/mL-h for conventional irinotecan; ref. 21).

Safety-related results indicate that the recommended dose for CPX-1 use in future trials should be no higher than 210 units/m^2; neutropenia and diarrhea were the dose-limiting toxicities. Adverse events were predictable, dose-related, and similar to conventional irinotecan and fluoropyrimidine treatment regimens. Previously reported prominent grade 3 or 4 adverse events following conventional irinotecan and fluoropyrimidine treatment (9–12) include neutropenia (21-40%), diarrhea (14-44.4%), and nausea (0-16%). In this study, the incidences of grade 3 neutropenia, diarrhea, and nausea in patients treated with the recommended phase II dose (210 units/m^2) were 15.4%, 15.4%, and 0.0%, respectively; 23.1% of patients had grade 3 hypokalemia, which may have been secondary to vomiting and/or diarrhea.

Ten patients (at starting doses of 100 units/m^2 or above) required 11 dose reductions due to toxicities related to the study treatment. Eight of the 11 dose reductions followed severe or persistent gastrointestinal toxicities, especially diarrhea and vomiting. The dose reductions were effective and reduced the diarrhea in six of seven patients and the vomiting in one patient. The effectiveness of dose reductions in reducing the incidence and severity of adverse events strongly suggests the dose relatedness of the major gastrointestinal toxicities and neutropenia. The every 2 weeks dosing schedule allows early recognition of toxicities and prompt dose reduction when needed.

The inclusion criteria for this study required a serum total bilirubin <1.25 times the upper limit of normal; thus, it is perhaps not surprising that we did not detect a correlation between bilirubin and toxicity.

As this was a small, dose-escalating study and the existing correlation between UGT1A1 genotype and irinotecan-associated toxicity may only be relevant at high (300-350 mg/m^2) doses (23), UGT1A1 genotyping was not conducted in this study nor were samples collected for this purpose. Future studies of CPX-1 should include pharmacogenomic analyses.

Evaluations of tumor response and progression-free survival suggest that CPX-1 is clearly active and remains active in patients with prior exposure to irinotecan and fluoropyrimidine treatment regimens. Comparisons of first- and second-line treatments with conventional irinotecan-based treatment regimens (FOLFIRI) in colorectal cancer patients reported precipitous drops in objective response rates [31-56% (9, 11, 12) versus 4% to 35% (9, 12), respectively]. Similarly, the rate of disease control (complete response + partial response + stable disease) also fell substantially [70-79% (9) versus 35% (12), respectively] between the first-line and second-line treatments. CPX-1 achieved partial response in 2 of 15 (13.3%) and disease control in 11 of 15 (73.3%) patients with colorectal cancer, 12 of whom were treated in the 3rd-, 4th-, or 5th-line setting. Because fluoropyrimidines and irinotecan are not typically used for the treatment of noncolorectal solid tumors, it is interesting to note that 1 partial response and 11 stable diseases were observed among the 18 patients with other tumor types treated with CPX-1. This suggests that there is potential for antitumor activity in other tumor types.

