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(54) Title: COMBINATION CHEMOTHERAPY WITH CHLOROTOXIN

(57) Abstract: This invention includes compositions and methods for combination chemotherapy, particularly involving at least one chemotherapeutic agent used in combination with chlorotoxin or a derivative thereof.

Combination Chemotherapy with Chlorotoxin

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Related Applications

This application claims the benefit of U.S. Provisional Application 60/406,033 (filed August 27, 2002) and U.S. Provisional Application 60/384,171 (filed May 31, 2002) both of which are hereby incorporated by reference in their entirety.

Field Of The Invention

The present invention relates generally to the fields of cell physiology and oncology. More specifically, the present invention relates to a novel method of treating cell proliferative disorders, such as cancers, with doses of chlorotoxin and/or derivatives thereof in combination with chemotherapeutic agents.

Background of the Invention

Tumors that originate in brain tissue are known as primary brain tumors as opposed to secondary brain tumors that develop when cancer metastatizes to the brain. Primary brain tumors are classified by the type of tissue in which they begin. The most common brain tumors are gliomas, which begin in the glial (supportive) tissue. Astrocytomas are a type of glioma that arise from small, star-shaped cells called astrocytes. They may grow anywhere in the brain or spinal cord but most often arise in the cerebrum in adults and the brain stem, the cerebrum, and the cerebellum in children. A grade III astrocytoma is sometimes called anaplastic astrocytoma while a grade IV astrocytoma is usually called glioblastoma multiforme. Brain stem gliomas occur in the lowest, stemlike part of the brain. Tumors in this area generally cannot be removed. Most brain stem gliomas are high-grade astrocytomas. Ependymomas are a type of glioma that usually develop in the lining of the ventricles and may also occur in the spinal cord. Although these tumors can develop at any age, they are most common in childhood and adolescence. Oligodendrogliomas arise in the cells that produce myelin, the fatty covering that protects nerves. These rare tumors usually arise in the cerebrum, grow slowly, usually do not spread into surrounding brain tissue and occur most often in middle-aged adults but have been found in people of all ages.

There are other types of brain tumors that do not originate in glial tissue.

Medulloblastomas were once thought to develop from glial cells. However, recent research suggests that these tumors develop from primitive (developing) nerve cells that normally do not remain in the body after birth. For this reason, medulloblastomas are sometimes called primitive neuroectodermal tumors. Most medulloblastomas arise in the cerebellum, however, they may occur in other areas as well. Meningiomas grow from the meninges and are usually benign. Because these tumors grow very slowly, the brain may be able to adjust to their presence and therefore these tumors often grow quite large before they cause symptoms. Schwannomas are benign tumors that begin in Schwann cells, which produce the myelin that protects the acoustic nerve. Acoustic neuromas are a type of schwannoma and occur mainly in adults. Craniopharyngiomas develop in the region of the pituitary gland near the hypothalamus and are usually benign, however, they are sometimes considered malignant because they can press on or damage the hypothalamus and affect vital functions. Germ cell tumors arise from primitive (developing) sex cells or germ cells. The most frequent type of germ cell tumor in the brain is the germinoma. Pineal region tumors occur in or around the pineal gland, a tiny organ near the center of the brain. The tumor can be slow growing (pineocytoma) or fast growing (pineoblastoma). The pineal region is very difficult to reach, and these tumors often cannot be removed.

Primitive neuroectodermal tumors are found both in the central and peripheral nervous systems. Primitive neuroectodermal tumors found only in the peripheral nervous system are referred to as peripheral primitive neuroectodermal tumors. Primitive neuroectodermal tumors manifest preferentially in children and have capacity for developing into a variety of neuronal, astrocytic, ependymal, muscular and melanotic lines. The conceptual basis of grouping these tumors together is based upon sharing common progenitor cells as well as sharing similar neoplastic transformations leading to tumors of similar morphological features and biological behavior. However, there remains controversy in placing all primitive neuroectodermal tumors into the same categories.

Supratentorial primitive neuroectodermal tumors include cerebral medulloblastomas, cerebral neuroblastomas, ependymoblastoma and other primitive neuroectodermal tumors, such as pineoblastomas. Peripheral neuroblastic tumors of the adrenal gland (medulla) and sympathetic nervous system are the most common type of childhood tumor outside of the central nervous system. Primary sites for these primitive neuroectodermal tumors are in the adrenals, abdominal, thoracic, cervical and pelvic sympathetic ganglia but include other primary sites as orbit, kidney, lung, skin, ovary, spermatic cord, and urinary bladder. Specific names of these related tumors are pheochromocytomas, paraganglioma,

neuroblastomas, ganglioneuromas, ganglioneuroblastomas, neurofibromas, schwannomas, and malignant peripheral nerve sheath tumors. These all share common origin in the neural crest. Medulloblastomas are members of the primitive neuroectodermal tumors that are described as highly malignant embryonal tumors of the central nervous system found in the cerebellum.

Currently, surgery is the treatment of choice for tumors of the central nervous system. Surgery provides a definite diagnosis, relieves the mass bulkiness of the tumor and extends survival of the patient. The only post-surgery adjuvant treatment which is known to work effectively on central nervous system tumors is radiation, and it can prolong survival. Radiation treatment, however, has many undesirable side effects. It can damage the normal tissue of the patient, including the neuronal tissue. Radiation also can cause severe side effects (*e.g.*, nausea, vomiting, hair loss).

The other common post-surgery adjuvant cancer treatment, chemotherapy, is relatively ineffective against neuroectodermal tumors. For example, chemotherapy against neuroectodermal tumors with nitrosourea agents is not curative. Many other cancer treating agents have been studied and tested, but generally they have a minimal effect on extending survival because many agents do not cross the blood-brain barrier. In view of these limited treatment options, the current prognosis for patients diagnosed with neuroectodermal tumors is not favorable. The median survival term for patients diagnosed with malignant astrocytomas having surgery and no adjuvant treatment is about fourteen weeks. Radiation therapy after surgery extends the median to about thirty-six weeks. The current two year survival rate for all forms of treatment is less than ten percent.

Other types of tumors are also difficult to combat by known cancer treatments. Lung cancer kills more Americans annually than the next four most frequently diagnosed neoplasms combined (Greenlee *et al.* (2001) CA Cancer J. Clin. 51, 15-36). Approximately eighty percent of primary lung tumors are of the non-small cell variety, which includes squamous cell and large cell carcinomas, as well as adenocarcinomas. Single-modality therapy is considered appropriate for most cases of early and late stage non-small cell lung cancer. Early stage tumors are potentially curable with surgery, chemotherapy, or radiotherapy, and late stage patients usually receive chemotherapy or best supportive care. Intermediate stage or locally advanced non-small cell lung cancer, which comprises twenty-five to thirty-five percent of all cases, is more typically treated with multi-modality therapy.

Breast cancer also presents treatment difficulties using known agents. The incidence of breast cancer in the United States has been rising at a rate of about two percent per year

since 1980, and the American Cancer Society estimated that 192,000 cases of invasive breast cancer were diagnosed in 2001. Breast cancer is usually treated with surgery, radiotherapy, chemotherapy, hormone therapy or combinations of the various methods. A major reason for the failure of cancer chemotherapy in breast cancer is the development of resistance to the cytotoxic drugs. Combination therapy using drugs with different mechanisms of action is an accepted method of treatment which prevents development of resistance by the treated tumor. Anti-angiogenic agents are particularly useful in combination therapy because they are not likely to cause resistance development since they do not act on the tumor, but on normal host tissue.

Compositions (see U.S. patent 5,905,027) and methods (see U.S. patent 6,028,174) for diagnosing and treating neuroectodermal tumors (*e.g.*, gliomas and meningiomas) have been developed based on the ability of chlorotoxin to bind to tumor cells of neuroectodermal origin (Soroceanu *et al.* (1998) Cancer Res. 58, 4871-4879; Ullrich *et al.* (1996) Neuroreport 7, 1020-1024; Ullrich *et al.* (1996) Am. J. Physiol. 270, C1511-C1521). Diagnosis of neuroectodermal tumors is accomplished by identification of labeled chlorotoxin bound to tumor cells while treatment of neuroectodermal tumors is accomplished by targeting tumors with cytotoxic agents linked to chlorotoxin. Chlorotoxin is a thirty-six amino acid protein naturally derived from *leiurus quinquestriatus* scorpion venom (DeBin *et al.* (1993) Am. J. Physiol. 264, C361-369). The present invention expands this area of therapeutics by providing a method for treating cancer using chlorotoxin, in combination with other, conventional cancer treating agents.

Summary of the Invention

The invention includes methods for treating cancer comprising administering chlorotoxin or a chlorotoxin derivative in combination with at least one chemotherapeutic agent. In some embodiments chlorotoxin or a chlorotoxin derivative is administered prior to administration of the chemotherapeutic agent, while in other embodiments it is administered simultaneously with the chemotherapeutic agent while in yet other embodiments, it is administered subsequent to the chemotherapeutic agent.

In another embodiment of the methods of the invention, the chemotherapeutic agent is selected from the group consisting of alkylating agents, purine antagonists, pyrimidine antagonists, plant alkaloids, intercalating antibiotics, aromatase inhibitors, anti-metabolites, mitotic inhibitors, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, biological response modifiers, anti-hormones and anti-androgens. Examples of

such chemotherapeutic agents to be used in the methods of the invention include, but are not limited to, BCNU, cisplatin, gemcitabine, hydroxyurea, paclitaxel, temozomide, topotecan, fluorouracil, vincristine, vinblastine, procarbazine, dacarbazine, altretamine, cisplatin, methotrexate, mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, fluorouracil, cytarabine, azacitidine, vinblastine, vincristine, etoposide, teniposide, irinotecan, docetaxel, doxorubicin, daunorubicin, dactinomycin, idarubicin, plicamycin, mitomycin, bleomycin, tamoxifen, flutamide, leuprolide, goserelin, aminoglutethimide, anastrozole, amsacrine, asparaginase, mitoxantrone, mitotane and amifostine.

In yet another embodiment, the methods of the invention are useful for treating types of cancer selected from the group consisting of lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma and pituitary adenoma.

The invention also includes compositions for treating cancer comprising chlorotoxin or a chlorotoxin derivative and at least one chemotherapeutic agent. In some embodiments, the chemotherapeutic agent is selected from the group consisting of alkylating agents, purine antagonists, pyrimidine antagonists, plant alkaloids, intercalating antibiotics, aromatase inhibitors, anti-metabolites, mitotic inhibitors, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, biological response modifiers, anti-hormones and anti-androgens. Examples of such chemotherapeutic agents to be used in the compositions of the invention include, but are not limited to, BCNU, cisplatin, gemcitabine, hydroxyurea, paclitaxel, temozomide, topotecan, fluorouracil, vincristine, vinblastine, procarbazine, dacarbazine, altretamine, cisplatin, methotrexate, mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, fluorouracil, cytarabine, azacitidine, vinblastine, vincristine, etoposide, teniposide, irinotecan, docetaxel, doxorubicin, daunorubicin, dactinomycin, idarubicin, plicamycin, mitomycin, bleomycin, tamoxifen, flutamide,

leuprolide, goserelin, aminoglutethimide, anastrozole, amsacrine, asparaginase, mitoxantrone, mitotane and amifostine.

The compositions of the invention are useful for treatment of one or more types of cancer selected from the group consisting of lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma and pituitary adenoma.

The invention also includes methods for detecting the presence of cancer in a patient comprising administering a detectable amount of labeled chlorotoxin or a chlorotoxin derivative, including radiolabeled chlorotoxin or a derivative thereof. Acceptable radiolabels include, but are not limited to, ^{3}H, ^{14}C, ^{18}F, ^{19}F, ^{31}P, ^{32}P, ^{35}S, ^{131}I, ^{125}I, ^{123}I, ^{64}Cu, ^{187}Re, ^{111}In, ^{90}Y, ^{99m}Tc and ^{177}Lu. Types of detectable cancer include, but are not limited to, lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma and pituitary adenoma.

Brief Description of Figures

Figure 1 depicts the effect of temodar in combination with chlorotoxin *in vitro*. D54 glioma cells were incubated with saline alone (control), temodar alone, temodar plus chlorotoxin, or pretreated with chlorotoxin twenty-four hours prior to temodar treatment.

Figure 2 depicts the effect of chlorotoxin on temodar efficacy *in vivo*. Nude mice with established U251 glioma flank tumors were treated with saline alone (control), temodar alone, or temodar plus chlorotoxin.

Figure 3 depicts the effect of chlorotoxin pretreatment on hydroxyurea efficacy *in vivo*. Nude mice with established D54 glioma flank tumors were treated with saline alone (control), hydroxyurea alone, or chlorotoxin plus hydroxyurea.

Figure 4 depicts a cytotoxicity assay in which low concentrations of chlorotoxin are shown to inhibit the growth and proliferation of glioblastoma cells.

Figure 5 depicts the effect of four day incubation and wash-out on the ability of chlorotoxin to inhibit abnormal cell growth.

Figure 6 depicts a cytotoxicity assay in which low concentrations of chlorotoxin are shown to inhibit the growth and proliferation of prostate cancer cells.

Figure 7 depicts an *in vivo* assay of the ability of chlorotoxin to inhibit the growth of glioblastoma tumor cells in athymic nude mice.

Figure 8 depicts an *in vivo* assay of the ability of chlorotoxin to enhance survival of athymic nude mice with intracranial glioblastoma tumors. Cessation of intravenous treatment indicated by arrow.

Figure 9 depicts an *in vivo* assay of the ability of chlorotoxin to inhibit growth of glioblastoma tumors in the flanks of athymic nude mice.

Figure 10 depicts a series of overlapping 10-mer peptides derived from chlorotoxin. Cysteine residues of SEQ ID NO: 1 are replaced in the 10-mers with serine to prevent cross-linking of peptides.

Figure 11 depicts binding of chlorotoxin and 10-mer peptides 1-15.

Figure 12 depicts binding of chlorotoxin and 10-mer peptides 16-27, 1, 5 and 10.

Figure 13 depicts binding of peptide 21, the native core 9-mer, and each alanine-substituted 9-mer peptide to both U251 and PC3 cells.

Figure 14 depicts binding of short scorpion toxins in PC3 human prostate cancer cells.

Figure 15 depictss the effect of peptide 21 on the proliferation of D54MG cells was studied by adding increasing doses of peptide 21 to the cells and then measuring the uptake of ^{3}H-thymidine.

Detailed Description

This invention relates to combination chemotherapy, particularly involving at least one chemotherapeutic agent used in combination with chlorotoxin or a derivative thereof. In one aspect, the invention includes compositions and methods for killing a cancer cell by first administering to a cancer cell chlorotoxin in combination with a chemotherapeutic agent. The present invention includes a method of retarding the growth of a tumor by administering chlorotoxin to tumor simultaneously with the chemotherapeutic agent. In another aspect, the invention includes compositions and methods for killing a cancer cell or retarding the growth of a tumor by first administering chlorotoxin (or a chlorotoxin derivative) and subsequently administering a chemotherapeutic agent. The present invention also includes a method of killing a cancer cell or retarding the growth of a tumor by first administering a chemotherapeutic agent and subsequently administering chlorotoxin (or a chlorotoxin derivative). The prior, simultaneous or subsequent administration of chlorotoxin or a derivative thereof may also have the effect of reducing the amount of chemotherapeutic agent necessary for successful treatment thus reducing the severe side effects associated with chemotherapeutic agents.

Combination Chemotherapeutic Compositions

This invention includes a pharmaceutical composition for the treatment of abnormal cell growth in a mammal, including a human, comprising an amount of chlorotoxin or a chlorotoxin derivative, in combination with an chemotherapeutic agent, that is effective in enhancing the effects of the chemotherapeutic agent in inhibiting abnormal cell growth (*i.e.*, acts as an adjuvant for the chemotherapeutic agent) and a pharmaceutically acceptable carrier. As used herein, the term "abnormal gell growth" unless otherwise indicated, refers to cell growth that is independent of normal regulatory mechanisms (*e.g.*, loss of contact inhibition). This includes the abnormal growth and/or proliferation of cells in both benign and malignant cells of neoplastic diseases. Chemotherapeutic-dependent inhibition of abnormal cell growth can occur by a variety of mechanisms including, but not limited to, cell death, apoptosis, inhibition of cell division, transcription, translation, transduction, etc.

In one embodiment of said composition, the abnormal cell growth is cancer. As used herein, the term "cancer" unless otherwise indicated, refers to diseases that are characterized by uncontrolled, abnormal cell growth and/or proliferation. Types of cancer where the composition is useful include, but are not limited to, lung cancer, bone cancer, liver cancer,

pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancers, spinal axis tumors, glioma, meningioma, pituitary adenoma, or a combination of one or more of the foregoing cancers. In another embodiment of said pharmaceutical composition, said abnormal cell growth is a benign proliferative disease, including, but not limited to, benign prostatic hyperplasia, hypertrophy or restinosis.

As discussed above, the invention includes a pharmaceutical composition for the treatment of abnormal cell growth in a mammal, including a human, which comprises an amount of a chlorotoxin, as defined above, in combination with at least one chemotherapeutic agent and a pharmaceutically acceptable carrier. As used herein, the term "chemotherapeutic agent" unless otherwise indicated, refers to any agent used in the treatment of cancer which inhibits, disrupts, prevents or interferes with abnormal cell growth and/or proliferation. Examples of chemotherapeutic agents include, but are not limited to, alkylating agents, purine antagonists, pyrimidine antagonists, plant alkaloids, intercalating antibiotics, aromatase inhibitors, anti-metabolites, mitotic inhibitors, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, biological response modifiers, steroid hormones and anti-androgens. In some embodiments, chlorotoxin or a derivative thereof can be combined with a single species of chemotherapeutic agent while in other embodiments, chlorotoxin can be combined with multiple species of chemotherapeutic agents.

Examples of alkylating agents include, but are not limited to, carmustine, lomustine, cyclophosphamide, ifosfamide, mechlorethamine and streptozotocin. Examples of antibiotics include, but are not limited to, bleomycin, dactinomycin, daunorubicin, doxorubicin, idarubicin and plicamycin. Examples of anti-metabolites include, but are not limited to, cytarabine, fludarabine, 5-fluorouracil, 6-mercaptopurine, methotrexate and 6-thioguanine. Examples of mitotic inhibitors include, but are not limited to, navelbine, paclitaxel, vinblastine and vincristine. Examples of steroid hormones and anti-androgens include, but

are not limited to, aminoglutethimides, estrogens, flutamide, goserelin, leuprolide, prednisone and tamoxifen.

In some aspects, the invention includes a population of conjugate molecules, said conjugate molecules comprising at least one chlorotoxin peptide or a derivative thereof and at least one chemotherapeutic agent, wherein the extent of conjugation of chlorotoxin and the chemotherapeutic agent is such that the effect of the chemotherapeutic agent in a mammal receiving the conjugate may be enhanced when compared to mixtures of the chemotherapeutic agent with chlorotoxin, or the chemotherapeutic agent alone. In another aspect, the invention includes compositions comprising a population of conjugate molecules wherein at least one chlorotoxin peptide or derivative thereof is conjugated to at least one chemotherapeutic agent and a pharmaceutically acceptable excipient. In some embodiments, chlorotoxin or a derivative thereof can be conjugated to a single species of chemotherapeutic agent while in other embodiments, chlorotoxin can be conjugated to multiple species of chemotherapeutic agents.

As used herein, the term "chlorotoxin" unless otherwise described, refers to the full-length, thirty-six amino acid polypeptide naturally derived from *Leiurus quinquestriatus* scorpion venom (DeBin *et al.* (1993) Am. J. Physiol. 264, C361-369) which comprises the amino acid sequence of native chlorotoxin as set forth in SEQ ID NO: 1. The term "chlorotoxin" includes polypeptides comprising SEQ ID NO: 1 which have been synthetically or recombinantly produced, such as those disclosed in U.S. Patent 6,319,891, which is herein incorporated by reference in its entirety.

As used herein, the term "chlorotoxin subunit" or "subunit of chlorotoxin" refers to a peptide comprising less than thirty-six contiguous amino acids of chlorotoxin and which is capable of specifically binding to cancer cells.

As used herein, the term "chlorotoxin derivative" refers to derivatives, analogs, variants, polypeptide fragments and mimetics of chlorotoxin and related peptides which retain the same activity as chlorotoxin, such as binding specifically binding to a cancer cell when compared to a normal cell, can also be used for practicing the methods of the invention. Examples of derivatives include, but are not limited to, peptide variants of chlorotoxin, peptide fragments of chlorotoxin, for example, fragments comprising or consisting of contiguous 10-mer peptides of SEQ ID NO: 1, 2, 3, 4, 5, 6 or 7 or comprising about residues 10-18 or 21-30 of SEQ ID NO: 1, core binding sequences, and peptide mimetics.

Chlorotoxin and peptide derivatives thereof can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the

nucleic acids encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included. The term "chlorotoxin derivative" as used herein is synonymous with "variant" also includes modifications to the chlorotoxin sequence by one or more deletions of up to 10 (*e.g.*, 1 to 7 or 1 to 5 amino acids; insertions of a total of up to 10 (*e.g.*, 1 to 5) amino acids internally within the amino acid sequence of chlorotoxin; or of up to a total of 100 amino acids at either terminus of the chlorotoxin sequence; or conservative substitutions of a total of up to 15 (*e.g.*, 1 to 5) amino acids.

Derivatives of chlorotoxin include polypeptides comprising a conservative or non-conservative substitution of at least one amino acid residue when the derivative sequence and the chlorotoxin sequence are maximally aligned. The substitution may be one which enhances at least one property or function of chlorotoxin, inhibits at least one property or function of chlorotoxin, or is neutral to at least one property or function of chlorotoxin. As used herein, a "property or function" of chlorotoxin includes, but is not limited to, at least one selected from the group consisting of the ability to arrest abnormal cell growth, cause paralysis of a subject, specific binding to a benign or malignant cancer cell when compared to a non-cancer cell (*i.e.*, normal), and killing of a benign or malignant cancer cell. In terms of the present disclosure, the cancer cell may be *in vivo*, *ex vivo*, *in vitro*, a primary isolate from a subject, a cultured cell or a cell line.

Derivatives of chlorotoxin further include polypeptides comprising the amino acid sequence KGRGKSY (SEQ ID NO: 8), corresponding to amino acid residues 23-29 of SEQ ID NO: 1. Derivatives of chlorotoxin also include polypeptides comprising the amino acid sequence $TTX_1X_2X_3MX_4X_5K$ (SEQ ID NO: 13) corresponding to amino acid residues 7-15 of SEQ ID NO: 1, wherein X_1 is an acidic amino acid selected from the group consisting of aspartic acid and glutamic acid; X_2 is an amino acid selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, proline, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine; X_3 is an amide amino acid selected from the group consisting of asparagine and glutamine; X_4 is an any amino acid but in a preferred embodiment is selected from the group consisting of serine, threonine and alanine; and X_5 is a basic amino acid selected from the group consisting of histine, lysine and arginine. In one embodiment,

X_1 is aspartic acid, X_2 is histidine or proline, X_3 is glutamine, X_4 is alanine and X_5 is arginine or lysine.

Peptide variants of chlorotoxin include, but are not limited to, deletion or conservative amino acid substitution variants of SEQ ID NO: 1. As used herein, a conservative variant refers to alterations in the amino acid sequence that do not adversely substantially affect the biological functions of the peptide. A substitution, insertion or deletion is said to adversely affect the peptide when the altered sequence substantially prevents or disrupts a biological function associated with the peptide (*e.g.*, binding to a cancer cell). For example, the overall charge, structure or hydrophobic/hydrophilic properties of the peptide can be altered without adversely affecting a biological activity. Accordingly, the amino acid sequence can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the biological activities of the peptide.

The methods of the invention include corresponding polypeptide toxins of other scorpion species that display similar or related activity to chlorotoxin for the diagnosis and treatment of diseases associated with abnormal cell proliferation as described herein, including cancer. For purposes of the specification, "similar or related activity to chlorotoxin" is defined as binding to cells displaying abnormal cell growth, including benign cells exhibiting abnormal growth and malignant cancer cells. Examples of such polypeptide toxins include, but are not limited to, toxins which contain one or more of the binding domains of chlorotoxin set forth in SEQ ID NO: 8 or SEQ ID NO: 13, and any of the consensus sequences set forth in Table 1.

Table 1 – Scorpion toxin alignments (sequence identifier in parenthesis)

Small Toxin (Peptide I) – *Mesobuthus tamulus sindicus*

```
                                  1                                   36
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
Small Toxin (SEQ ID NO: 15)  RCKPCFTTDPQMSKKCADCCGGKGKGKCYGPQCLC-
Consensus   (SEQ ID NO: 16)   C PCFTTD QMAKKC DCCGGKGKGKCYGPQCLC
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Probable Toxin LQH 8/6 – *Leiurus quinquestriatus hebraeus*

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                                  1                                     38
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR--
Toxin LQH   (SEQ ID NO: 17)  RCSPCFTTDQQMTKKCYDCCGGKGKGKCYGPQCICAPY
Consensus   (SEQ ID NO: 18)   C PCFTTD QM KKC DCCGGKGKGKCYGPQCIC
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Chinese Scorpion – *Mesobuthus martensii*

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                                  1                                   35
Chlorotoxin (SEQ ID NO: 1)   ----------------------MCMPCFTTDHQM
Chinese     (SEQ ID NO: 19)  MKFLYGIVFIALFLTVMFATQTDGCGPCFTTDANM
Consensus   (SEQ ID NO: 20)                          C PCFTTD NM
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                                    36                          61
Chlorotoxin (SEQ ID NO: 1)   ARKCDDCCGGKGRGKCYGPQCLCR--
Chinese     (SEQ ID NO: 19) ARKCRECCGGIG--KCFGPQCLCNRI
Consensus   (SEQ ID NO: 20) ARKC DCCGG G  KCFGPQCLC

                                    1                                35
Chlorotoxin (SEQ ID NO: 1)   ------------------------MCMPCFTTDHQM
Chinese     (SEQ ID NO: 21) MKFLYGIVFIALFLTVMFATQTDGCGPCFTTDANM
Consensus   (SEQ ID NO: 22)                            C PCFTTD NM

                                    36                          59
Chlorotoxin (SEQ ID NO: 1)   ARKCDDCCGGKGRGKCYGPQCLCR
Chinese     (SEQ ID NO: 21) ARKCRECCGGIGKCFGPQCLCNRI
Consensus   (SEQ ID NO: 22) ARKC DCCGG GK       C
```

Insect toxin I5 (lesser Asian Scorpion) – *Mesobuthus eupeus*

```
                                    1                                   37
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR-
toxin I5    (SEQ ID NO: 23) MCMPCFTTDPNMANKCRDCCGG-GK-KCFGPQCLCNR
Consensus   (SEQ ID NO: 24) MCMPCFTTD NMA KC DCCGG GK KCFGPQCLC

                                    1                                   36
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
toxin I5    (SEQ ID NO: 25) MCMPCFTTDPNMANKCRDCCGGGKKCFGPQCLCNR-
Consensus   (SEQ ID NO: 26) MCMPCFTTD NMA KC DCCGG  K       C
```

Insectotoxin I1 (lesser Asian Scorpion) – *Mesobuthus eupeus*

```
                                    1                                   38
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR--
toxin I1    (SEQ ID NO: 27) MCMPCFTTRPDMAQQCRACCKG--RGKCFGPQCLCGYD
Consensus   (SEQ ID NO: 28) MCMPCFTT   MA  C  CC G  RGKCFGPQCLC

                                    1                                   36
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
toxin I1    (SEQ ID NO: 29) MCMPCFTTRPDMAQQCRACCKGRGKCFGPQCLCGYD
Consensus   (SEQ ID NO: 30) MCMPCFTT   MA  C  CC GKGK       C
```

Insectotoxin 15A (lesser Asian Scorpion) – *Mesobuthus eupeus*

```
                                    1                                   37
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR-
toxin 15A   (SEQ ID NO: 31) MCMPCFTTDPNMAKKCRDCCGGNG--KCFGPQCLCNR
Consensus   (SEQ ID NO: 32) MCMPCFTTD NMAKKC DCCGG G  KCFGPQCLC

                                    1                                   36
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
toxin 15A   (SEQ ID NO: 33) MCMPCFTTDPNMAKKCRDCCGGNGKCFGPQCLCNR-
Consensus   (SEQ ID NO: 34) MCMPCFTTD NMAKKC DCCGG GK       C
```

Neurotoxin P2 (Moroccan scorpion) – *Androctonus mauretanicus*

```
                                    1                                   38
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR--
Neurotoxin  (SEQ ID NO: 35) -CGPCFTTDPYTESKCATCCGG--RGKCVGPQCLCNRI
Consensus   (SEQ ID NO: 36)  C PCFTTD     KC  CCGG  RGKC GPQCLC

                                    1                                   36
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
Neurotoxin  (SEQ ID NO: 37) -CGPCFTTDPYTESKCATCCGGRGKCVGPQCLCNRI
Consensus   (SEQ ID NO: 38)  C PCFTTD     KC  CCGGKGK       C
```

Alignment of Chlorotoxin with all above scorpion toxins

```
                  1                                                  50
Chlorotoxin       ----------------------MCMPCFTTDHQMARKCDDCCGGKGRGK
Small Toxin       ----------------------RCKPCFTTDPQMSKKCADCCGGKGKGK
Toxin LQH         ----------------------RCSPCFTTDQQMTKKCYDCCGGKGKGK
Chinese           MKFLYGIVFIALFLTVMFATQTDGCGPCFTTDANMARKCRECCGGIG--K
toxin I5          ----------------------MCMPCFTTDPNMANKCRDCCGGGK--K
toxin I1          ----------------------MCMPCFTTRPDMAQQCRACCKGRG--K
toxin 15A         ----------------------MCMPCFTTDPNMAKKCRDCCGGNG--K
Neurotoxin        ----------------------CGPCFTTDPYTESKCATCCGGRG--K
Consensus                               MCMPCFTTDPNMAKKCRDCCGGKG  K

                             51       61
Chlorotoxin (SEQ ID NO: 1)   CYGPQCLCR--
Small Toxin (SEQ ID NO: 15)  CYGPQCLC---
Toxin LQH   (SEQ ID NO: 17)  CYGPQCICAPY
Chinese     (SEQ ID NO: 19)  CFGPQCLCNRI
toxin I5    (SEQ ID NO: 25)  CFGPQCLCNR-
toxin I1    (SEQ ID NO: 29)  CFGPQCLCGYD
toxin 15A   (SEQ ID NO: 33)  CFGPQCLCNR-
Neurotoxin  (SEQ ID NO: 37)  CVGPQCLCNRI
Consensus   (SEQ ID NO: 39)  CFGPQCLCNR
```

Alignment of Chlorotoxin with toxins not requiring gaps to align

```
                             1                                      38
Chlorotoxin (SEQ ID NO: 1)   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR--
Small Toxin (SEQ ID NO: 15)  RCKPCFTTDPQMSKKCADCCGGKGKGKCYGPQCLC---
Toxin LQH   (SEQ ID NO: 17)  RCSPCFTTDQQMTKKCYDCCGGKGKGKCYGPQCICAPY
Consensus   (SEQ ID NO: 40)  RC PCFTTD QMSKKC DCCGGKGKGKCYGPQCLC
```

Alignment of Chlorotoxin with toxins requiring gaps (gaps removed)

```
                  1                                                  50
Chlorotoxin       ----------------------MCMPCFTTDHQMARKCDDCCGGKGRGK
CT-Neurotox       MKFLYGIVFIALFLTVMFATQTDGCGPCFTTDANMARKCRECCGGIGKCF
toxin I5          ----------------------MCMPCFTTDPNMANKCRDCCGGGKKCF
toxin I1          ----------------------MCMPCFTTRPDMAQQCRACCKGRGKCF
toxin 15A         ----------------------MCMPCFTTDPNMAKKCRDCCGGNGKCF
Neurotoxin        ----------------------CGPCFTTDPYTESKCATCCGGRGKCV
Consensus                               MCMPCFTTDPNMARKCRDCCGGRGKCF

                             51
Chlorotoxin (SEQ ID NO: 1)   CYGPQCLCR
CT-Neurotox (SEQ ID NO: 19)  GPQCLCNRI
toxin I5    (SEQ ID NO: 25)  GPQCLCNR-
toxin I1    (SEQ ID NO: 29)  GPQCLCGYD
toxin 15A   (SEQ ID NO: 33)  GPQCLCNR-
Neurotoxin  (SEQ ID NO: 37)  GPQCLCNRI
Consensus   (SEQ ID NO: 41)  GPQCLCNR
```

Chlorotoxin Peptide 8 alignment with other scorpion toxins

```
                             1
      Pep8-Ctlx   (SEQ ID NO: 42)  CGGKGRGKCY
Pep8-SCX1_BUTSI   (SEQ ID NO: 43)  CGGKGKGKCY
      Consensus   (SEQ ID NO: 43)  CGGKGKGKCY
```

```
                                                1
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY
Pep8-SCX8_LEIQH    (SEQ ID NO: 43) CGGKGKGKCY
      Consensus    (SEQ ID NO: 43) CGGKGKGKCY

                                                1          12
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY--
Pep8-AF079059_2    (SEQ ID NO: 44) CGGIG--KCFGP
      Consensus    (SEQ ID NO: 45) CGG GRGKCFGP

                                                1
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY
Pep8-AF079059_2    (SEQ ID NO: 44) CGGIGKCFGP
      Consensus    (SEQ ID NO: 46) CGG GK

                                     1          12
  Pep8-Ctlx        (SEQ ID NO: 42) CGGKGRGKCY--
Pep8-JN0361        (SEQ ID NO: 47) CGG-GK-KCFGP
  Consensus        (SEQ ID NO: 48) CGGKGKGKCFGP

                                     1
  Pep8-Ctlx        (SEQ ID NO: 42) CGGKGRGKCY
Pep8-JN0361        (SEQ ID NO: 47) CGGGKKCFGP
  Consensus        (SEQ ID NO: 49) CGG  K

                                                1          12
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY--
Pep8-SCX1_BUTEU    (SEQ ID NO: 50) --CKGRGKCFGP
      Consensus    (SEQ ID NO: 51) CG KGRGKCFGP

                                                1
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY
Pep8-SCX1_BUTEU    (SEQ ID NO: 50) CKGRGKCFGP
      Consensus    (SEQ ID NO: 52) C GKGK

                                                1          12
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY--
Pep8-SCX5_BUTEU    (SEQ ID NO: 53) CGGNG--KCFGP
      Consensus    (SEQ ID NO: 54) CGG GRGKCFGP

                                                1
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY
Pep8-SCX5_BUTEU    (SEQ ID NO: 53) CGGNGKCFGP
      Consensus    (SEQ ID NO: 55) CGG GK

                                                1          12
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY--
Pep8-SCXP_ANDMA    (SEQ ID NO: 56) CGG--RGKCVGP
      Consensus    (SEQ ID NO: 57) CGGKGRGKC GP

                                                1
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY
Pep8-SCXP_ANDMA    (SEQ ID NO: 56) CGGRGKCVGP
      Consensus    (SEQ ID NO: 58) CGGKGK

                                                1          12
```

```
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY--
  Pep8-SCX1_BUTSI  (SEQ ID NO: 43) CGGKGKGKCY--
  Pep8-SCX8_LEIQH  (SEQ ID NO: 43) CGGKGKGKCY--
  Pep8-AF079059_2  (SEQ ID NO: 44) CGG--IGKCFGP
     Pep8-JN0361   (SEQ ID NO: 47) CGG--GKKCFGP
  Pep8-SCX1_BUTEU  (SEQ ID NO: 50) CKG--RGKCFGP
  Pep8-SCX5_BUTEU  (SEQ ID NO: 53) CGG--NGKCFGP
  Pep8-SCXP_ANDMA  (SEQ ID NO: 56) CGG--RGKCVGP
      Consensus    (SEQ ID NO: 59) CGG  RGKCFGP
```

```
                                          1
      Pep8-Ctlx    (SEQ ID NO: 42) CGGKGRGKCY
  Pep8-SCX1_BUTSI  (SEQ ID NO: 43) CGGKGKGKCY
  Pep8-SCX8_LEIQH  (SEQ ID NO: 43) CGGKGKGKCY
  Pep8-AF079059_2  (SEQ ID NO: 44) CGGIGKCFGP
     Pep8-JN0361   (SEQ ID NO: 47) CGGGKKCFGP
  Pep8-SCX1_BUTEU  (SEQ ID NO: 50) CKGRGKCFGP
  Pep8-SCX5_BUTEU  (SEQ ID NO: 53) CGGNGKCFGP
  Pep8-SCXP_ANDMA  (SEQ ID NO: 56) CGGRGKCVGP
      Consensus    (SEQ ID NO: 60) CGGKGKCFGP
```

Chlorotoxin Peptide 21 alignment with other scorpion toxins

```
                                          1
      Pep21-cltx   (SEQ ID NO: 61) TTDHQMARKC
  Pep21-SCX1_BUTSI (SEQ ID NO: 62) TTDPQMSKKC
       Consensus   (SEQ ID NO: 63) TTD QMAKKC
```

```
                                          1
      Pep21-cltx   (SEQ ID NO: 61) TTDHQMARKC
  Pep21-SCX8_LEIQH (SEQ ID NO: 64) TTDQQMTKKC
       Consensus   (SEQ ID NO: 65) TTD QM KKC
```

```
                                          1
      Pep21-cltx   (SEQ ID NO: 61) TTDHQMARKC
  Pep21-AF079059_2 (SEQ ID NO: 66) TTDANMARKC
       Consensus   (SEQ ID NO: 67) TTD NMARKC
```

```
                                 1
    Pep21-cltx     (SEQ ID NO: 61) TTDHQMARKC
  Pep21-JN0361     (SEQ ID NO: 68) TTDPNMANKC
     Consensus     (SEQ ID NO: 69) TTD NMA KC
```

```
                                          1
      Pep21-cltx   (SEQ ID NO: 61) TTDHQMARKC
  Pep21-SCX1_BUTEU (SEQ ID NO: 70) TTRPDMAQQC
       Consensus   (SEQ ID NO: 71) TT   MA  C
```

```
                             1
      Pep21-cltx   (SEQ ID NO: 61) TTDHQMARKC
  Pep21-SCX5_BUTEU (SEQ ID NO: 72) TTDPNMAKKC
       Consensus   (SEQ ID NO: 73) TTD NMAKKC
```

```
                                          1
      Pep21-cltx   (SEQ ID NO: 61) TTDHQMARKC
  Pep21-SCXP_ANDMA (SEQ ID NO: 74) TTDPYTESKC
       Consensus   (SEQ ID NO: 75) TTD     KC
```

					1
	Pep21-cltx	(SEQ	ID	NO: 61)	TTDHQMARKC
	Pep21-SCX1_BUTSI	(SEQ	ID	NO: 62)	TTDPQMSKKC
5	Pep21-SCX8_LEIQH	(SEQ	ID	NO: 64)	TTDQQMTKKC
	Pep21-AF079059_2	(SEQ	ID	NO: 66)	TTDANMARKC
	Pep21-JN0361	(SEQ	ID	NO: 68)	TTDPNMANKC
	Pep21-SCX1_BUTEU	(SEQ	ID	NO: 70)	TTRPDMAQQC
	Pep21-SCX5_BUTEU	(SEQ	ID	NO: 72)	TTDPNMAKKC
10	Pep21-SCXP_ANDMA	(SEQ	ID	NO: 74)	TTDPYTESKC
	Consensus	(SEQ	ID	NO: 76)	TTDPNMAKKC

As used herein, the term "related scorpion toxin" refers to any of the toxins or related peptides, such as those disclosed in Table 1, displaying amino acid and/or nucleotide sequence identity to chlorotoxin. Examples of related scorpion toxins include, but are not limited to, CT neurotoxin from *Mesobuthus martensii* (GenBank Accession AAD47373), Neurotoxin BmK 41-2 from *Buthus martensii karsch* (GenBank Accession A59356), Neurotoxin Bm12-b from *Buthus martensii* (GenBank Accession AAK16444), Probable Toxin LQH 8/6 from *Leiurus quinquestriatus hebraeu* (GenBank Accession P55966), Small toxin from *Mesobuthus tamulus sindicus* (GenBank Accession P15229), the sequences of which are all herein incorporated by reference in their entirety.

Homology or sequence identity at the nucleotide or amino acid sequence level is determined by **BLAST** (**B**asic **L**ocal **A**lignment **S**earch **T**ool) analysis using the algorithm employed by the programs **blastp, blastn, blastx, tblastn** and **tblastx** (Altschul *et al.* (1997) Nucleic Acids Res. 25, 3389-3402 and Karlin *et al.* (1990) Proc. Natl. Acad. Sci. USA 87, 2264-2268, both fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is to first consider similar segments, with gaps (non-contiguous) and without gaps (contiguous), between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (1994) Nature Genetics 6, 119-129 which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (*i.e.*, the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter (low complexity) are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the **BLOSUM62** matrix (Henikoff *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 10915-10919, fully incorporated by reference), recommended for query sequences over eighty-five nucleotides or amino acids in length.

For **blastn**, the scoring matrix is set by the ratios of **M** (*i.e.*, the reward score for a pair of matching residues) to **N** (*i.e.*, the penalty score for mismatching residues), wherein the default values for **M** and **N** are +5 and -4, respectively. Four **blastn** parameters were adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every winkth position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent **Blastp** parameter settings were Q=9; R=2; wink=1; and gapw=32. A **Bestfit** comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2.

The present invention encompasses the allelic variants, conservative substitution variants, and the members of the scorpion toxin peptide family, having an amino acid sequence of at least about seventy-five percent, at least about eighty-five percent, at least about ninety percent sequence, at least about ninety-five percent, or at least about ninety-nine percent sequence identity with the entire chlorotoxin sequence set forth in SEQ ID NO: 1. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after alignment the sequences.

Fusion proteins, or N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology. Examples of such extensions include, but are not limited to, the following sequences:

HHHHHHMCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR SEQ ID NO: 2),

YMCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR (SEQ ID NO: 3),

YSYMCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR (SEQ ID NO: 4).

The chlorotoxin peptide variants include peptides having a fragment of the amino acid sequence set forth in SEQ ID NO: 1, having at least about 7, 8, 9, 10, 15, 20, 25, 30, or 35 contiguous amino acid residues. The peptide variants further include those fragments associated with the activity of chlorotoxin. Such fragments, also referred to as polypeptides, may contain functional regions of the chlorotoxin peptide identified as regions of the amino acid sequence which correspond to known peptide domains, as well as regions of pronounced hydrophilicity. Variants may also include peptide with at least two core sequences linked to one another, in any order, with intervening amino acids removed or replaced by a linker

sequence. The regions are all easily identifiable by using commonly available protein sequence analysis software such as MacVector (Oxford Molecular).

Contemplated peptide variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the alleles or other naturally occurring variants of the family of peptides; and derivatives wherein the peptide has been covalently modified by substitution, chemical, enzymatic or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope). Examples of chlorotoxin variant peptides include, but are not limited to the following sequences:

MCMPCFTTDHQMARKCDDCCGGKGRGKCFGPQCLCR (SEQ ID NO: 5),
RCKPCFTTDPQMSKKCADCCGGKGKGKCYGPQCLC (SEQ ID NO: 6),
RCSPCFTTDQQMTKKCYDCCGGKGKGKCYGPQCICAPY (SEQ ID NO: 7).

Peptide Mimetics

In another class of chlorotoxin derivatives, the present invention includes peptide mimetics that mimic the three-dimensional structure of chlorotoxin. Such peptide mimetics may have significant advantages over naturally occurring peptides including, for example, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity and others.

In one form, mimetics are peptide-containing molecules that mimic elements of chlorotoxin peptide secondary structure. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. In another form, peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compounds are also referred to as peptide mimetics or peptidomimetics (Fauchere (1986) Adv. Drug Res. 15, 29-69; Veber & Freidinger (1985) Trends Neurosci. 8, 392-396; Evans *et al.* (1987) J. Med. Chem. 30, 1229-1239 which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling.

Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptide

mimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biochemical property or pharmacological activity), but have one or more peptide linkages optionally replaced by a linkage by methods known in the art. Labeling of peptide mimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering positions on the peptide mimetic that are predicted by quantitative structure-activity data and molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules to which the peptide mimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptide mimetics should not substantially interfere with the desired biological or pharmacological activity of the peptide mimetic.

The use of peptide mimetics can be enhanced through the use of combinatorial chemistry to create drug libraries. The design of peptide mimetics can be aided by identifying amino acid mutations that increase or decrease binding of a peptide to, for instance, a tumor cell. Approaches that can be used include the yeast two hybrid method (see Chien *et al.* (1991) Proc. Natl. Acad. Sci. USA 88, 9578-9582) and using the phage display method. The two hybrid method detects protein-protein interactions in yeast (Fields *et al.* (1989) Nature 340, 245-246). The phage display method detects the interaction between an immobilized protein and a protein that is expressed on the surface of phages such as lambda and M13 (Amberg *et al.* (1993) Strategies 6, 2-4; Hogrefe *et al.* (1993) Gene 128, 119-126). These methods allow positive and negative selection for peptide-protein interactions and the identification of the sequences that determine these interactions.

Pharmaceutical Compositions

Pharmaceutical compositions of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal or buccal routes. For example, an agent may be administered locally to a tumor via microinfusion. Alternatively, or concurrently, administration may be by the oral route. For example, chlorotoxin or a derivative thereof could be administered locally to the site of a tumor, followed by oral administration of at least one chemotherapeutic agent. The prior administration of chlorotoxin may have the effect of reducing the amount of chemotherapeutic agent necessary for successful treatment thus reducing the severe side effects associated with chemotherapeutic agents. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further includes compositions containing chlorotoxin or derivatives thereof and one or more chemotherapeutic agents that are useful in the treatment of cancer. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 1.0 pg/kg body weight to 100 mg/kg body weight. The preferred dosages for systemic administration comprise 100.0 ng/kg body weight to 10.0 mg/kg body weight. The preferred dosages for direct administration to a site via microinfusion comprise 1 ng/kg body weight to 1 mg/kg body weight.