The identification of chemotherapy doublets with efficacy dependent on particular molar ratios and the encapsulation of those doublets within a drug carrier to enable delivery of the synergistic molar ratio to the tumor target (CombiPlex Technology) may be a means of improving the efficacy of chemotherapy combinations in the future. CPX-1 is the first combination treatment regimen that has succeeded in maintaining plasma concentrations of infused drugs at a molar ratio determined to be synergistic *in vitro*. A phase II trial evaluating the efficacy and safety of CPX-1 in treating advanced colorectal cancer is ongoing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Frei E III. Clinical studies of combination chemotherapy for cancer. In: Chou TC, Rideout DC, editors. Synergism and antagonism in chemotherapy. California: Academic Press; 1991. p. 103–8.
2. DeVita VT, Jr. Principles of cancer management: Chemotherapy. In: DeVita, Jr., Hellman S, Rosenberg SA, editors. Cancer: principles & practice of oncology. Pennsylvania: Lippincott-Raven; 1997. vol. 1. p. 333–47.
3. Tallarida RJ. Drug synergism: its detection and applications. J Pharmacol Exp Ther 2001;298:865–72.
4. Harasym TO, Tardi PG, Johnstone SA, Bally MB, Janoff AS, Mayer LD. Fixed drug ratio liposome formulations of combination cancer therapeutics. In: Gregoriadis G, editor. Liposome technology, 3rd ed. vol. III. Informa Healthcare; 2007. p. 25–48.
5. Mayer LD, Harasym TO, Tardi PG, et al. Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice. Mol Cancer Ther 2006;5:1854–63.
6. Tsai CM, Gazdar AF, Venzon DJ, et al. Lack of *in vitro* synergy between etoposide and *cis*-diamminedichloroplatinum (II). Cancer Res 1989;49:2390–7.
7. Harasym TO, Tardi PG, Harasym NL, Harvie P, Johnstone SA, Mayer LD. Increased preclinical efficacy of irinotecan and floxuridine coencapsulated inside liposomes is associated with tumor delivery of synergistic drug ratios. Oncol Res 2007;16:361–74.
8. Mayer LD, Janoff AS. Optimizing combination chemotherapy by controlling drug ratios. Mol Interventions 2007;7:216–23.
9. Douillard JY, Cunningham D, Roth AD, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000;355:1041–7.
10. Douillard JY, Sobrero A, Carnaghi C, et al. Metastatic colorectal cancer: integrating irinotecan into combination and sequential chemotherapy. Ann Oncol 2003;14 Suppl 2:ii7–12.
11. Goldberg RM, Sargent DJ, Morton RF, et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004;22:23–30.
12. Tournigand C, André T, Achille E, et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. J Clin Oncol 2004;22:229–37.
13. Grothey E, Chu E. The clinical efficacy of FOLFIRI and bavacizumab in combination as first-line therapy of metastatic colorectal cancer. Clin Colorectal Cancer 2007;6:621–4.
14. Lee JJ, Chu E. An update on treatment advances for the first-line therapy of metastatic colorectal cancer. Cancer J 2007;13:276–81.
15. Lee JJ, Chu E. First-line use of anti-epidermal growth factor receptor monoclonal antibodies in metastatic colorectal cancer. Clin Colorectal Cancer 2007;6 Suppl 2:S42–6.
16. Ansfield FJ, Curreri AR. Further Clinical Comparison between 5-fluorouracil (5-Fu) and 5-fluoro-2′-deoxyuridine (5-Fudr). Cancer Chemother Rep 1963;32:101–5.
17. Reitemeier RJ, Moertel CG, Hahn RG. Comparison of 5-fluorouracil (Nsc-19893) and 2′-deoxy-5-fluorouridine

(Nsc-27640) in treatment of patients with advanced adenocarcinoma of colon or rectum. Cancer Chemother Rep 1965;44:39–43.

18. Division of Oncology and Pulmonary Drug Products, FDA. Review and evaluation of pharmacology and toxicology data 1995; NDA 20–571.

19. Hansen HH, Selawry OS, Muggia FM, Walker MD. Clinical studies with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037). Cancer Res 1971;31:223–7.

20. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.

21. Pitot HC, Goldberg RM, Reid JM, et al. Phase I dose-finding and pharmacokinetic trial of irinotecan hydrochloride (CPT-11) using a once-every-three-week dosing schedule for patients with advanced solid tumor malignancy. Clin Cancer Res 2000;6:2236–44.

22. Creaven PJ, Rustum YM, Petrelli NJ, et al. Phase I and pharmacokinetic evaluation of floxuridine/leucovorin given on the Roswell Park weekly regimen. Cancer Chemother Pharmacol 1994;34:261–5.

23. Hoskins JM, Goldberg RM, Qu P, Ibrahim JG, McLeod HL. UGT1A1 *28 genotype and irinotecan-induced neutropenia: dose matters. J Natl Cancer Inst 2007;99:1290–5.