In addition to chlorotoxin and chemotherapeutic agents, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol and dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

As mentioned above, topical administration may be used. Any common topical formulation such as a solution, suspension, gel, ointment or salve and the like may be employed. Preparation of such topical formulations are described in the art of pharmaceutical formulations as exemplified, for example, by Gennaro *et al.* (1995) Remington's Pharmaceutical Sciences, Mack Publishing. For topical application, the compositions could also be administered as a powder or spray, particularly in aerosol form. In a some embodiments, the compositions of this invention may be administered by inhalation. For inhalation therapy the active ingredients may be in a solution useful for administration by

metered dose inhalers or in a form suitable for a dry powder inhaler. In another embodiment, the compositions are suitable for administration by bronchial lavage.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof. In another embodiment, the pharmaceutical composition comprises chlorotoxin or derivatives thereof in combination with at least one sustained release form of chemotherapeutic agent. In such formulations, chlorotoxin or derivatives thereof will be distributed throughout the body, prior to release of the chemotherapeutic agents, allowing for binding of chlorotoxin to the cancer cells prior to binding of the chemotherapeutic agent to the cancer cells. Upon the delayed release of the chemotherapeutic agent from such formulations, and subsequent distribution to the site of the cancer cells, the effects of the chemotherapeutic agent may be enhanced by the earlier binding of chlorotoxin to the cancer cells. Such delayed release formulations may have the same effect as sequential administration of chlorotoxin followed by one or more chemotherapeutic agents.

Labeled Chlorotoxin and Chlorotoxin Derivatives

The invention also includes isotopically-labeled chlorotoxin or derivatives thereof, that have one or more atoms are replaced by an atom having an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes that can be incorporated into compounds of the invention include isotopes of hydrogen, carbon, fluorine, phosphorous, iodine, copper, rhenium, indium, yttrium, technecium and lutetium (*i.e.*, ^{3}H, ^{14}C, ^{18}F, ^{19}F, ^{31}P, ^{32}P, ^{35}S, ^{131}I, ^{125}I, ^{123}I, ^{64}Cu, ^{187}Re, ^{111}In, ^{90}Y, ^{99m}Tc, ^{177}Lu). In some embodiments, isotopes which are metals (*e.g.*, copper, rhenium, indium, yttrium, technecium and lutectium) are non-covalently attached to the chlorotoxin or derivatives thereof by chelation. Examples of chelation included in the invention are chelation of a metal isotope to a polyHis region fused to chlorotoxin or a derivative thereof. Non-metal isotopes may be covalently attached to chlorotoxin or derivatives thereof using any means acceptable.

The invention also includes chlorotoxin or derivatives thereof labeled with a metal such as gadolinium (Gd). In some embodiments, a metal such as gadolinium is covalently attached to chlorotoxin or derivative thereof by chelation. Examples of chelation included in the invention are chelation of a metal such as gadolinium to a polyHis region fused to chlorotoxin or a derivative thereof.

Labeled chlorotoxin and derivatives thereof provided by this invention are also useful as radiotracers for positron emission tomography (PET) imaging or for single photon emission computerized tomography (SPECT).

Agents of the present invention, prodrugs thereof, and pharmaceutically acceptable salts of said agents or of said prodrugs which contain the aforementioned isotopes and/or other isotopes of other atoms are within the scope of this invention. Trititium and carbon-14 isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with heavier isotopes such as deuterium can afford certain therapeutic advantages resulting from greater metabolic stability, for example increased *in vivo* half-life or reduced dosage requirements and, hence, may be preferred in some circumstances.

Methods of Treatment Using Combination Chemotherapy with Chlorotoxin

This invention also includes methods for the treatment of abnormal cell growth in a mammal, including a human, comprising administering to said mammal an amount of chlorotoxin or derivative thereof, or a pharmaceutical composition comprising an amount of chlorotoxin or a derivative thereof, that is effective in enhancing the effect of a chemotherapeutic agent (*i.e.*, acts as an adjuvant for the chemotherapeutic agent) when administered prior to, or subsequent to, a chemotherapeutic agent. In one embodiment of this method, the abnormal cell growth is cancer, including, but not limited to, lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma, pituitary adenoma, or a combination of one or more of the foregoing cancers. In another embodiment of said method, said abnormal cell growth is a benign proliferative disease, including, but not limited to, psoriasis, benign prostatc hyperplasia, hypertrophy or restinosis.

This invention also includes methods for the treatment of abnormal cell growth in a mammal which comprises administering to said mammal, including a human, a pharmaceutical composition comprising amount of chlorotoxin or a chlorotoxin derivative and one or more chemotherapeutic agents, that is effective in enhancing the effects of the chemotherapeutic agent in inhibiting abnormal cell growth. This includes the abnormal growth and/or proliferation of cancer cells including benign and malignant cells of neoplastic diseases. Inhibition of abnormal cell growth can occur by a variety of mechanism including, but not limited to, cell death, apoptosis, inhibition of cell division, transcription, translation, transduction, etc.

As discussed above, chlorotoxin and derivatives thereof can be provided in combination, or in sequential combination with other chemotherapeutic agents that are useful in the treatment of abnormal cell growth (*e.g.*, cancer). As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time. For example, chlorotoxin or chlorotoxin derivatives can be used in combination with one or more chemotherapeutic agents selected from the following types of chemotherapeutic agents including, but not limited to, mitotic inhibitors, alkylating agents, anti-metabolites, intercalating antibiotics, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, biological response modifiers, anti-hormones, and anti-androgens.

Examples of alkylating agents include, but are not limited to, carmustine, lomustine, cyclophosphamide, ifosfamide, mechlorethamine and streptozotocin. Examples of antibiotics include, but are not limited to, bleomycin, dactinomycin, daunorubicin, doxorubicin, idarubicin and plicamycin. Examples of anti-metabolites include, but are not limited to, cytarabine, fludarabine, 5-fluorouracil, 6-mercaptopurine, methotrexate and 6-thioguanine. Examples of mitotic inhibitors include, but are not limited to, navelbine, paclitaxel, vinblastine and vincristine. Examples of steroid hormones and anti-androgens include, but are not limited to, aminoglutethimides, estrogens, flutamide, goserelin, leuprolide, prednisone and tamoxifen.

Examples of pharmaceutical formulations of the above chemotherapeutic agents include, but are not limited to, BCNU (*i.e.*, carmustine, 1,3-bis(2-chloroethyl)-l-nitrosurea, BiCNU®), cisplatin (cis-platinum, cis-diamminedichloroplatinum, Platinol®), doxorubicin (hydroxyl daunorubicin, Adriamycin®), gemcytabine (difluorodeoxycytidine, Gemzar®), hyrdoxyurea (hyroxycarbamide, Hydrea®), paclitaxel (Taxol®), temozolomide (TMZ,

Temodar®), topotecan (Hycamtin®), fluorouracil (5-fluorouracil, 5-FU, Adrucil®), vincristine (VCR, Oncovin®) and vinblastine (Velbe® or Velban®).

In practicing the methods of this invention, chlorotoxin or derivatives thereof may be used alone or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, chlorotoxin or derivatives thereof may be co-administered along with other chemotherapeutic agents typically prescribed for various types of cancer according to generally accepted oncology medical practice. The compositions of this invention can be utilized *in vivo*, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice or *in vitro*. The invention is particularly useful in the treatment of human subjects.

Methods of Treatment Using Chlorotoxin in Combination with Radiation

The invention includes a therapeutic method comprising administration of chlorotoxin or a derivative thereof in combination with radiation therapy for the treatment of diseases associated with abnormal cell growth, such as cancer. In particular, the therapy is designed to induce apoptosis (cell death) in cancer cells, although reducing the incidence or number of metastases, and reducing tumor size also are contemplated. Tumor cell resistance to radiotherapy agents represents a major problem in clinical oncology. Thus, in the context of the present invention, it also is contemplated that combination therapy with chlorotoxin could be used on radiation resistant tumors to improve the efficacy of the radiation therapy.

As discussed above, the invention includes a method of treating cancer comprising administering to a mammal with cancer an amount of chlorotoxin or a derivative thereof in combination with ionizing radiation, both in sufficient doses that, when combined, cancer cell death is induced. In one embodiment, the presence of the chlorotoxin reduces the amount of radiation required to treat the cancer when compared to radiation treatment alone. Chlorotoxin or derivatives thereof can be provided prior to said radiation, after said radiation or concurrent with said radiation.

Radiation that causes DNA damage has been used extensively and includes what are commonly known as gamma-rays, X-rays (*e.g.*, external beam radiation generated by a linear accelerator), and the directed delivery of radioisotopes to tumor cells. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. For external beam radiation treatment in combination with chlorotoxin, treatment is usually given as one treatment per day. Occasionally two treatments per day will be given, where a day has

been missed, or with certain cancer therapy indications. The standard dosing ranges from about 1.8 Gy to about 2.0 Gy per day, with weekly doses ranging from about 9 Gy to about 10 Gy per week. Treatment is usually given five days per week with two days off for recovery time from the preceding week of treatment.

Methods of Diagnosis Using Chlorotoxin

The invention includes diagnostic methods for the determination of the presence and location of abnormal cell growth in an organ or body area of a patient. In one embodiment of this method, the abnormal cell growth is cancer, including, but not limited to, lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma, pituitary adenoma, or a combination of one or more of the foregoing cancers.

The present methods comprise administration of a detectable quantity of a composition comprising a detectable amount of chlorotoxin or a derivative thereof to a patient. As used herein, the term "detectable amount" refers to the amount of labeled chlorotoxin or derivative thereof administered to a patient that is sufficient to enable detection of binding of the labeled chlorotoxin or derivative thereof to one or more abnormal cells including malignant cancer cells in a tumor. As used herein, the term "imaging effective amount" refers to the amount of the labeled chlorotoxin or derivative thereof administered to a patient that is sufficient to enable imaging of binding of the labeled chlorotoxin or derivative thereof to one or more abnormal cells including malignant cancer cells in a tumor.

The invention employs isotopically-labeled chlorotoxin or derivatives thereof which, in conjunction with non-invasive neuroimaging techniques such as magnetic resonance spectroscopy (MRS) or imaging (MRI), or gamma imaging such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT), are used to

identify and quantify abnormal cells *in vivo* including malignant cells in tumors. The term "*in vivo* imaging" refers to any method which permits the detection of labeled chlorotoxin or a derivative thereof as described above. For gamma imaging, the radiation emitted from the tumor or area being examined is measured and expressed either as total binding, or as a ratio in which total binding in one tissue is normalized to (for example, divided by) the total binding in another tissue or the entire body of the same subject during the same *in vivo* imaging procedure. Total binding *in vivo* is defined as the entire signal detected in a tumor or tissue by an *in vivo* imaging technique without the need for correction by a second injection of an identical quantity of labeled compound along with a large excess of unlabeled, but otherwise chemically identical compound. As used herein, the terms "subject" or "patient" refers to a mammal, preferably a human, and most preferably a human suspected of having abnormal cells, including malignant cells in a tumor.

For purposes of *in vivo* imaging, the type of detection instrument available is a major factor in selecting a given label. For instance, radioactive isotopes are particularly suitable for *in vivo* imaging in the methods of the present invention. The type of instrument used will guide the selection of the radioisotope. For instance, the radioisotope chosen must have a type of decay detectable by a given type of instrument. Another consideration relates to the half-life of the radioisotope. The half-life should be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that the host does not sustain deleterious radiation. The isotopically-labeled chlorotoxin or derivative thereof can be detected using gamma imaging where emitted gamma irradiation of the appropriate wavelength is detected. Methods of gamma imaging include, but are not limited to, positron emission tomography (PET) imaging or for single photon emission computerized tomography (SPECT). Preferably, for SPECT detection, the chosen radiolabel will lack a particulate emission, but will produce a large number of photons. For PET detection, the radiolabel will be a positron-emitting radioisotope which will be detected by the PET camera.

In the present invention, chlorotoxin or derivatives thereof are made which are useful for *in vivo* detection and imaging of tumors. These compounds are to be used in conjunction with non-invasive neuroimaging techniques such as magnetic resonance spectroscopy (MRS) or imaging (MRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT). In accordance with this invention, chlorotoxin or derivatives thereof may be labeled with any acceptable radioisotope described above by general organic chemistry techniques known to the art (see March (1992) Advanced Organic Chemistry: Reactions, Mechanisms & Structure, Wiley). Chlorotoxin and derivatives thereof

also may be radiolabeled with isotopes of copper, fluorine, carbon, bromine, etc. for PET by techniques well known in the art and are described (see Phelps (1986) Positron Emission Tomography and Autoradiography, Raven Press pages 391-450). Chlorotoxin and derivatives thereof also may be radiolabeled with acceptable isotopes such as iodine for SPECT by any of several techniques known to the art (see Kulkarni (1991) Int. J. Rad. Appl. Inst. 18, 647-648).

For example, chlorotoxin and derivatives thereof may be labeled with any suitable radioactive iodine isotope, such as, but not limited to ^{131}I or ^{123}I by iodination of a diazotized amino derivative directly via diazonium iodide (see Greenbaum (1936) Am. J. Pharm. 108, 17-18), or by conversion of the unstable diazotized amine to the stable triazene, or by conversion of a non-radioactive halogenated precursor to a stable tri-alkyl tin derivative which then can be converted to the iodo compound by several methods well known to the art (see Chumpradit *et al.* (1991) J. Med. Chem. 34, 877-878 and Zhuang *et al.* (1994) J. Med. Chem. 37, 1406-1407).

Chlorotoxin and derivatives thereof also may be radiolabeled with known metal radiolabels, such as ^{64}Cu or ^{99m}Tc. Modification of the substituents to introduce ligands that bind such metal ions can be effected without undue experimentation by one of ordinary skill in the radiolabeling art including covalent attachment to a polyHis region in a modified chlorotoxin peptide or derivative thereof. The metal radiolabeled chlorotoxin or derivatives thereof can then be used to detect and image tumors.

The diagnostic methods of the present invention may use isotopes detectable by nuclear magnetic resonance spectroscopy for purposes of *in vivo* imaging and spectroscopy. Elements particularly useful in magnetic resonance spectroscopy include, but are not limited to, ^{19}F and ^{13}C. Suitable radioisotopes for purposes of this invention include beta-emitters, gamma-emitters, positron-emitters and x-ray emitters. These radioisotopes include, but are not limited to, ^{131}I, ^{123}I, ^{18}F, ^{11}C, ^{75}Br and ^{76}Br.

Suitable stable isotopes for use in Magnetic Resonance Imaging (MRI) or Spectroscopy (MRS), according to this invention include, but are not limited to, ^{19}F and ^{13}C. Suitable radioisotopes for *in vitro* identification and quantification of abnormal cells including tumor cells, in a tissue biopsy or post-mortem tissue include ^{125}I, ^{14}C and ^{3}H. The preferred radiolabels are ^{64}Cu or ^{18}F for use in PET *in vivo* imaging, ^{123}I or ^{131}I for use in SPECT imaging *in vivo*, ^{19}F for MRS and MRI and ^{3}H or ^{14}C for *in vitro* methods. However, any conventional method for visualizing diagnostic probes can be utilized in accordance with this invention.

Generally, the dosage of the isotopically-labeled chlorotoxin and derivatives thereof will vary depending on considerations such as age, condition, sex, and extent of disease in the patient, contraindications, if any, concomitant therapies and other variables, to be adjusted by the skilled artisan. Dosage can vary from 0.001 mg/kg to 1000 mg/kg, preferably 0.1 mg/kg to 100 mg/kg. Administration to the patient may be local or systemic and accomplished intravenous, intra-arterial, intra-thecal (via the spinal fluid), intra-cranial or the like. Administration may also be intra-dermal or intra-cavitary, depending upon the body site under examination.

After a sufficient time has elapsed for the labeled chlorotoxin or derivative thereof to bind with the abnormal cells, for example thirty minutes to forty-eight hours, the area of the subject under investigation is examined by routine imaging techniques such as MRS/MRI, SPECT, planar scintillation imaging, PET, and emerging imaging techniques, as well. The exact protocol will necessarily vary depending upon factors specific to the patient, as noted above, and depending upon the body site under examination, method of administration and type of label used; the determination of specific procedures would be routine to the skilled artisan. For brain imaging, preferably, the amount (total or specific binding) of the bound isotopically-labeled chlorotoxin or derivatives thereof is measured and compared (as a ratio) with the amount of isotopically-labeled chlorotoxin or derivatives thereof bound to the cerebellum of the patient. This ratio is then compared to the same ratio in age-matched normal brain.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples describe embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1

Determination of chemotherapeutic agent activity *in vitro*

A tissue culture method was optimized to test the effects of various chemotherapeutic agents on multiple cancer cell lines (see Table 1). Cells were plated on 96-well microtiter tissue culture plates at a density of approximately 1000-2000 cells per well, depending on the specific cell line. Cells were allowed to adhere twenty-four hours in a 37°C, humidified cell culture incubator supplied with five percent carbon dioxide. In order to achieve a dose-

response curve for each drug in each cell line, cells were treated with decreasing concentrations of a specific cytotoxic compound for two to five days. Following treatment, the cytotoxic effect of the drug was quantified using the Cell Counting Kit-8 (CCK-8) (Dojindo Inc.) according to the manufacturer's instructions. In brief, following the treatment period with the cytotoxic drug, cells were incubated with CCK-8 reagent and incubated at 37°C for one to four hours, depending on the specific cell type. After incubation, plates were read on a microplate reader at a wavelength of 490 nM. The IC_{50} of each drug was calculated from a X-Y scatter plot of the negative log concentration of drug versus mean optical density (Table 2).

Table 2. Cell Line Designation, Source and Tissue Origin

Cell Designation	Cell Line Source	Tissue Origin
D54-MG		Human glioblastoma multiforme
U251-MG		Human glioblastoma multiforme
SKMEL28	ATCC (HTB-72)	Human malignant melanoma
SKMEL31	ATCC (HTB-73)	Human malignant melanoma
PC-3	ATCC (CRL-1435)	Human prostate tumor
LNCaP	ATCC (CRL-1740)	Human prostate tumor
NCI-H187	ATCC (CRL-5804)	Human small cell lung carcinoma

Table 3. IC_{50} of Chemotherapeutic Agents in Multiple Cell Lines

Cell Line	Drug	IC_{50}
D54-MG	Doxorubicin	60.0 ng/ml
D54-MG	Paclitaxel	10.5 nM
D54-MG	Temodar	0.12 mM
D54-MG	Cisplatin	0.010 mg/ml
D54-MG	5-Fluorouracil	0.0015 mg/ml
U251	Doxorubicin	30.0 ng/ml
U251	Paclitaxel	8.0 nM
U251	Temodar	0.15 mM
SKMEL28	Doxorubicin	40.0 ng/ml

Table 3. IC_{50} of Chemotherapeutic Agents in Multiple Cell Lines		
SKMEL28	Temodar	0.03 mM
SKMEL28	Cisplatin	0.008 mg/ml
SKMEL31	Doxorubicin	35.0 ng/ml
SKMEL31	Paclitaxel	25 nM
SKMEL31	Temodar	0.15 mM
PC-3	Hydroxyurea	35 mM

Example 2

Effect of chlorotoxin on chemotherapeutic agent activity *in vitro*

For measurement of pharmacologic effect of chlorotoxin on chemotherapeutic agents, the cell culture methodology in Example 1 was employed with the following modifications: a concentration of the chemotherapeutic agent approaching the IC_{50} but usually just below was used in each assay. Various amounts of chlorotoxin were then titrated in combination with a concentration of chemotherapeutic agent near or below its IC_{50} and the effect of chlorotoxin on the cytotoxic effects of the chemotherapeutic agent measured two to three days following adminstration. Concentration of chlorotoxin employed in this assay ranged from micoromolar down to nanomolar concentrations.

The effect of adding chlorotoxin in combination with Temodar on D54-MG cell proliferation is shown in Figure 1. The level of Temodar used in this experiment (0.050 mM) is about thirty-fold lower than the concentration necessary to kill these cells and produce a lower optical density value (see Table 2). Chloroxotoxin (TM-601) alone had no effect on cell growth. Chlorotoxin when added at the same time as Temodar did not produce any effect but when chlorotoxin was added twenty-four hours prior to Temodar, a concentration of 0.050 mM Temodar reduced cell proliferation equivalent to a level usually observed with a thirty-fold higher concentration of Temodar. These results demonstrate that administration of chlorotoxin, prior to administration of Temodar, sensitized cancer cells to the effects of Temodar.

Example 3

Effect of chlorotoxin on chemotherapeutic agent activity *in vivo*

The purpose of this study was to determine whether hydroxyurea or temodar combined with chlorotoxin were sufficient to inhibit tumor growth as indicated from *in vitro* studies with glioma cell lines. Other studies indicated that chlorotoxin, pre-incubated with

human cancer cell lines, greatly sensitized the cells to temodar, a chemotherapeutic, tumor cell killing agent. Combination treatment with chlorotoxin with hydroxyurea or temodar in mice with glioma flank tumors was compared to the treatment group of hydroxyurea or temodar alone and saline alone. Hydroxyurea and temodar dosage was based on the lowest dosage (10 mg/kg body weight) used in previous studies to determine clearance from the body in the treatment of sickle cell disease paradigm in nude mice (Iyamu *et al.* (2001) Chemotherapy 47, 270-278).

Nude mice were ear-tagged and given an identification number were inoculated with five million U251 glioma cells in 0.10 ml mixture with five percent methyl cellulose under light anesthesia according to standard operating procedures for flank tumor inoculations (Iyamu *et al.* (2001) Chemotherapy 47, 270-278). Flank tumors had developed and were established approximately thirty days following inoculation.

Mice with established flank tumors were each treated with 0.100 ml injection (i.p) of sterilized solutions consisting of either of saline, saline and hydroxyurea or temodar (13.2 mg/kg body weight), or saline, hydroxyurea or temodar (13.2 mg/kg) and chlorotoxin (0.080 mg/kg body weight). Tumor volume was calculated based on the measurements with the same set of calipers on the indicated days by determining the length × width × height of the tumor of non-anesthetized mice. As each animal had different-sized tumors at the beginning of the experiment, the data is presented in final form as percent change of the tumor growth from the initial date of the injection protocol. Statistical significance was determined according to a one-way ANOVA test. At a level where temodar alone has little effect on the growth of the xenografted tumor, temodar combined with chlorotoxin dramatically decreased the growth of the tumor (Figure 2).

As mentioned above, the efficacy of hyrdroxyurea combined with chlorotoxin was also assessed in the same mouse flank tumor model with the exception that D54 glioma cells were used to establish the glioma flank tumor. Mice treated with chlorotoxin in combination with hydroxyurea had tumors significantly ($p=0.01$ at day 29 and $p=0.005$ at day 32) smaller in size than mice treated with either hydroxyurea alone or saline alone indicating that chlorotoxin in combination with hydroxyurea reduced tumor growth significantly more than hydroxyurea alone (Figure 3).

Example 4

PET imaging studies with labled chlorotoxin

The following illustrative procedure may be utilized when performing PET imaging studies on patients in the clinic. The patient is fasted for at least twelve hours allowing water intake *ad libitum* and is premedicated with 0.3-0.4 ml Acepromazine injected intra-muscular on the day of the experiment. A twenty-gauge, two inch intravenous catheter is inserted into the contra-lateral ulnar vein for administration of radiolabeled chlorotoxin.

The patient is positioned in the PET camera and a tracer dose of $[^{15}O]H_2O$ is administered via the intravenous catheter. The image thus obtained is used to insure that the patient is positioned correctly to include complete imaging of the desired areas including the tumor. Subsequently, $[^{64}Cu]$ radiolabeled chlorotoxin (<20 mCi) is administered via the intravenous catheter. Following the acquisition of the total radiotracer image, an infusion is begun of the radiolabeled chlorotoxin which is evaluated at multiple dose rates (0.1, 1.0 or 10 mpk/day). After infusion for two hours, the $[^{64}Cu]$ radiolabeled chlorotoxin is again injected via the catheter. Images are again acquired for up to ninety minutes. Within ten minutes of the injection of radiotracer and at the end of the imaging session, 1.0 ml blood samples are obtained for determining the plasma concentration of the radiolabeled chlorotoxin.

Example 5

D54 glioblastoma cells were plated at a density of about 1000 cells/well in a 96-well flat bottom plate and incubated in 5% CO_2 at 37°C. After twenty-four hours chlorotoxin was added at 1:4 limiting dilutions to a final concentration of 20, 5, 1.25, 0.313, 0.078, 0.0195, 0.0049, 0.0012, 0.00031 or 0.00008 nM. Control cells received vehicle only. Twenty-four hours after treatment, the effect of chlorotoxin was quantified using the MTT mitochondrial enzyme substrate with the Cell Counting Kit-8 (CCK-8) (Dojindo Inc.) according to the manufacturer's instructions. In brief, following the treatment period with chlorotoxin, cells were incubated with CCK-8 reagent. After incubation, plates were read on a microplate reader at a wavelength of 490 nm, with higher absorbance indicating greater cell viability. Figure 4 shows that chlorotoxin incubation inhibited proliferation of the D54 cells at all concentrations tested down through 0.00120 nM as evidenced by the lower number of viable cells/well versus PBS control.

Example 6

D54 glioblastoma cells were plated at a density of about 1000 cells/well in a 96-well flat bottom plate and incubated in 5% CO_2 at 37°C. After twenty-four hours chlorotoxin was added at 1:4 limiting dilutions to a final concentration (in nM) of 20, 5, 1.25, 0.313, 0.078,

0.02, 0.0049, 0.0012, 0.0003, or 0.00008. Control cells received vehicle only. After twenty-four hours, half of the cells were washed free of chlorotoxin, the medium replaced with fresh medium. Cells in both conditions, chlorotoxin left on and chlorotoxin removed, were incubated for an additional four days. Following incubation, the effect of chlorotoxin was quantified using the MTT mitochondrial enzyme substrate with the CCK-8 as in Example 1. Figure 5 shows that the long incubation time allowed the cells to overcome the effects of chlorotoxin with the additional days of proliferation and chlorotoxin did not appear to inhibit cell proliferation in this instance.

Example 7

PC3 prostate cancer cells were plated at a density of about 1000 cells/well in a 96-well flat bottom plate and incubated in 5% CO_2 at 37°C. After twenty-four hours chlorotoxin was added at 1:2 limiting dilutions to a final concentration (nM) of 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0.039. Control cells received PBS vehicle only. Twenty-four hours after treatment, the effect of chlorotoxin was quantified using the MTT mitochondrial enzyme substrate with the CCK-8 as in Example 1. Figure 6 shows that chlorotoxin incubation inhibited proliferation of the D54 cells at all concentrations tested as evidenced by the lower number of viable cells/well versus PBS control

Example 8

Three groups of eight athymic nude mice received a subcutaneous injection of 5×10^7 human D54 glioblastoma cells in their right flank to produce human glioma flank xenografts in these mice. Animals in Groups I and III received 2.6 μg chlorotoxin (SEQ ID NO: 1) in 100 μl phosphate-buffered saline intravenously at 14, 21, 28, 35, 42 and 49 days after D54 injection. Animals in Groups II and III received 2 Gy ^{60}C whole-body irradiation at 15, 22, 29, 36, 43 and 50 days after D54 injection. Tumor size was measured three times weekly and is depicted in Figure 7.

Example 9

Intracranial D54MG glioma xenografts were established in athymic nude mice by the implantation of 1×10^6 D54MG cells in the brain of each subject. A treatment regimen was begun 14 days post-implantation with tail vein intravenous injections two times per week. The control group of seven mice were administered saline vehicle only. A second group of mice, comprising eight animals, were each administered a low dose of chlorotoxin of 0.2

μg/dose and a third group of mice, comprising eight animals, were administered a high dose of chlorotoxin of 2.0 μg/dose. Animals were followed until death and survival time was plotted on a Kaplan-Meier chart, indicating median survival (Figure 8). These results indicate that treatment with chlorotoxin alone substantially extends the life of a subject in an intracranial model and that this enhanced survival may be dose dependent. It is notable that administration of chlorotoxin was intravenous, demonstrating that chlorotoxin crosses the blood-brain barrier to exert its effect.

Example 10

In a separate investigation, D54MG glioma xenografts were established peripherally by implanting 10×10^6 D54MG cells in the flanks of athymic nude mice. Tumors were palpable at 14 days, with individual tumor volumes of approximately 43 mm^3. Again, the treatment regimen was begun 14 days post-implantation with tail vein intravenous injections two times per week. The control group of seven mice were administered saline vehicle only. A second group of mice, comprising eight animals, were each administered a low dose of chlorotoxin of 0.2 μg/dose. Tumor size was measured at the time of each injection, and plotted as a percent of original tumor size (Figure 9). Intravenous treatment was ended at 42 days and the measurement of the tumors was continued for several weeks. These results demonstrate that low-dose chlorotoxin alone can dramatically decrease the tumor growth in this flank model.

Example 11

To identify core binding site sequences of chlorotoxin, twenty-seven overlapping 10-mers derived from SEQ ID NO: 1 were synthesized, starting from the C-terminus of the peptide as indicated in Figure 10. Each peptide had a biotin attached at the amino terminus to facilitate detection and each cysteine residue was replaced with a serine in order to prevent cross-linking.

Binding of the 10-mer peptides to PC3 prostate cancer cells *in vitro* was measured by incubating cultured PC3 cells with individual peptides. Binding was detected and quantified by incubating the peptide exposed cells with HRP-avidin using a commercial kit according to manufacturer's instructions.

Figure 11 shows that the 10-mer peptide 4 of SEQ ID NO:1 does not bind to PC3, indicating that the lysine residue which starts peptide 5 must be the start of the binding site. Peptides 5-8 bind, but the binding is lost in peptide 9. This suggests that the tyrosine residue

is another key, since this is present in peptide 8 but lost in peptide 9. This indicated that a first binding region of chlorotoxin resides within the 7-mer sequence KGRGKSY (SEQ ID NO: 8) residing at amino acid residues 23-29 of SEQ ID NO: 1 which are common to peptides 5-8.

Figure 12 shows that peptide 19 of SEQ ID NO: 1 does not bind PC3 cells but peptide 20 does, indicating that the threonine residue which starts peptide 20 may be the start of a second binding site because peptides 20-24 bind most strongly. Binding decreases again in peptide 25, suggesting that the peptide 24 terminal arginine residue is another key, since this is present in peptide 24 but lost in peptide 25. This indicates that a second binding region of chlorotoxin resides within the 9-mer sequence TDHQMAR (SEQ ID NO: 9) residing at amino acid residues 8-14 of SEQ ID NO: 1 which are common to peptides 20-24. The binding in this second core sequence is broader, which may be a reflection of very similar amino acids present at the ends of the region. For example, there are two threonine residues at peptides 20 and 21, and there is a lysine at the end of peptide 22 next to the arginine residue.

Example 12

To determine the *in vivo* activity of these identified binding regions, 10-mer peptides 5 (amino acid residues 23-32), 12 (amino acid residues 16-25; as a negative control) and 21 (amino acid residues 7-16) of SEQ ID NO: 1 were used in a crayfish paralysis assay, an assay which is commonly used to determine the bio-activity of chlorotoxin (see DeBin *et al.* (1993) Am. J. Physiol. 264, C361-369). Peptides 5 and 12 failed to paralyze crayfish, while peptide 21 was effective, indicating that the site which is responsible for the paralytic effect of chlorotoxin is the region defined by peptide 21.

Additionally, several of the chlorotoxin derivatives were each analyzed in the crayfish assay and compared to chlorotoxin (Table 4). Each of these derivatives comprises the putative end-amino acids, the T and the R within the sequence corresponding to peptide 21.

Table 4

Peptide	SEQ ID	Crayfish Assay	Identity	Sequence Comparison
Cltx	1	Yes	100 %	TDHQMAR (SEQ ID NO: 9)
Cltx (Y/F)	5	Yes	100 %	TDHQMAR (SEQ ID NO: 9)
STP-1	6	Yes	71.4 %	TDPQMSR (SEQ ID NO: 77)
6xH-Cltx	2	Yes	100 %	TDHQMAR (SEQ ID NO: 9)
Y-Cltx	3	Yes	100 %	TDHQMAR (SEQ ID NO: 9)

Table 4				
YSY-Cltx	4	Yes	100 %	TDHQMAR (SEQ ID NO: 9)

Example 13

Chlorotoxin is a 36-amino acid peptide with 8 cysteines, depicted below in bold type with the sequences of peptide number 8 (beta-region peptide) and peptide number 21 (alpha-region peptide) identified using the overlapping 10-mers in Example 12 underlined below:

MCMPCFTTDHQMARKCDDCCGGKGRCKCYGPQCLCR (SEQ ID NO: 1)

In order to confirm the identify the minimal binding sequences within the alpha and beta peptides, the entire peptides were synthesized as a 10-mer with a biotin at the amino terminus as well as shorter sequences reducing the size of the peptide by one amino acid at the amino terminus each time.

For the beta peptide, the sequences of peptide 8 noted in Table 5 were evaluated and probed for binding to U251 glioma cells:

Table 5	
Peptide	**Sequence**
8	Biotin-GGKGRGKSYG (SEQ ID NO: 78)
8a	Biotin-GKGRGKSYG (SEQ ID NO: 79)
8b	Biotin-KGRGKSYG (SEQ ID NO: 80)
8c	Biotin-GRGKSYG (SEQ ID NO: 81)

For the alpha peptide, the sequences of peptide 21 noted in Table 6 were evaluated and probed for binding to U251 glioma cells:

Table 6	
Peptide	**Sequence**
21	Biotin-TTDHQMARKS (SEQ ID NO: 82)
21a	Biotin-TDHQMARKS (SEQ ID NO: 10)
21b	Biotin-DHQMARKS (SEQ ID NO: 83)
21c	Biotin-HQMARKS (SEQ ID NO: 84)
21d	Biotin-QMARKS (SEQ ID NO: 85)

Results demonstrated that the initial threonine residue of the alpha-region peptide is detrimental to binding but that the second threonine is crucial to binding. It was also

discovered that none of the smaller peptides exhibit binding as strong as the 9-mer of peptide 21a.

Example 14

To determine the contribution of each residue to the binding properties of the alpha peptide, alanine scan variants were synthesized by replacing each amino acid of the 9-mer peptide TDHQMARKS (SEQ ID NO: 10) sequentially as depicted in Table 7. Peptide 21, the native core 9-mer, and each alanine-substituted 9-mer peptide was synthesized with a biotin at the amino terminus and evaluated for their binding versus both U251 and PC3 cells (Figure 13).

Table 7

Peptide	Sequence
21	Biotin-TTDHQMARKS (SEQ ID NO: 82)
21a	Biotin-TDHQMARKS (SEQ ID NO: 10)
21a-A1	Biotin-**A**DHQMARKS (SEQ ID NO: 86)
21a-A2	Biotin-T**A**HQMARKS (SEQ ID NO: 87)
21a-A3	Biotin-TD**A**QMARKS (SEQ ID NO: 88)
21a-A4	Biotin-TDH**A**MARKS (SEQ ID NO: 89)
21a-A5	Biotin-TDHQ**A**ARKS (SEQ ID NO: 90)
21a-A6	Biotin-TDHQM**A**RKS (SEQ ID NO: 10)
21a-A7	Biotin-TDHQMA**A**KS (SEQ ID NO: 91)
21a-A8	Biotin-TDHQMAR**A**S (SEQ ID NO: 92)
21a-A9	Biotin-TDHQMARK**A** (SEQ ID NO: 93)

The pattern for U251 and PC3 binding are generally similar. Replacement of the aspartic acid (D) residue in the second position of the 9-mer increased binding of the peptide to cells and replacement of the Q residue in the fourth position produced a large increase of peptide binding to cells. Accordingly, the peptide TAHAMARKS (SEQ ID NO: 11) should be more active than the parent peptide TDHQMARKS (SEQ ID NO: 10). Based on the binding of the peptide TDHAMARKS, this binding may be equal to or greater than chlorotoxin itself.

Based upon this finding, it is expected that a variant peptide of chlorotoxin of the sequence below may be stronger in binding than the native chlorotoxin polypeptide.

MCMPCFTT**A**H**A**MARKCDDCCGGKGRCKCYGPQCLCR (SEQ ID NO: 12)

Example 15

In order to compare binding of the short scorpion toxins, the regions homologous to peptide 21 of small toxin and probable toxin LQH-8/6 were synthesized and biotinylated for analysis in the chlorotoxin binding assay (see Table 8 for amino acid sequences of the peptides).

Table 8

Scorpion Toxin	Peptide 21
Chlorotoxin	TTDHQMARKS (SEQ ID NO: 82)
Small Toxin	TTDPQMSKK (SEQ ID NO: 94)
Probable Toxin LQH-8/6	TTDQQMTKK (SEQ ID NO: 95)

As shown in Figure 14, and in accordance with previous results, chlorotoxin exhibited significant binding in PC3 human prostate cancer cells (221.93% of background levels) and peptide 21 binding paralleled that of chlorotoxin (232.50% of background levels). Additionally, peptide 21 of small toxin peptide (21ST) and peptide 21 of probable toxin LQH-8/6 (21LQ) demonstrated binding levels equivalent to that of full-length chlorotoxin and chlorotoxin peptide 21 (225.26% and 242.32%, respectively). Furthermore, a negative peptide containing amino acids 26-35 of chlorotoxin (SEQ ID NO: 1) exhibited binding levels comparable to background (110%). Similar results were obtained in D54 glioblastoma cells (data not shown).

The results from this study using the chlorotoxin binding assay indicate that chlorotoxin, small toxin peptide, and probable toxin LQH-8/6 bind similarly to human cancer cells *in vitro*. Table 9 below highlights amino acids conserved within the putative primary binding domain (amino acids 7-16) of the three toxin peptides.

Table 9

Scorpion toxin	Amino acid sequence
Chlorotoxin	**TTDHQMARK**C (SEQ ID NO: 61)
Small toxin peptide	**TTDPQMSKK** (SEQ ID NO: 94)
Probable toxin LQH-8/6	**TTDQQMTKK** (SEQ ID NO: 95)

Example 16

The purpose of this experiment was to determine if the proliferation D54MG Glioblastoma cells, as measured by ^{3}H-thymidine uptake, is effected by Peptide 21, a segment of the full chlorotoxin sequence. The sequence of peptide 21 and its relation to chlorotoxin is shown in the sequence below:

```
Chlorotoxin:    MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
Peptide 21:             TTDHQMARK (SEQ ID NO: 82)
```

Peptide 21 (SEQ ID NO: 82) has been identified in several other reports as having binding and biological activity comparable to the full length chlorotoxin.

D54MG cells were plated in a 24 well plate at 100,000 cells/ml/well using five rows of four wells for each concentration. The cells were allowed to adhere in normal media for twenty-four hours at 37°C and 5% carbon dioxide. TM-701 was diluted to a 1 nM stock solution and added to each row at the concentrations of 0, 20, 80, 160 and 320 nM.

The cells and peptide 21 were allowed to incubate for 24 hours at 37°C and 5% carbon dioxide. After twenty-four hours, the cells were rinsed two times with warm PBS. Normal media was added back to the cells at 1 ml/well. One μCi of ^{3}H-thymidine was added to each well (1 μl of 1 mCi/ml ^{3}H-thymidine to each well). The plate was incubated for two hours at 37°C. The media and thymidine were removed and the wells were rinsed with ice-cold phosphate-buffered saline three times. To each well was added 1 ml of 0.3 N NaOH. The plate was incubated in the 37°C incubator for thirty minutes. Each well of 0.3 N NaOH was pipetted up and down three to four times and removed from the plate and the solution was placed in scintillation vials for counting. Scintillation fluid at four times the amount of sample was added to the vials (4 ml). Each vial was counted on the scintillation counter for one minute. The results are shown in Table 10 and Figure 15. The data demonstrates that peptide 21 behaves similar to chlorotoxin, in that the uptake of ^{3}H-thymidine decreases in a does-dependent manner. This data also indicates that peptide 21 has an effect on the DNA synthesis in these cells.

Table 10

[Peptide 21] (nM)	^{3}H-Thymidine uptake ± SD (CPM)	
0	8645 ± 1218	1218
20	7795 ± 634	634
80	7412 ± 630	630
160	6983 ± 329	329
320	5782 ± 886	886

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, patent applications and publications referred to in this application are herein incorporated by reference in their entirety.

What is claimed:

1. A method for treating cancer comprising administering chlorotoxin or a chlorotoxin derivative in combination with at least one chemotherapeutic agent.

2. A method according to claim 1 wherein the chlorotoxin or chlorotoxin derivative is administered prior to administration of the chemotherapeutic agent.

3. A method according to claim 1 wherein the chlorotoxin or chlorotoxin derivative is administered subsequent to administration of the chemotherapeutic agent.

4. A method according to claim 1 wherein chlorotoxin or chlorotoxin derivative is administered simultaneously with the chemotherapeutic agent.

5. A method according to claim 1, wherein the chemotherapeutic agent is selected from the group consisting of alkylating agents, purine antagonists, pyrimidine antagonists, plant alkaloids, intercalating antibiotics, aromatase inhibitors, anti-metabolites, mitotic inhibitors, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, biological response modifiers, anti-hormones and anti-androgens.

6. A method according to claim 1, wherein the chemotherapeutic agent is selected from the group consisting of BCNU, cisplatin, gemcitabine, hydroxyurea, paclitaxel, temozomide, topotecan, fluorouracil, vincristine, vinblastine, procarbazine, dacarbazine, altretamine, cisplatin, methotrexate, mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, fluorouracil, cytarabine, azacitidine, vinblastine, vincristine, etoposide, teniposide, irinotecan, docetaxel, doxorubicin, daunorubicin, dactinomycin, idarubicin, plicamycin, mitomycin, bleomycin, tamoxifen, flutamide, leuprolide, goserelin, aminoglutethimide, anastrozole, amsacrine, asparaginase, mitoxantrone, mitotane and amifostine.

7. A method according to claim 1, wherein the cancer is a neuroectodermal cancer.

8. A method according to claim 1, wherein the cancer is selected from the group consisting of lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of

the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma and pituitary adenoma.

9. A composition for treating cancer comprising chlorotoxin or a chlorotoxin derivative and at least one chemotherapeutic agent.

10. A composition according to claim 9, wherein the chemotherapeutic agent is selected from the group consisting of alkylating agents, purine antagonists, pyrimidine antagonists, plant alkaloids, intercalating antibiotics, aromatase inhibitors, anti-metabolites, mitotic inhibitors, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, biological response modifiers, anti-hormones and anti-androgens.

11. A composition according to claim 9, wherein the chemotherapeutic agent is selected from the group consisting of BCNU, cisplatin, gemcitabine, hydroxyurea, paclitaxel, temozomide, topotecan, fluorouracil, vincristine, vinblastine, procarbazine, dacarbazine, altretamine, cisplatin, methotrexate, mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, fluorouracil, cytarabine, azacitidine, vinblastine, vincristine, etoposide, teniposide, irinotecan, docetaxel, doxorubicin, daunorubicin, dactinomycin, idarubicin, plicamycin, mitomycin, bleomycin, tamoxifen, flutamide, leuprolide, goserelin, aminoglutethimide, anastrozole, amsacrine, asparaginase, mitoxantrone, mitotane and amifostine.

12. A composition according to claim 9, wherein the cancer is a neuroectodermal cancer.

13. A composition according to claim 9, wherein the cancer is selected from the group consisting of lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma and pituitary adenoma.

14. A method for detecting the presence of cancer in a patient comprising administering a detectable amount of labeled chlorotoxin or chlorotoxin derivative.

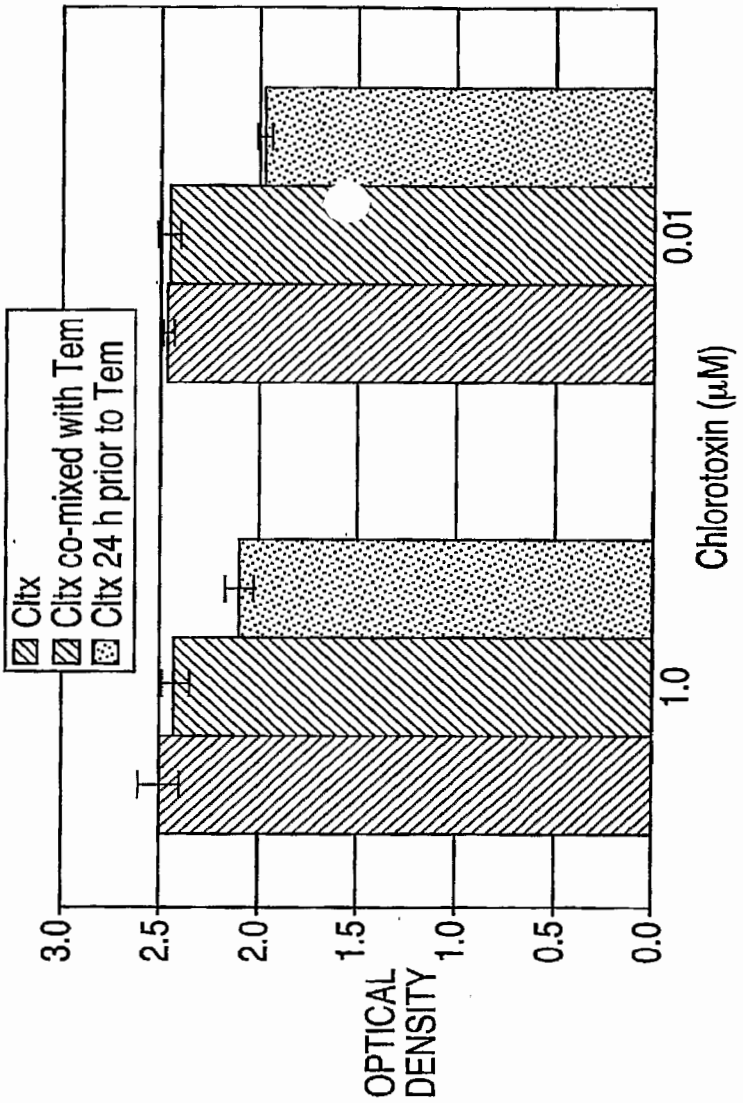
15. The method according to claim 14, wherein the cancer is selected from the group consisting of lung cancer, bone cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, chronic or acute leukemia, lymphocytic lymphomas, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma and pituitary adenoma.

16. The method according to claim 14, wherein the label is a radiolabel.

17. The method according to claim 16, wherein the radiolabel is selected from the group consisting of ^{3}H, ^{14}C, ^{18}F, ^{19}F, ^{31}P, ^{32}P, ^{35}S, ^{131}I, ^{125}I, ^{123}I, ^{64}Cu, ^{187}Re, ^{111}In, ^{90}Y, ^{99m}Tc and ^{177}Lu.

FIG. 1

Cytotoxicity Assay
Chlorotoxin +/- Temodar (50 μM) in
D54 Glioblastoma Cells



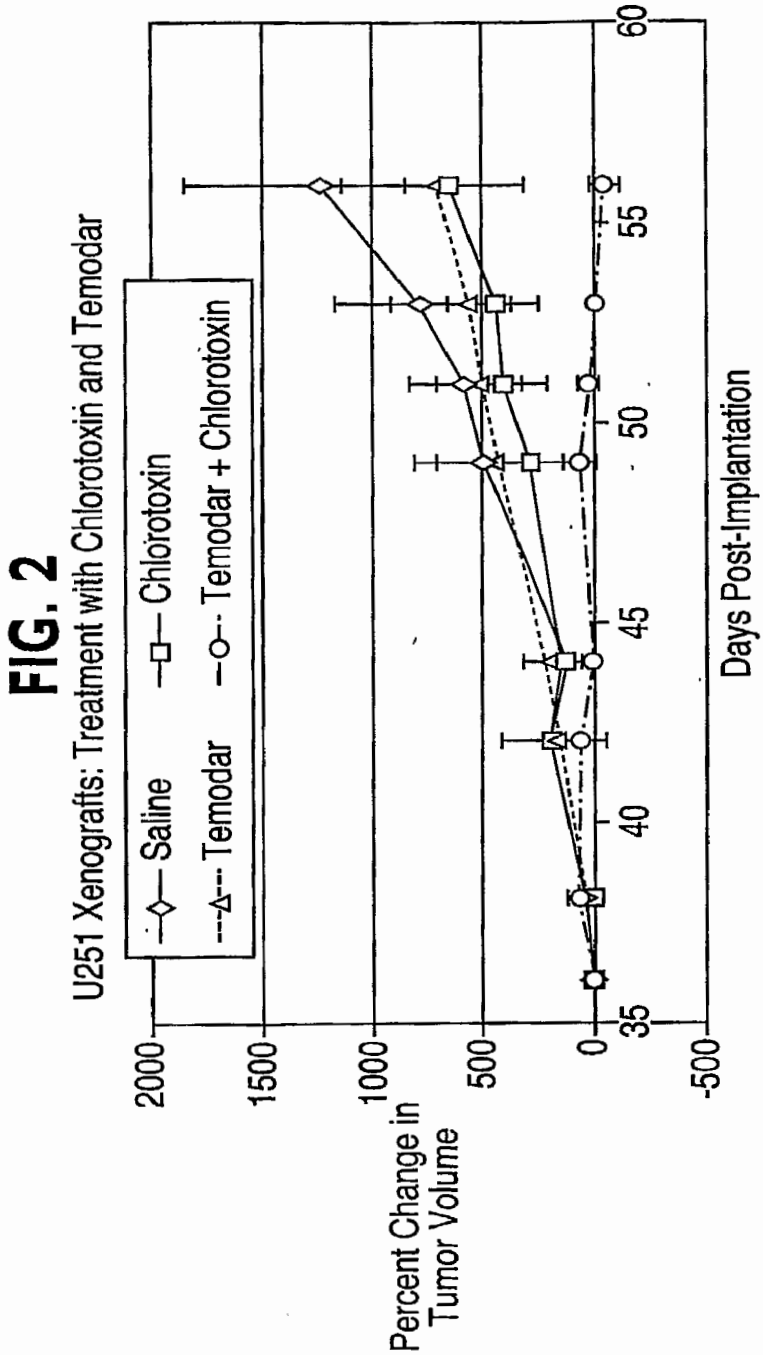

U251 Xenografts: Treatment with Chlorotoxin and Temodar
Saline
Chlorotoxin
Temodar
Temodar + Chlorotoxin
Percent Change in Tumor Volume
2000
1500
1000
500
0
-500
35
40
45
50
55
60
Days Post-Implantation

FIG. 2

FIG. 3

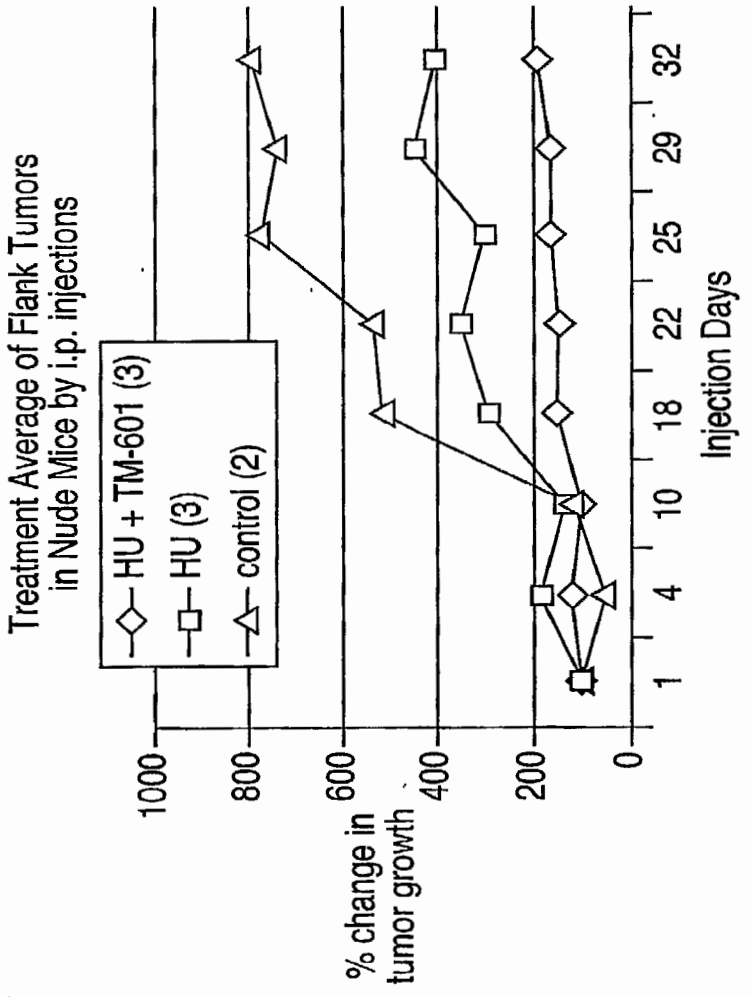

Treatment Average of Flank Tumors
in Nude Mice by i.p. injections
HU + TM-601 (3)
HU (3)
control (2)
% change in
tumor growth
1000
800
600
400
200
0
1
4
10
18
22
25
29
32
Injection Days

FIG. 4

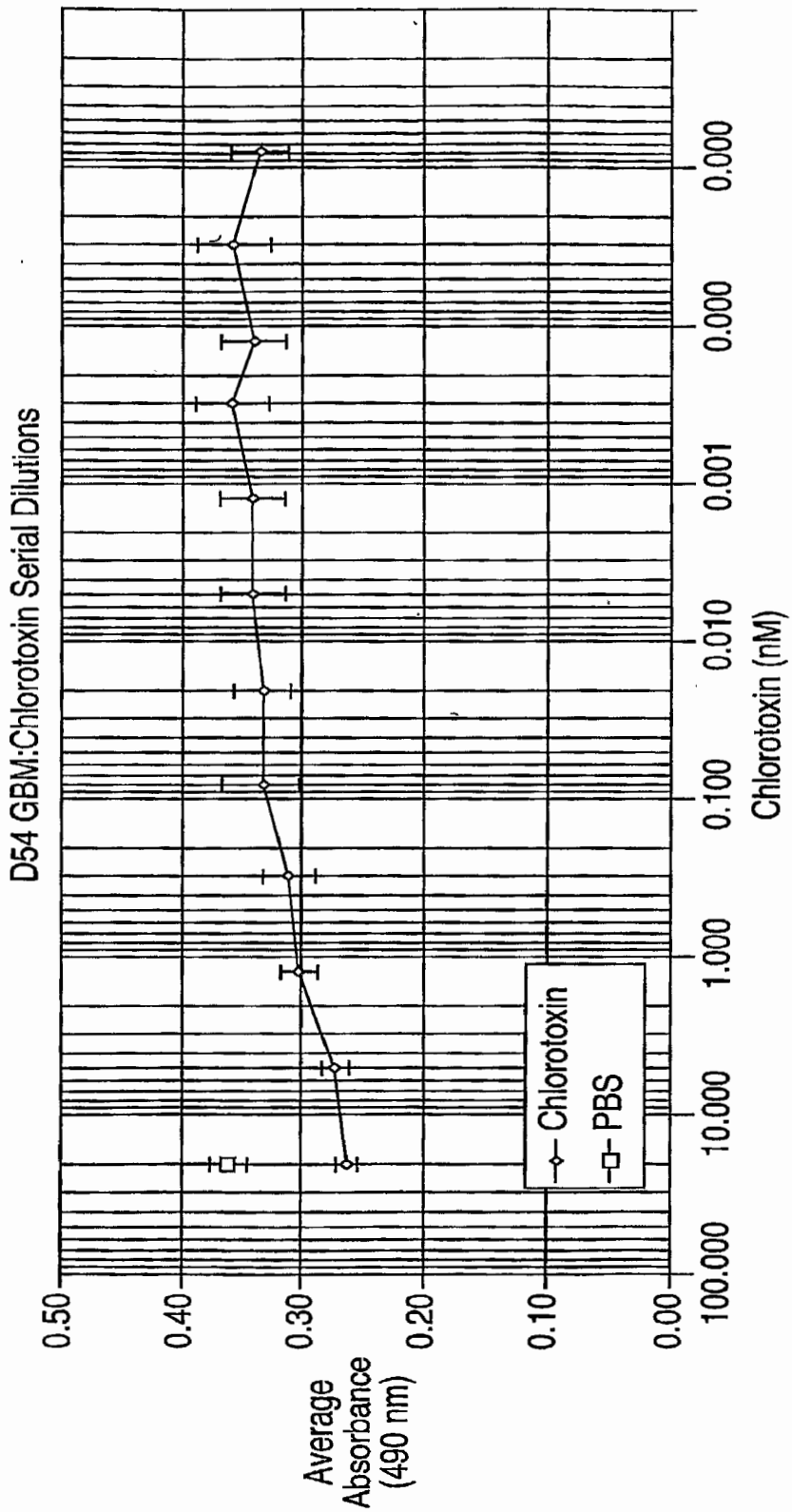

D54 GBM:Chlorotoxin Serial Dilutions
Average Absorbance (490 nm)
0.50
0.40
0.30
0.20
0.10
0.00
Chlorotoxin (nM)
100.000
10.000
1.000
0.100
0.010
0.001
0.000
0.000
Chlorotoxin
PBS

FIG. 5

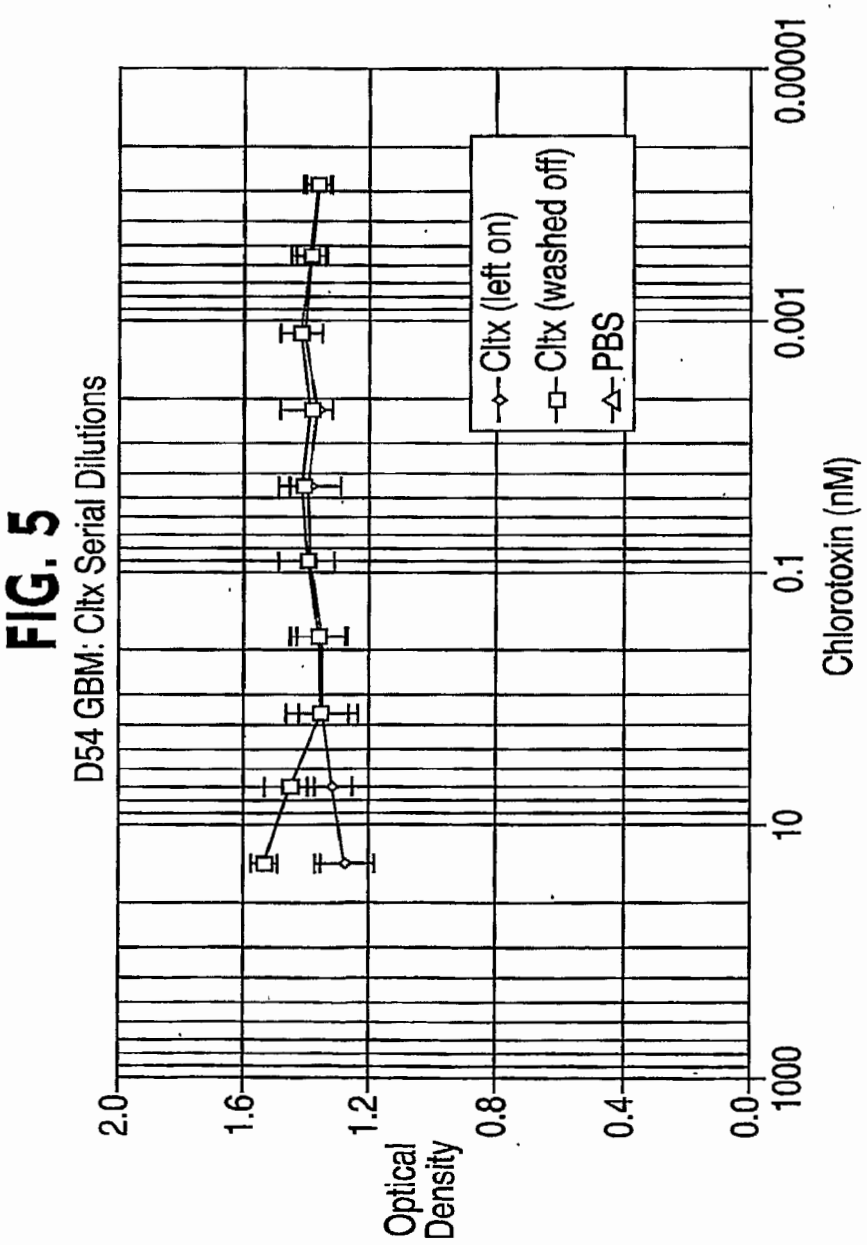
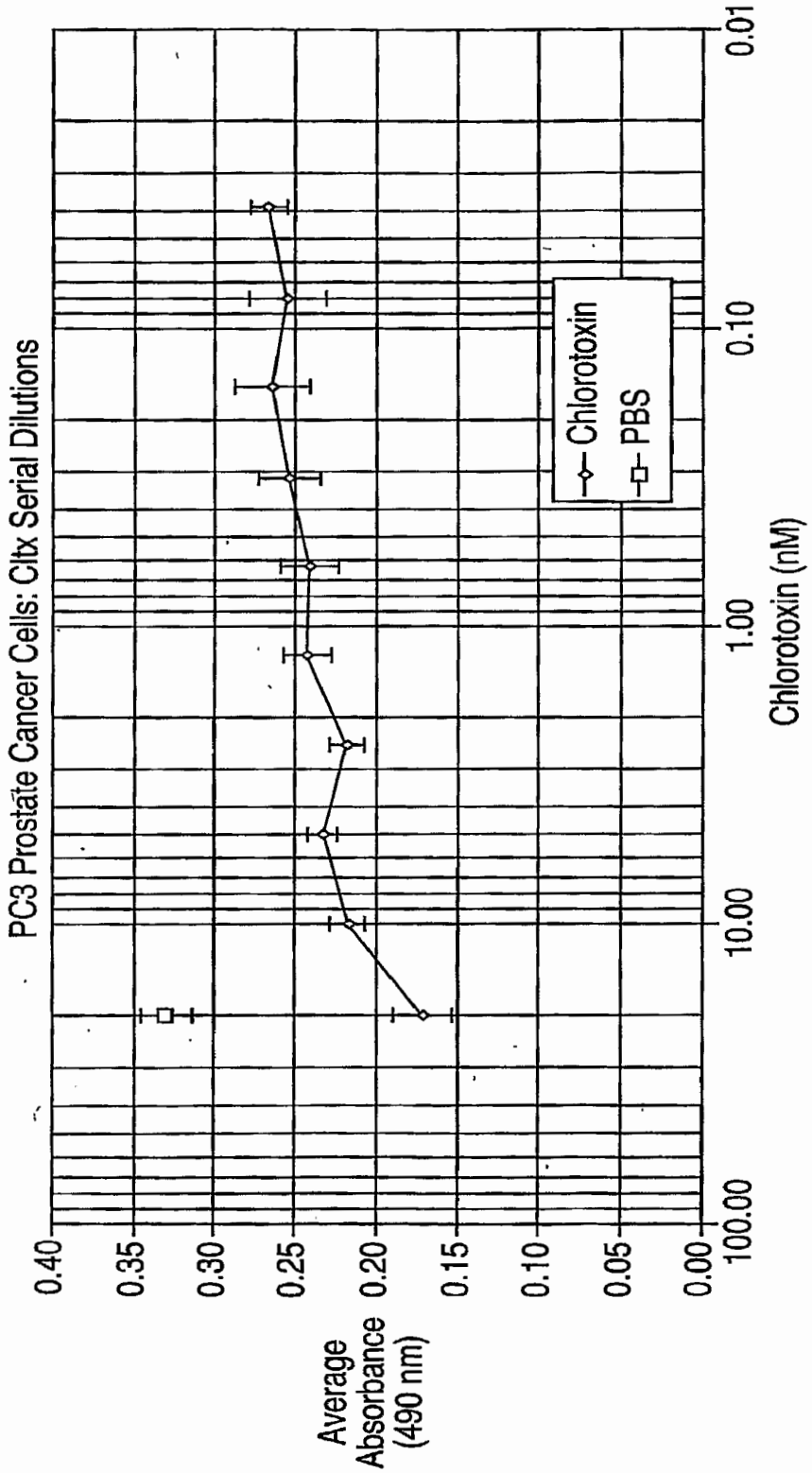

D54 GBM: Cltx Serial Dilutions
Optical Density
2.0
1.6
1.2
0.8
0.4
0.0
1000
10
0.1
0.001
0.00001
Chlorotoxin (nM)
Cltx (left on)
Cltx (washed off)
PBS

FIG. 6


PC3 Prostate Cancer Cells: Cltx Serial Dilutions
Average Absorbance (490 nm)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
100.00
10.00
1.00
0.10
0.01
Chlorotoxin (nM)
Chlorotoxin
PBS

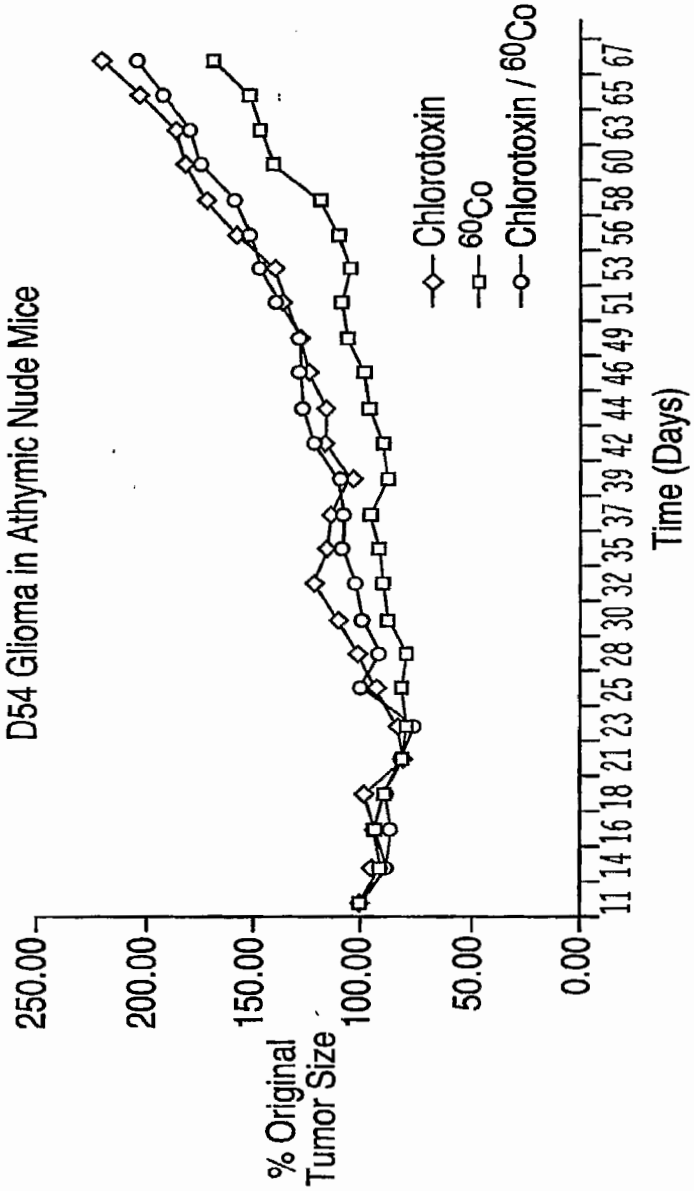

D54 Glioma in Athymic Nude Mice
% Original Tumor Size
250.00
200.00
150.00
100.00
50.00
0.00
11 14 16 18 21 23 25 28 30 32 35 37 39 42 44 46 49 51 53 56 58 60 63 65 67
Time (Days)
Chlorotoxin
60Co
Chlorotoxin / 60Co

FIG. 7

FIG. 8

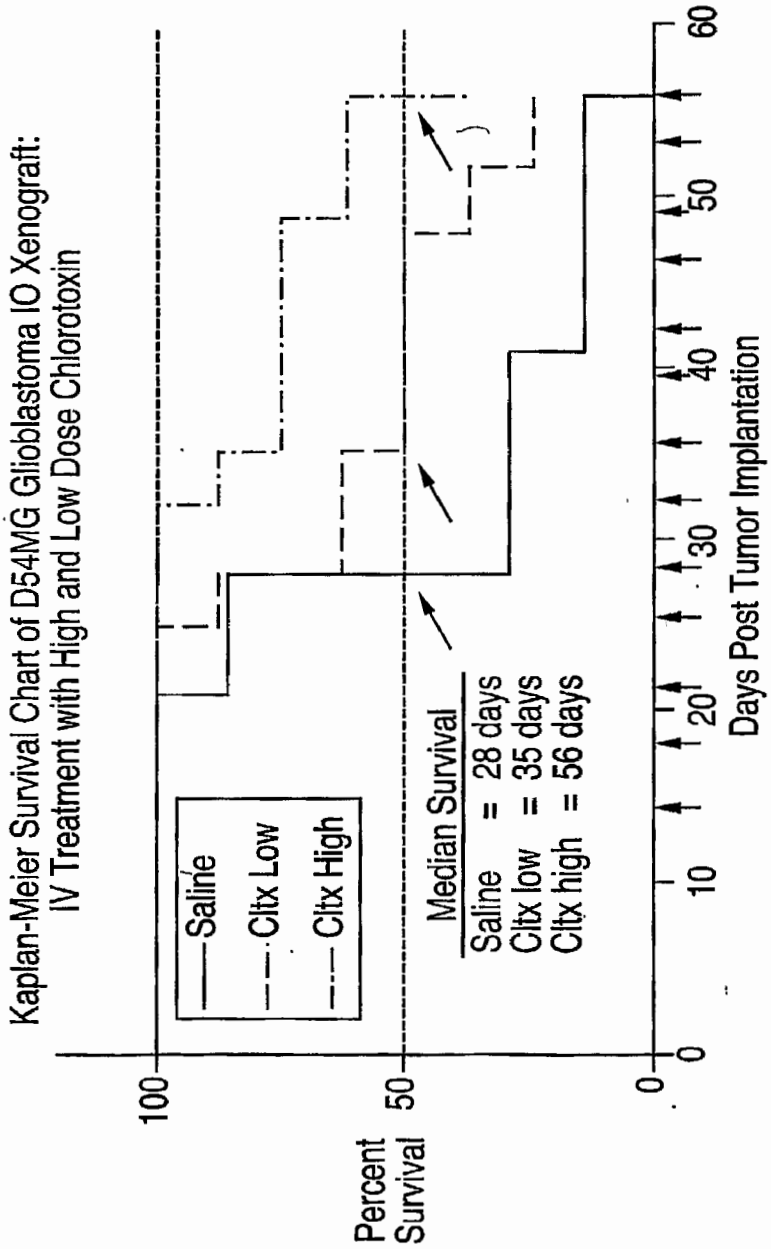

Kaplan-Meier Survival Chart of D54MG Glioblastoma IO Xenograft:
IV Treatment with High and Low Dose Chlorotoxin
Saline
Ctx Low
Ctx High
Median Survival
Saline = 28 days
Ctx low = 35 days
Ctx high = 56 days
Percent Survival
100
50
0
0
10
20
30
40
50
60
Days Post Tumor Implantation

FIG. 9

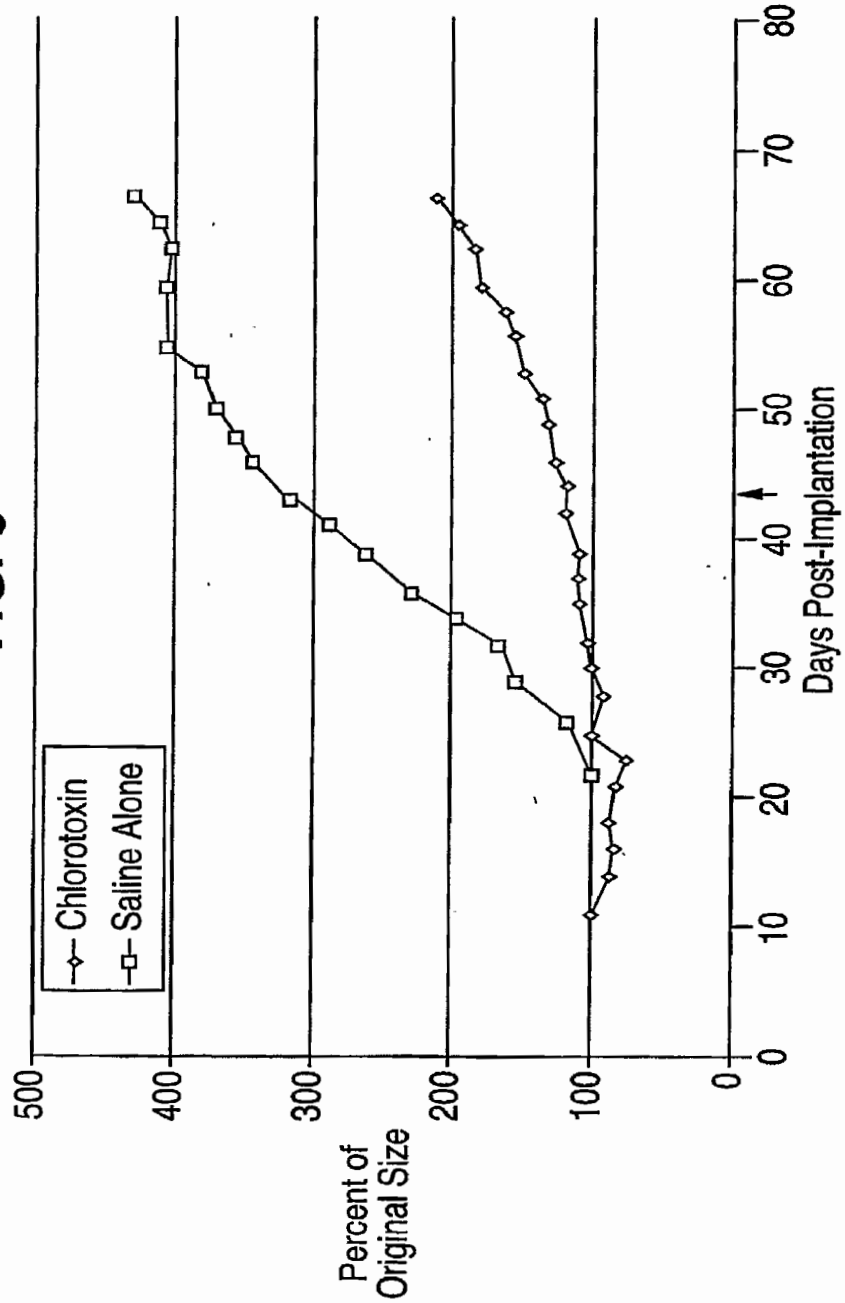
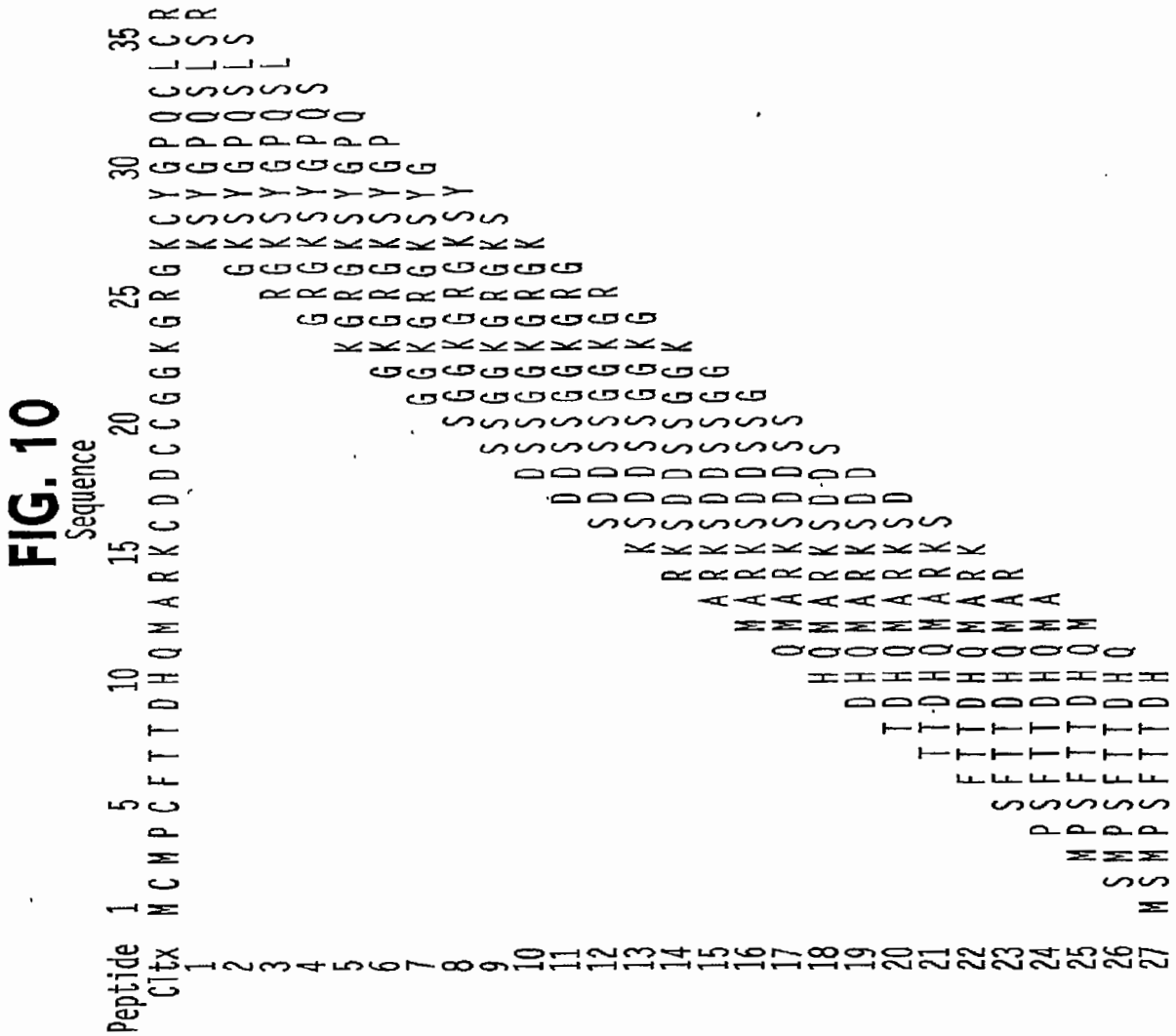

Chlorotoxin
Saline Alone
Percent of Original Size
500
400
300
200
100
0
0
10
20
30
40
50
60
70
80
Days Post-Implantation

FIG. 10


Sequence
Peptide
Ctlx
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2 KSSYGPQSSLSR
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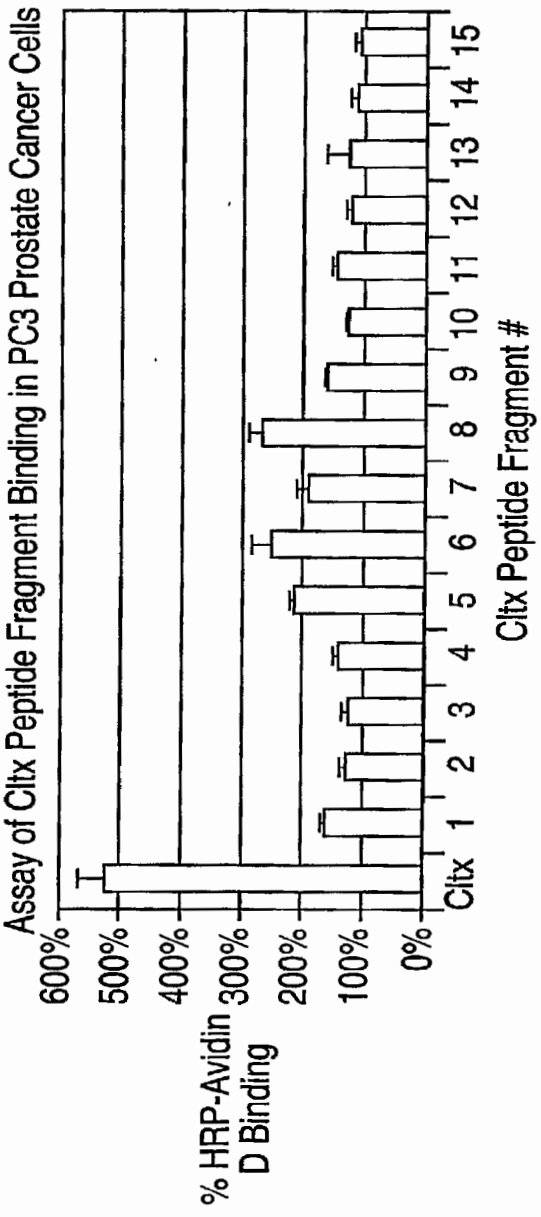
4 RGKSSYGPQSSL
5 GRGKSSYGPQSS
6 KGRGKSSYGPQS
7 GKGRGKSSYGPQ
8 GGKGRGKSSYGP
9 SGGKGRGKSSYG
10 SSGGKGRGKSSY
11 DSSGGKGRGKSS
12 DDSSGGKGRGKS
13 SDDSSGGKGRGK
14 KSDDSSGGKGRG
15 RKSDDSSGGKGR
16 ARKSDDSSGGKG
17 MARKSDDSSGGK
18 QMARKSDDSSGG
19 HQMARKSDDSSG
20 DHQMARKSDDSS
21 TDHQMARKSDDS
22 TTDHQMARKSDD
23 FTTDHQMARKSD
24 SFTTDHQMARKS
25 PSFTTDHQMARK
26 MPSFTTDHQMAR
27 SMPSFTTDHQMA
MSMPSFTTDHQM

FIG. 11


Assay of Cltx Peptide Fragment Binding in PC3 Prostate Cancer Cells
% HRP-Avidin D Binding
600%
500%
400%
300%
200%
100%
0%
Cltx
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Cltx Peptide Fragment #

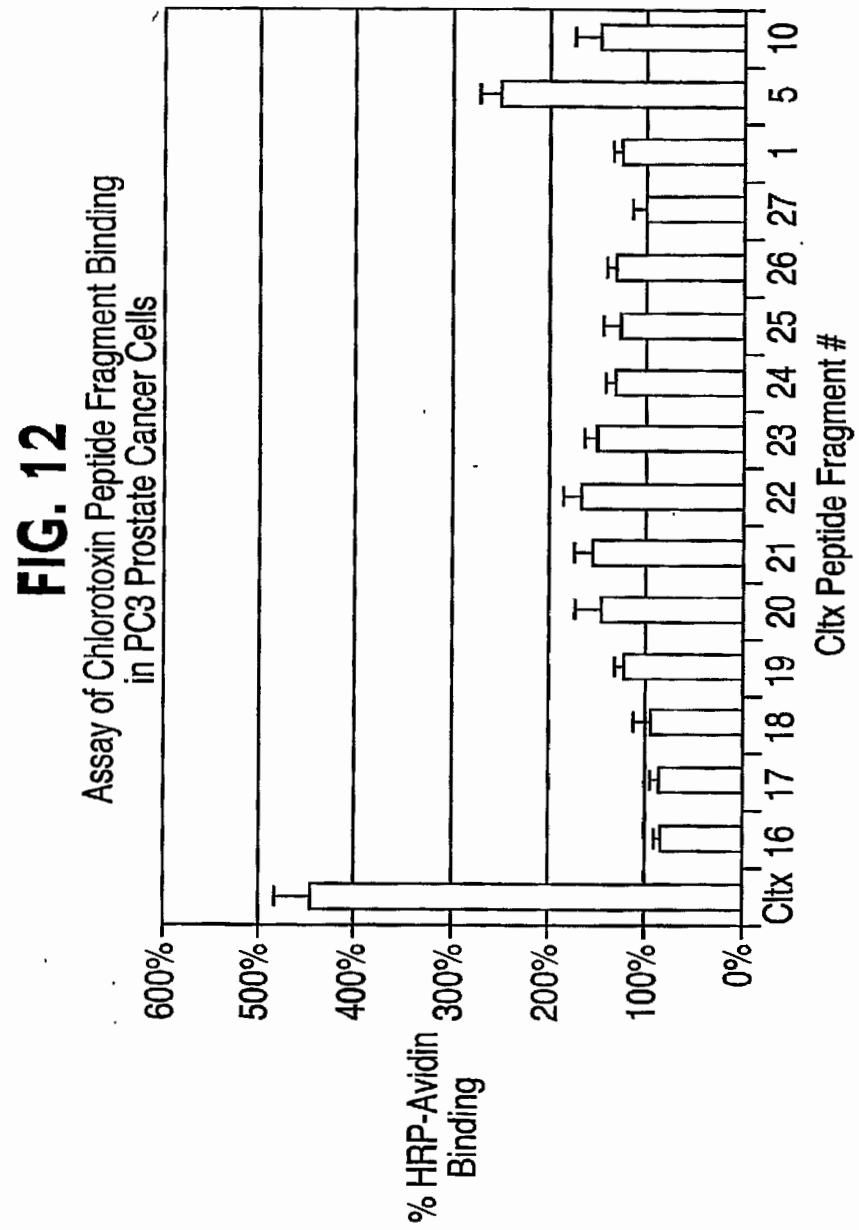
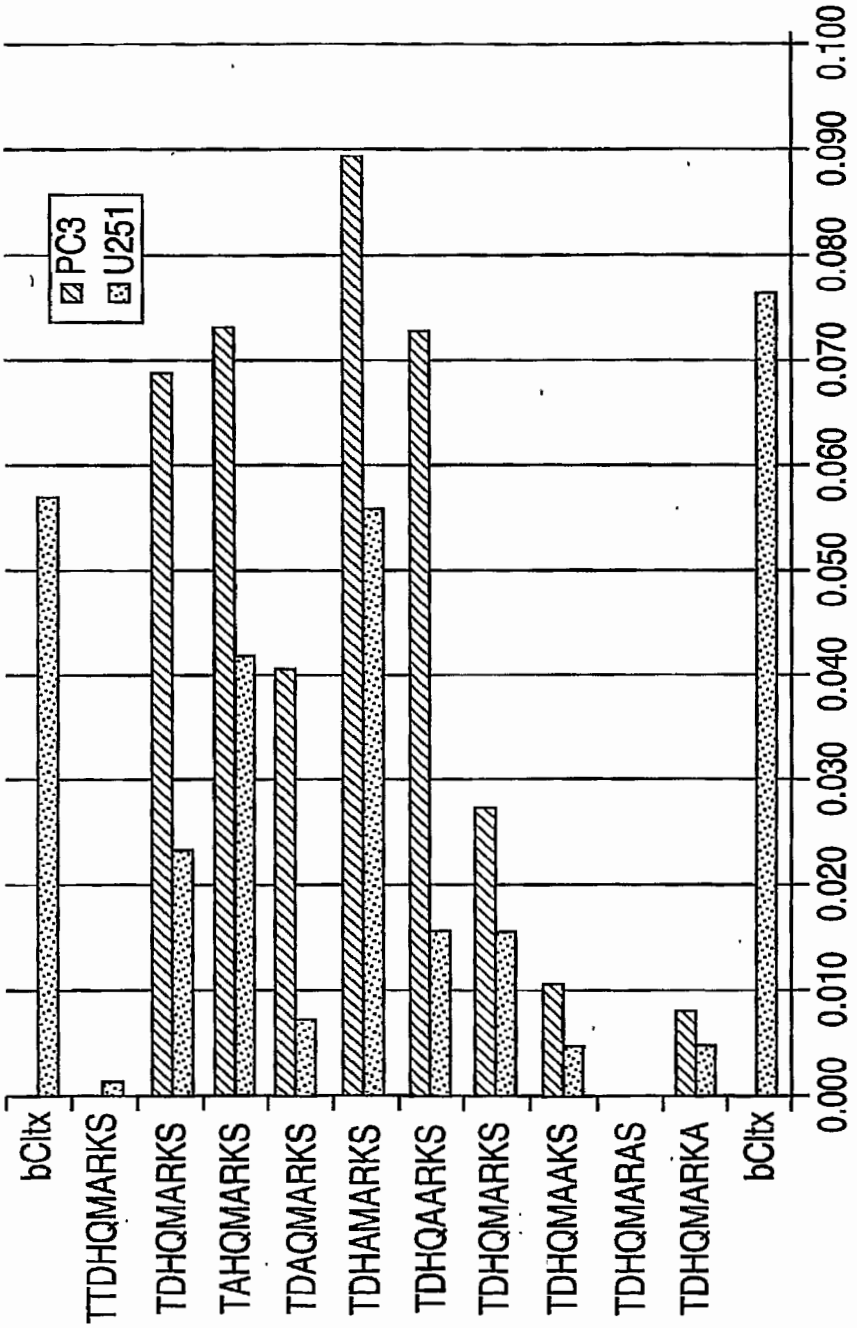

Assay of Chlorotoxin Peptide Fragment Binding in PC3 Prostate Cancer Cells
% HRP-Avidin Binding
600%
500%
400%
300%
200%
100%
0%
Cltx 16 17 18 19 20 21 22 23 24 25 26 27 1 5 10
Cltx Peptide Fragment #

FIG. 12

FIG. 13


PC3
U251
bCltx
TTDHQMARKS
TDHQMARKS
TAHQMARKS
TDAQMARKS
TDHAMARKS
TDHQAARKS
TDHQMARKS
TDHQMAAKS
TDHQMARAS
TDHQMARKA
bCltx
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0.020
0.030
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0.050
0.060
0.070
0.080
0.090
0.100

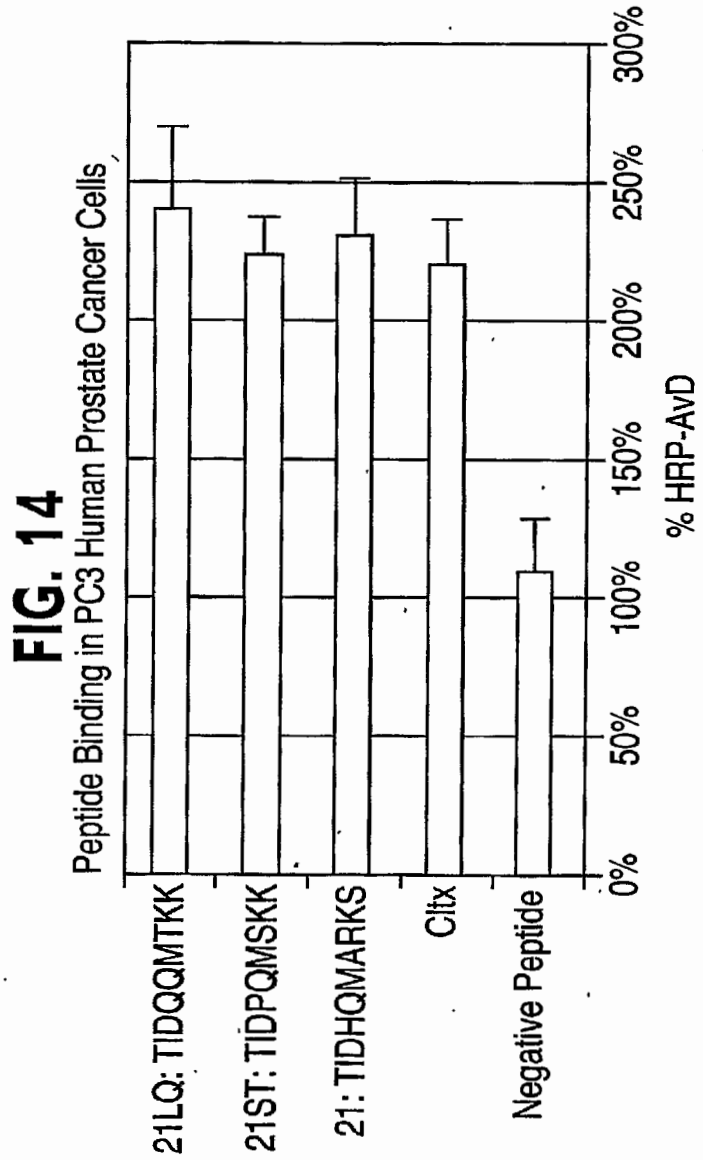
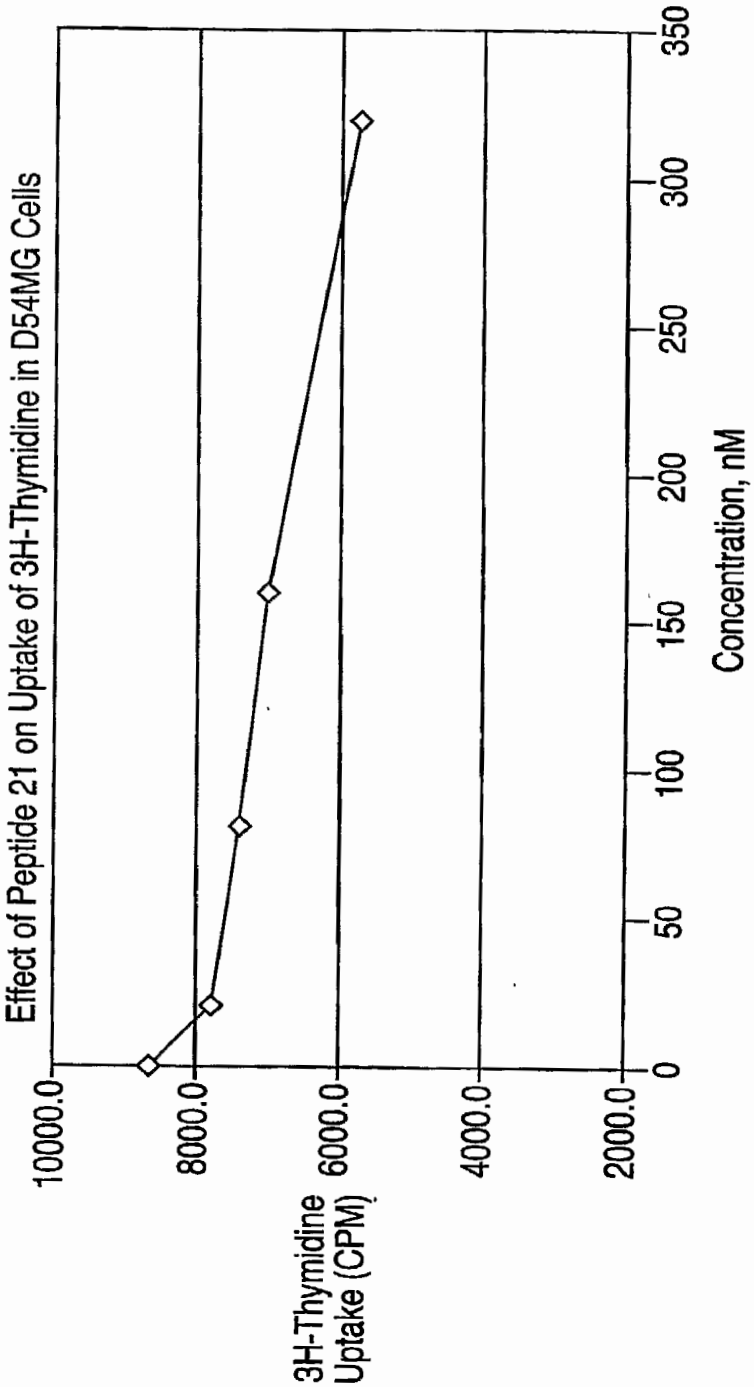

Peptide Binding in PC3 Human Prostate Cancer Cells
% HRP-AvD
0%
50%
100%
150%
200%
250%
300%
21LQ: TIDQQMTKK
21ST: TIDPQMSKK
21: TIDHQMARKS
Citx
Negative Peptide

FIG. 14

FIG. 15


Effect of Peptide 21 on Uptake of 3H-Thymidine in D54MG Cells
3H-Thymidine Uptake (CPM)
10000.0
8000.0
6000.0
4000.0
2000.0
0
50
100
150
200
250
300
350
Concentration, nM

SEQUENCE LISTING

<110> ALVAREZ, Vernon L.
GRIMES, Carol A.
GONDA, Matthew A.

<120> Combination chemotherapy with chlorotoxin

<130> 51530-5006-WO

<150> US 60/406,033
<151> 2002-08-27

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1 5 10 15

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20 25 30

Cys Leu Cys Arg
35

<210> 2
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<400> 2

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1 5 10 15

Gln Met Ala Arg Lys Cys Asp Asp Cys Cys Gly Gly Lys Gly Arg Gly
20 25 30

Lys Cys Tyr Gly Pro Gln Cys Leu Cys Arg
35 40

<210> 3
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<400> 3

Tyr Met Cys Met Pro Cys Phe Thr Thr Asp His Gln Met Ala Arg Lys
1 5 10 15

Cys Asp Asp Cys Cys Gly Gly Lys Gly Arg Gly Lys Cys Tyr Gly Pro
20 25 30

Gln Cys Leu Cys Arg
35

<210> 4
<211> 39
<212> PRT
<213> Leiurus quinquestriatus

<400> 4

Tyr Ser Tyr Met Cys Met Pro Cys Phe Thr Thr Asp His Gln Met Ala
1 5 10 15

Arg Lys Cys Asp Asp Cys Cys Gly Gly Lys Gly Arg Gly Lys Cys Tyr
20 25 30

Gly Pro Gln Cys Leu Cys Arg
35

<210> 5
<211> 36
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<213> Artificial sequence

<220>
<223> Chlorotoxin variant

<400> 5

Met Cys Met Pro Cys Phe Thr Thr Asp His Gln Met Ala Arg Lys Cys
1 5 10 15

Asp Asp Cys Cys Gly Gly Lys Gly Arg Gly Lys Cys Phe Gly Pro Gln
20 25 30

Cys Leu Cys Arg
35

<210> 6
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<223> Chlorotoxin variant

<400> 6

Arg Cys Lys Pro Cys Phe Thr Thr Asp Pro Gln Met Ser Lys Lys Cys
1 5 10 15

Ala Asp Cys Cys Gly Gly Lys Gly Lys Gly Lys Cys Tyr Gly Pro Gln
20 25 30

Cys Leu Cys
35

<210> 7
<211> 38
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<220>
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<400> 7

Arg Cys Ser Pro Cys Phe Thr Thr Asp Gln Gln Met Thr Lys Lys Cys
1 5 10 15

Tyr Asp Cys Cys Gly Gly Lys Gly Lys Gly Lys Cys Tyr Gly Pro Gln
20 25 30

Cys Ile Cys Ala Pro Tyr
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<210> 8
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<223> Derivative of Chlorotoxin: amino acid residues 23-29

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Lys Gly Arg Gly Lys Ser Tyr
1 5

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<213> Leiurus quinquestriatus

<220>
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<223> Derivative of Chlorotoxin: amino acid residues 8-14

<400> 9

Thr Asp His Gln Met Ala Arg
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<220>
<223> Chlorotoxin alpha peptide

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Thr Asp His Gln Met Ala Arg Lys Ser
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<223> Variant of chlorotoxin alpha peptide

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Thr Ala His Ala Met Ala Arg Lys Ser
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Met Cys Met Pro Cys Phe Thr Thr Ala His Ala Met Ala Arg Lys Cys
1 5 10 15

Asp Asp Cys Cys Gly Gly Lys Gly Arg Cys Lys Cys Tyr Gly Pro Gln
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Cys Leu Cys Arg
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<400> 13

Thr Thr Xaa Xaa Xaa Met Xaa Xaa Lys
1 5

<210> 14
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<213> Leiurus quinquestriatus

<400> 14

Thr Thr Asp His Gln Met Ala Arg Lys
1 5

<210> 15
<211> 35
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<213> Mesobuthus tamulus

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Arg Cys Lys Pro Cys Phe Thr Thr Asp Pro Gln Met Ser Lys Lys Cys
1 5 10 15

Ala Asp Cys Cys Gly Gly Lys Gly Lys Gly Lys Cys Tyr Gly Pro Gln
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Cys Leu Cys
35

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<223> Xaa can be His or Pro

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<223> Xaa can be Asp or Ala

<400> 16

Cys Xaa Pro Cys Phe Thr Thr Asp Xaa Gln Met Ala Lys Lys Cys Xaa
1 5 10 15

Asp Cys Cys Gly Gly Lys Gly Lys Gly Lys Cys Tyr Gly Pro Gln Cys
20 25 30

Leu Cys

<210> 17
<211> 38
<212> PRT
<213> Leiurus quinquestriatus

<400> 17

Arg Cys Ser Pro Cys Phe Thr Thr Asp Gln Gln Met Thr Lys Lys Cys
1 5 10 15

Tyr Asp Cys Cys Gly Gly Lys Gly Lys Gly Lys Cys Tyr Gly Pro Gln
20 25 30

Cys Ile Cys Ala Pro Tyr
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<210> 18
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Asp Cys Cys Gly Gly Lys Gly Lys Gly Lys Cys Tyr Gly Pro Gln Cys
20 25 30

Ile Cys

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Met Lys Phe Leu Tyr Gly Ile Val Phe Ile Ala Leu Phe Leu Thr Val
1 5 10 15

Met Phe Ala Thr Gln Thr Asp Gly Cys Gly Pro Cys Phe Thr Thr Asp
20 25 30

Ala Asn Met Ala Arg Lys Cys Arg Glu Cys Cys Gly Gly Ile Gly Xaa
35 40 45

Xaa Lys Cys Phe Gly Pro Gln Cys Leu Cys Asn Arg Ile
50 55 60

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<223> Xaa can be Lys or Ile

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Cys Xaa Pro Cys Phe Thr Thr Asp Xaa Asn Met Ala Arg Lys Cys Xaa
1 5 10 15

Asp Cys Cys Gly Gly Xaa Gly Xaa Xaa Lys Cys Phe Gly Pro Gln Cys
20 25 30

Leu Cys

<210> 21
<211> 59
<212> PRT
<213> Mesobuthus martensii

<400> 21

Met Lys Phe Leu Tyr Gly Ile Val Phe Ile Ala Leu Phe Leu Thr Val
1 5 10 15

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Met Phe Ala Thr Gln Thr Asp Gly Cys Gly Pro Cys Phe Thr Thr Asp
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Ala Asn Met Ala Arg Lys Cys Arg Glu Cys Cys Gly Gly Ile Gly Lys
        35                  40                  45

Cys Phe Gly Pro Gln Cys Leu Cys Asn Arg Ile
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<223> Xaa can be Lys or Phe

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Asp Cys Cys Gly Gly Xaa Gly Lys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
20 25 30

<210> 23
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<400> 23

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Arg Asp Cys Cys Gly Gly Xaa Gly Lys Xaa Lys Cys Phe Gly Pro Gln
20 25 30

Cys Leu Cys Asn Arg
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<210> 24
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<223> Xaa can be Asp or Arg

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<223> Xaa can be any amino acid

<400> 24

Met Cys Met Pro Cys Phe Thr Thr Asp Xaa Asn Met Ala Xaa Lys Cys
1 5 10 15

Xaa Asp Cys Cys Gly Gly Xaa Gly Lys Xaa Lys Cys Phe Gly Pro Gln
20 25 30

Cys Leu Cys
35

<210> 25
<211> 35
<212> PRT
<213> Mesobuthus eupeus

<400> 25

Met Cys Met Pro Cys Phe Thr Thr Asp Pro Asn Met Ala Asn Lys Cys
1 5 10 15

Arg Asp Cys Cys Gly Gly Gly Lys Lys Cys Phe Gly Pro Gln Cys Leu
20 25 30

Cys Asn Arg
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<210> 26
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Xaa Asp Cys Cys Gly Gly Xaa Xaa Lys Xaa Xaa Xaa Xaa Xaa Xaa Xaa
20 25 30

Cys

<210> 27
<211> 38
<212> PRT
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<400> 27

Met Cys Met Pro Cys Phe Thr Thr Arg Pro Asp Met Ala Gln Gln Cys
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Arg Ala Cys Cys Lys Gly Xaa Xaa Arg Gly Lys Cys Phe Gly Pro Gln
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Cys Leu Cys Gly Tyr Asp
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<210> 28
<211> 35
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Xaa Xaa Cys Cys Xaa Gly Xaa Xaa Arg Gly Lys Cys Phe Gly Pro Gln
20 25 30

Cys Leu Cys
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<210> 29
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<212> PRT
<213> Mesobuthus eupeus

<400> 29

Met Cys Met Pro Cys Phe Thr Thr Arg Pro Asp Met Ala Gln Gln Cys
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Arg Ala Cys Cys Lys Gly Arg Gly Lys Cys Phe Gly Pro Gln Cys Leu
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Cys Gly Tyr Asp
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<400> 30

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Xaa Xaa Cys Cys Xaa Gly Lys Gly Lys Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Cys

<210> 31
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<400> 31

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1 5 10 15

Arg Asp Cys Cys Gly Gly Asn Gly Xaa Xaa Lys Cys Phe Gly Pro Gln
20 25 30

Cys Leu Cys Asn Arg
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<210> 32
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<223> Xaa can be Lys or Asn

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<400> 32

Met Cys Met Pro Cys Phe Thr Thr Asp Xaa Asn Met Ala Lys Lys Cys
1 5 10 15

Xaa Asp Cys Cys Gly Gly Xaa Gly Xaa Xaa Lys Cys Phe Gly Pro Gln
20 25 30

Cys Leu Cys
35

<210> 33
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<400> 33

Met Cys Met Pro Cys Phe Thr Thr Asp Pro Asn Met Ala Lys Lys Cys
1 5 10 15

Arg Asp Cys Cys Gly Gly Asn Gly Lys Cys Phe Gly Pro Gln Cys Leu
20 25 30

Cys Asn Arg
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<400> 34

Met Cys Met Pro Cys Phe Thr Thr Asp Xaa Asn Met Ala Lys Lys Cys .
1 5 10 15

Xaa Asp Cys Cys Gly Gly Xaa Gly Lys Xaa Xaa Xaa Xaa Xaa Xaa Xaa
20 25 30

Cys

<210> 35
<211> 37
<212> PRT
<213> Androctonus mauretanicus

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Cys Gly Pro Cys Phe Thr Thr Asp Pro Tyr Thr Glu Ser Lys Cys Ala
1 5 10 15

Thr Cys Cys Gly Gly Xaa Xaa Arg Gly Lys Cys Val Gly Pro Gln Cys
20 25 30

Leu Cys Asn Arg Ile
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<223> Xaa can be any amino acid

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<222> (28)..(28)
<223> Xaa can be Tyr or Val

<400> 36

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1 5 10 15

Xaa Cys Cys Gly Gly Xaa Xaa Arg Gly Lys Cys Xaa Gly Pro Gln Cys
20 25 30

Leu Cys

<210> 37
<211> 35
<212> PRT
<213> Androctonus mauretanicus

<400> 37

Cys Gly Pro Cys Phe Thr Thr Asp Pro Tyr Thr Glu Ser Lys Cys Ala
1 5 10 15

Thr Cys Cys Gly Gly Arg Gly Lys Cys Val Gly Pro Gln Cys Leu Cys
20 25 30

Asn Arg Ile
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<210> 38
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<400> 38

Cys Xaa Pro Cys Phe Thr Thr Asp Xaa Xaa Xaa Xaa Xaa Lys Cys Xaa
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Xaa Cys Cys Gly Gly Lys Gly Lys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
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<223> Xaa can be any amino acid

<400> 39

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Arg Asp Cys Cys Gly Gly Lys Gly Xaa Xaa Lys Cys Phe Gly Pro Gln
20 25 30

Cys Leu Cys Asn Arg
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<210> 40
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<220>
<223> Toxin consensus sequence

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<222> (3)..(3)
<223> Xaa can be Met, Lys or Ser

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<223> Xaa can be His, Pro, or Gln

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/17410

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/00
US CL : 514/12
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. : 514/12, 13, 2

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.
Y	US 5,905,027 A (ULLRICH et al.) 18 May 1999 (18.05.1999), see column 26, lines 12-40).	1-17
Y	US 6,028,174 A (ULLRICH et al.) 22 February 2000 (22.02.2000), see column 26 lines 18-29.	1-17
Y	US 2002/0065216 A1 (SONTHEIMER et al) 30 May 2002 (30.05.2002), see pages 14-15.	1-17

☐ 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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 2003 (20.10.2003)	13 NOV 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer B. Dell Chism Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
STN, BIOSIS, MEDLINE, SCISEARCH, WEST
search terms: chlorotoxin, cancer, therapeutic, agent, tumor, blastoma, neoplasm

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(30) Priority Data:
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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ZHU, Guidong** [US/US]; 1395 Almaden Ln, Gurnee, IL 60031 (US). **GONG, Jianchun** [CN/US]; 130 Ferndale Rd, Deerfield, IL 60015 (US). **GANDHI, Virajkumar, B.** [IN/US]; 4123 Greenleaf Ct, Apt 205, Park City, IL 60085 (US). **PENNING, Thomas, D.** [US/US]; 321 Highview, Elmhurst, IL 60126 (US). **GIRANDA, Vincent** [US/US]; 272 S Fork Dr, Gurnee, IL 60031 (US).

(74) Agents: **CORBIN, Johanna, M.** et al.; Dept. 377 Bldg AP6A-1, 100 Abbott Park Road, Abbott Park, IL 60064-6008 (US).

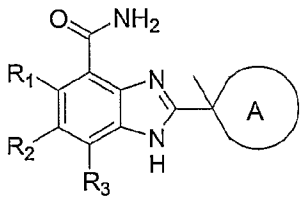
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: 1H-BENZIMIDAZOLE-4-CARBOXAMIDES SUBSTITUTED WITH A QUATERNARY CARBON AT THE 2-POSITION ARE POTENT PARP INHIBITORS



(I)

(57) Abstract: Compounds of Formula (I) (I) inhibit the PARP enzyme and are useful for treating a disease or a disorder associated with PARP. Also disclosed are pharmaceutical compositions comprising compounds of Formula (I), methods of treatment comprising compounds of Formula (I), and methods of inhibiting the PARP enzyme comprising compounds of Formula (I).

WO 2006/110816 A2

1H-BENZIMIDAZOLE-4-CARBOXAMIDES SUBSTITUTED WITH A QUATERNARY CARBON AT THE 2-POSITION ARE POTENT PARP INHIBITORS

Technical Field

The present invention relates to 1H-benzimidazole-4-carboxamides substituted at the 2-position with a quaternary carbon, their preparation, and their use as inhibitors of the enzyme poly(ADP-ribose)polymerase for the preparation of drugs.

Background

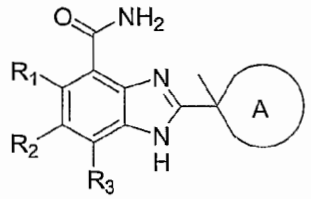
Poly(ADP-ribose)polymerase (PARP) or poly(ADP-ribose)synthase (PARS) has an essential role in facilitating DNA repair, controlling RNA transcription, mediating cell death, and regulating immune response. These actions make PARP inhibitors targets for a broad spectrum of disorders. PARP inhibitors have demonstrated efficacy in numerous models of disease, particularly in models of ischemia reperfusion injury, inflammatory disease, degenerative diseases, protection from adverse effects of cytoxic compounds, and the potentiation of cytotoxic cancer therapy. PARP has also been indicated in retroviral infection and thus inhibitors may have use in antiretroviral therapy. PARP inhibitors have been efficacious in preventing ischemia reperfusion injury in models of myocardial infarction, stroke, other neural trauma, organ transplantation, as well as reperfusion of the eye, kidney, gut and skeletal muscle. Inhibitors have been efficacious in inflammatory diseases such as arthritis, gout, inflammatory bowel disease, CNS inflammation such as MS and allergic encephalitis, sepsis, septic shock, hemmorhagic shock, pulmonary fibrosis, and uveitis. PARP inhibitors have also shown benefit in several models of degenerative disease including diabetes (as well as complications) and Parkinsons disease. PARP inhibitors can ameliorate the liver toxicity following acetominophen overdose, cardiac and kidney toxicities from doxorubicin and platinum based antineoplastic agents, as well as skin damage secondary to sulfur mustards. In various cancer models, PARP inhibitors have been shown to potentiate radiation and chemotherapy by increasing apoptosis of cancer cells, limiting tumor growth, decreasing metastasis, and prolonging the survival of tumor-bearing animals.

The present invention describes the finding that 1H-benzimidazole-4-carboxamides substituted with a quaternary carbon at the 2-position increases affinity for the PARP enzyme. The present invention describes benzimidazole derivatives of Formula (I) which have increased affinity and constitute potent PARP inhibitors.

5

Summary of the Invention

In one embodiment, the present invention provides compounds of Formula (I)



(I),

10 or a therapeutically acceptable salt thereof, wherein

R_1, R_2, and R_3 are independently selected from the group consisting of hydrogen, alkenyl, alkoxy, alkoxycarbonyl, alkyl, alkynyl, cyano, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, nitro, NR_AR_B, and (NR_AR_B)carbonyl;

A is a nonaromatic 4, 5, 6, 7, or 8-membered ring that contains 1 or 2 nitrogen atoms

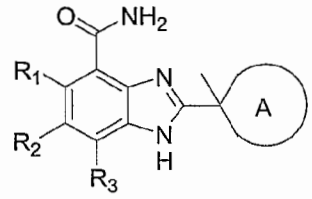
15 and, optionally, one sulfur or oxygen atom, wherein the nonaromatic ring is optionally substituted with 1, 2, or 3 substituents selected from the group consisting of alkenyl, alkoxy, alkoxyalkyl, alkoxycarbonyl, alkoxycarbonylalkyl, alkyl, alkynyl, aryl, arylalkyl, cycloalkyl, cycloalkylalkyl, cyano, haloalkoxy, haloalkyl, halogen, heterocycle, heterocyclealkyl, heteroaryl, heteroarylalkyl, hydroxy, hydroxyalkyl, nitro, oxo, NR_CR_D, (NR_CR_D)alkyl,

20 (NR_CR_D)carbonyl, (NR_CR_D)carbonylalkyl, and (NR_CR_D)sulfonyl; and

R_A, R_B, R_C, and R_D are independently selected from the group consisting of hydrogen, alkyl, and alkycarbonyl.

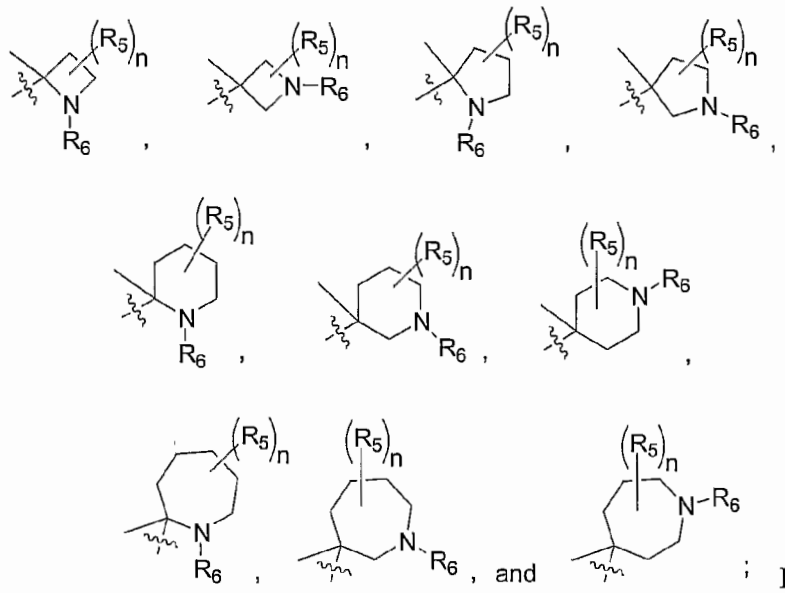
Detailed Description of the Invention

25 In another embodiment, the present invention provides compounds of Formula (I)



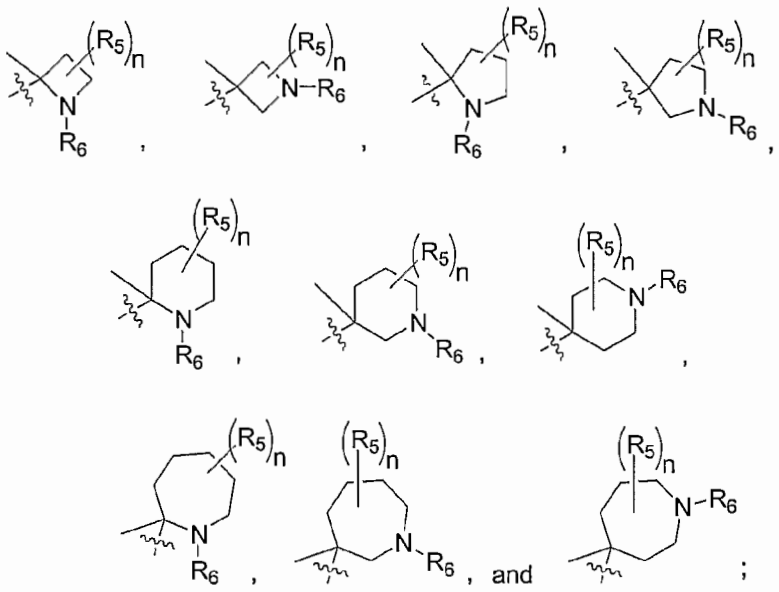
(I),

or a therapeutically acceptable salt thereof wherein R_1, R_2, and R_3 are independently selected from the group consisting of hydrogen, alkenyl, alkoxy, alkoxycarbonyl, alkyl, alkynyl, cyano, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, nitro, NR_AR_B, and (NR_AR_B)carbonyl; A is selected from the group consisting of



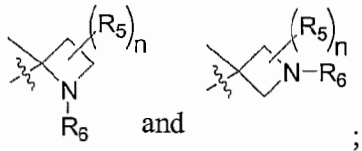
; R_5 is independently selected from the group consisting of alkenyl, alkoxy, alkoxycarbonyl, alkyl, alkynyl, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, NR_CR_D, and (NR_CR_D)carbonyl; n is 0, 1, 2, or 3; R_6 is selected from the group consisting of hydrogen, alkenyl, alkoxyalkyl, alkoxycarbonyl, alkoxycarbonylalkyl, alkyl, alkynyl, aryl, arylalkyl, cycloalkyl, cycloalkylalkyl, heterocycle, heterocyclealkyl, heteroaryl, heteroarylalkyl, hydroxyalkyl, oxo, (NR_CR_D)alkyl, (NR_CR_D)carbonyl, (NR_CR_D)carbonylalkyl, and (NR_CR_D)sulfonyl; R_A and R_B are independently selected from the group consisting of hydrogen, alkyl, and alkycarbonyl; and R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

In another embodiment, the present invention provides compounds of Formula (I) or a therapeutically acceptable salt thereof wherein R_1, R_2, and R_3 are hydrogen; A is selected from the group consisting of



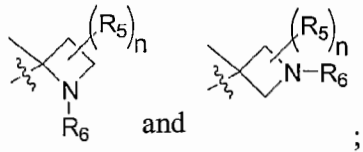
; n is 0; R_6 is selected from the group consisting of hydrogen, alkenyl, alkoxyalkyl, alkoxycarbonyl, alkoxycarbonylalkyl, alkyl, alkynyl, aryl, arylalkyl, cycloalkyl, cycloalkylalkyl, heterocycle, heterocyclealkyl, heteroaryl, heteroarylalkyl, hydroxyalkyl, (NR_CR_D)alkyl, (NR_CR_D)carbonyl, (NR_CR_D)carbonylalkyl, and (NR_CR_D)sulfonyl; and R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of



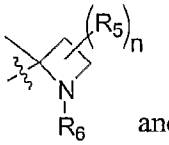
; and n, R_1, R_2, R_3, R_5, and R_6 are as defined in Formula (I).

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of



; n is 0; R_1, R_2, and R_3 are hydrogen; R_6 is selected from the group consisting of hydrogen, alkyl, (NR_CR_D)sulfonyl, and arylalkyl; and R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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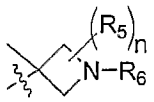
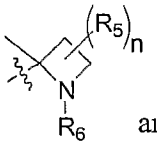

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is selected from the group consisting of cycloalkyl and cycloalkylalkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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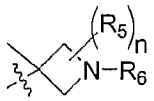
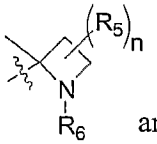

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heterocycle.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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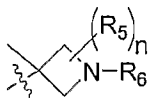
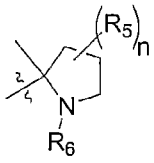

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heteroarylalkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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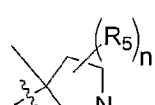
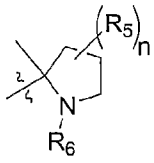

; and n, R_1, R_2, R_3, R_5, and R_6 are as defined in Formula (I).

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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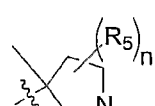
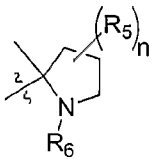

; n is 0; R_1, R_2, and R_3 are hydrogen; R_6 is selected from the group consisting of hydrogen, alkyl, (NR_CR_D)sulfonyl, and arylalkyl; and R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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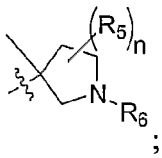
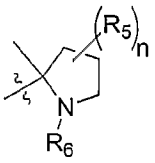

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is selected from the group consisting of cycloalkyl and cycloalkylalkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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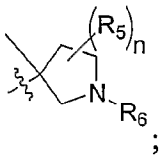
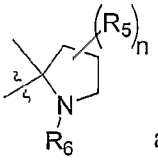

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heterocycle.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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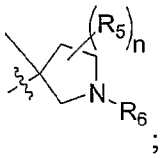
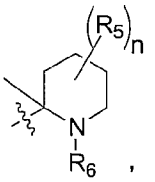

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heteroarylalkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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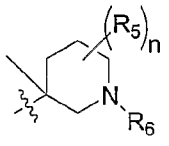
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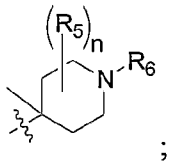
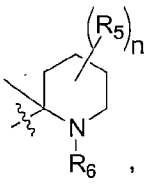

; and n, R_1, R_2, R_3, R_5, and R_6 are as defined in Formula (I).

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of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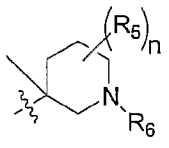
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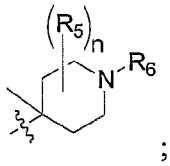
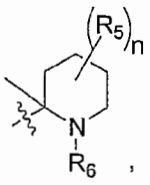
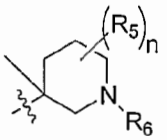
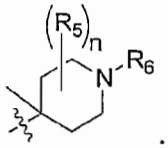
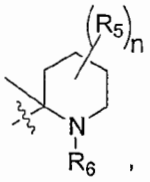
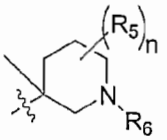
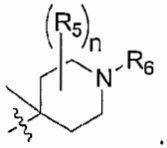

; n is 0; R_1, R_2, and R_3 are hydrogen; R_6 is selected from the group consisting of hydrogen, alkyl, (NR_CR_D)sulfonyl, and arylalkyl; and R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

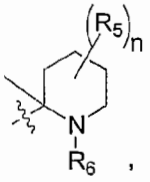
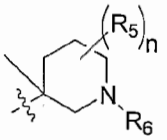
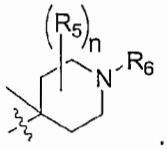
In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of




; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is selected from the group consisting of cycloalkyl and cycloalkylalkyl.

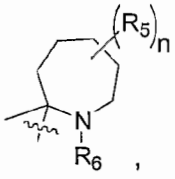
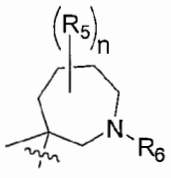
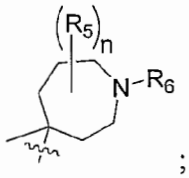
In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of




; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heterocycle.

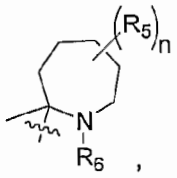
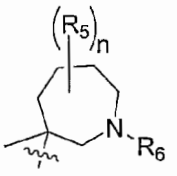
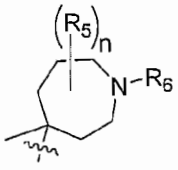
In another embodiment, the present invention provides compounds of Formula (II) wherein A is selected from the group consisting of




; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heteroarylalkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of

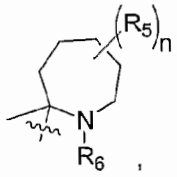
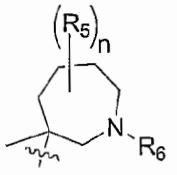
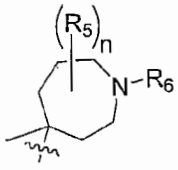



; and n, R_1, R_2, R_3, R_5, and R_6 are as defined in Formula (I).

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of

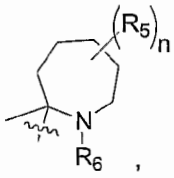
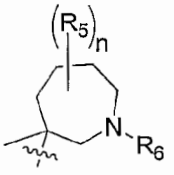
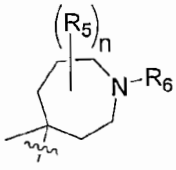
; n is 0; R_1, R_2, and R_3 are hydrogen; R_6 is selected from the group consisting of hydrogen, alkyl, (NR_CR_D)sulfonyl, and arylalkyl; and R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of

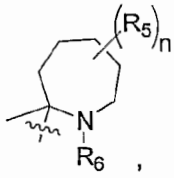
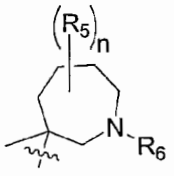
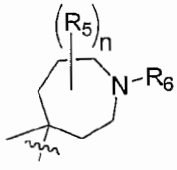
; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is selected from the group consisting of cycloalkyl and cycloalkylalkyl.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heterocycle.

In another embodiment, the present invention provides compounds of Formula (I) wherein A is selected from the group consisting of

; n is 0; R_1, R_2, and R_3 are hydrogen; and R_6 is heteroarylalkyl.

In another embodiment, the present invention provides a pharmaceutical composition comprising a compound of Formula (I), or a therapeutically acceptable salt thereof, in combination with a therapeutically acceptable carrier.

In another embodiment, the present invention provides a method of inhibiting PARP in a mammal in recognized need of such treatment comprising administering to the mammal

a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating cancer in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating leukemia, colon cancer, glioblastomas, lymphomas, melanomas, carcinomas of the breast, or cervical carcinomas in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of potentiation of cytotoxic cancer therapy in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating ischemia reperfusion injury associated with, but not limited to, myocardial infarction, stroke, other neural trauma, and organ transplantation, in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of reperfusion including, but not limited to, reperfusion of the eye, kidney, gut and skeletal muscle, in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating inflammatory diseases including, but not limited to, arthritis, gout, inflammatory bowel disease, CNS inflammation, multiple sclerosis, allergic encephalitis, sepsis, septic shock, hemmorhagic shock, pulmonary fibrosis, and uveitis in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating immunological diseases or disorders such as rheumatoid arthritis and septic shock in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating degenerative disease including, but not limited to, diabetes and Parkinsons disease, in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating hypoglycemia in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating retroviral infection in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating liver toxicity following acetominophen overdose in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating cardiac and kidney toxicities from doxorubicin and platinum based antineoplastic agents in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a method of treating skin damage secondary to sulfur mustards in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for inhibiting the PARP enzyme in a mammal in recognized need of such treatment.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for inhibiting tumor growth in a mammal in recognized need of such treatment.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating cancer in a mammal in recognized need of such treatment.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating leukemia, colon cancer, glioblastomas, lymphomas, melanomas, carcinomas of the breast, or cervical carcinomas in a mammal in a mammal in recognized need of such treatment.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for potentiation of cytotoxic cancer therapy in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating ischemia reperfusion injury associated with, but not limited to, myocardial infarction, stroke, other neural trauma, and organ transplantation, in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating reperfusion including, but not limited to, reperfusion of the eye, kidney, gut and skeletal muscle, in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating

inflammatory diseases including, but not limited to, arthritis, gout, inflammatory bowel disease, CNS inflammation, multiple sclerosis, allergic encephalitis, sepsis, septic shock, hemmorhagic shock, pulmonary fibrosis, and uveitis in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating immunological diseases or disorders such as rheumatoid arthritis and septic shock in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating degenerative disease including, but not limited to, diabetes and Parkinsons disease, in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating hypoglycemia in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating retroviral infection in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating liver toxicity following acetominophen overdose in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating cardiac and kidney toxicities from doxorubicin and platinum based antineoplastic agents in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

In another embodiment, the present invention provides a use of a compound of Formula (I), or a therapeutically acceptable salt thereof, to prepare a medicament for treating skin damage secondary to sulfur mustards in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

Definitions

As used throughout this specification and the appended claims, the following terms have the following meanings:

The term "alkenyl" as used herein, means a straight or branched chain hydrocarbon containing from 2 to 10 carbons and containing at least one carbon-carbon double bond formed by the removal of two hydrogens. Representative examples of alkenyl include, but are not limited to, ethenyl, 2-propenyl, 2-methyl-2-propenyl, 3-butenyl, 4-pentenyl, 5-hexenyl, 2-heptenyl, 2-methyl-1-heptenyl, and 3-decenyl.

The term "alkoxy" as used herein, means an alkyl group, as defined herein, appended to the parent molecular moiety through an oxygen atom. Representative examples of alkoxy include, but are not limited to, methoxy, ethoxy, propoxy, 2-propoxy, butoxy, tert-butoxy, pentyloxy, and hexyloxy.

The term "alkoxyalkyl" as used herein, means at least one alkoxy group, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein. Representative examples of alkoxyalkyl include, but are not limited to, tert-butoxymethyl, 2-ethoxyethyl, 2-methoxyethyl, and methoxymethyl.

The term "alkoxycarbonyl" as used herein, means an alkoxy group, as defined herein, appended to the parent molecular moiety through a carbonyl group, as defined herein. Representative examples of alkoxycarbonyl include, but are not limited to, methoxycarbonyl, ethoxycarbonyl, and tert-butoxycarbonyl.

The term "alkoxycarbonylalkyl" as used herein, means an alkoxycarbonyl group, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein.

The term "alkyl" as used herein, means a straight or branched chain hydrocarbon containing from 1 to 10 carbon atoms. Representative examples of alkyl include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, iso-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, n-hexyl, 3-methylhexyl, 2,2-dimethylpentyl, 2,3-dimethylpentyl, n-heptyl, n-octyl, n-nonyl, and n-decyl.

The term "alkylcarbonyl" as used herein, means an alkyl group, as defined herein, appended to the parent molecular moiety through a carbonyl group, as defined herein. Representative examples of alkylcarbonyl include, but are not limited to, acetyl, 1-oxopropyl, 2,2-dimethyl-1-oxopropyl, 1-oxobutyl, and 1-oxopentyl.

The term "alkylcarbonyloxy" as used herein, means an alkylcarbonyl group, as defined herein, appended to the parent molecular moiety through an oxygen atom. Representative examples of alkylcarbonyloxy include, but are not limited to, acetyloxy, ethylcarbonyloxy, and tert-butylcarbonyloxy.

The term "alkylthio" as used herein, means an alkyl group, as defined herein, appended to the parent molecular moiety through a sulfur atom. Representative examples of alkylthio include, but are not limited, methylthio, ethylthio, tert-butylthio, and hexylthio.

The term "alkylthioalkyl" as used herein, means an alkylthio group, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein. Representative examples of alkylthioalkyl include, but are not limited, methylthiomethyl and 2-(ethylthio)ethyl.

The term "alkynyl" as used herein, means a straight or branched chain hydrocarbon group containing from 2 to 10 carbon atoms and containing at least one carbon-carbon triple bond. Representative examples of alkynyl include, but are not limited, to acetylenyl, 1-propynyl, 2-propynyl, 3-butynyl, 2-pentynyl, and 1-butynyl.

The term "aryl," as used herein, means a phenyl group or a naphthyl group.

The aryl groups of the present invention can be optionally substituted with one, two, three, four, or five substituents independently selected from the group consisting of alkenyl, alkoxy, alkoxyalkyl, alkoxycarbonyl, alkyl, alkylcarbonyl, alkylcarbonyloxy, alkylthio,

alkylthioalkyl, alkynyl, carboxy, cyano, formyl, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, mercapto, nitro, $-NR_ER_F$, and (NR_ER_F)carbonyl.

The term "arylalkyl" as used herein, means an aryl group, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein. Representative examples of arylalkyl include, but are not limited to, benzyl, 2-phenylethyl, 3-phenylpropyl, 1-methyl-3-phenylpropyl, and 2-naphth-2-ylethyl.

The term "carbonyl" as used herein, means a -C(O)- group.

The term "carboxy" as used herein, means a $-CO_2H$ group.

The term "cyano" as used herein, means a -CN group.

The term "cycloalkyl" as used herein, means a saturated cyclic hydrocarbon group containing from 3 to 8 carbons, examples of cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl.

The cycloalkyl groups of the present invention are optionally substituted with 1, 2, 3, or 4 substituents selected from alkenyl, alkoxy, alkoxyalkyl, alkoxycarbonyl, alkyl, alkylcarbonyl, alkylcarbonyloxy, alkylthio, alkylthioalkyl, alkynyl, carboxy, cyano, formyl, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, mercapto, oxo, $-NR_ER_F$, and (NR_ER_F)carbonyl.

The term "cycloalkylalkyl" as used herein, means a cycloalkyl group, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein. Representative examples of cycloalkylalkyl include, but are not limited to, cyclopropylmethyl, 2-cyclobutylethyl, cyclopentylmethyl, cyclohexylmethyl, and 4-cycloheptylbutyl.

The term "formyl" as used herein, means a -C(O)H group.

The term "halo" or "halogen" as used herein, means -Cl, -Br, -I or -F.

The term "haloalkoxy" as used herein, means at least one halogen, as defined herein, appended to the parent molecular moiety through an alkoxy group, as defined herein. Representative examples of haloalkoxy include, but are not limited to, chloromethoxy, 2-fluoroethoxy, trifluoromethoxy, and pentafluoroethoxy.

The term "haloalkyl" as used herein, means at least one halogen, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein. Representative examples of haloalkyl include, but are not limited to, chloromethyl, 2-fluoroethyl, trifluoromethyl, pentafluoroethyl, and 2-chloro-3-fluoropentyl.

The term "heteroaryl," as used herein, means a monocyclic heteroaryl ring or a bicyclic heteroaryl ring. The monocyclic heteroaryl ring is a 5 or 6 membered ring. The 5 membered ring has two double bonds and contains one, two, three or four heteroatoms independently selected from the group consisting of N, O, and S. The 6 membered ring has three double bonds and contains one, two, three or four heteroatoms independently selected from the group consisting of N, O, and S. The bicyclic heteroaryl ring consists of the 5 or 6 membered heteroaryl ring fused to a phenyl group or the 5 or 6 membered heteroaryl ring is fused to another 5 or 6 membered heteroaryl ring. Nitrogen heteroatoms contained within the heteroaryl may be optionally oxidized to the N-oxide. The heteroaryl is connected to the parent molecular moiety through any carbon atom contained within the heteroaryl while maintaining proper valence. Representative examples of heteroaryl include, but are not limited to, benzothienyl, benzoxadiazolyl, cinnolinyl, furopyridinyl, furyl, imidazolyl, indazolyl, indolyl, isoxazolyl, isoquinolinyl, isothiazolyl, naphthyridinyl, oxadiazolyl, oxazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, pyrazolyl, pyrrolyl, pyridinium N-oxide, quinolinyl, tetrazolyl, thiadiazolyl, thiazolyl, thienopyridinyl, thienyl, triazolyl, and triazinyl.

The heteroaryl groups of the present invention are substituted with 0, 1, 2, 3, or 4 substituents independently selected from alkenyl, alkoxy, alkoxyalkyl, alkoxycarbonyl, alkyl, alkylcarbonyl, alkylcarbonyloxy, alkylthio, alkylthioalkyl, alkynyl, carboxy, cyano, formyl, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, mercapto, nitro, $-NR_ER_F$, and (NR_ER_F)carbonyl.

The term "heteroarylalkyl" as used herein, means a heteroaryl, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein. Representative examples of heteroarylalkyl include, but are not limted to, pyridinymethyl.

The term "heterocycle" or "heterocyclic" as used herein, means a monocyclic or bicyclic heterocyclic ring. The monocyclic heterocyclic ring consists of a 3, 4, 5, 6, 7, or 8 membered ring containing at least one heteroatom independently selected from O, N, and S. The 3 or 4 membered ring contains 1 heteroatom selected from the group consisting of O, N and S. The 5 membered ring contains zero or one double bond and one, two or three heteroatoms selected from the group consisting of O, N and S. The 6 or 7 membered ring contains zero, one or two double bonds and one, two or three heteroatoms selected from the group consisting of O, N and S. The bicyclic heterocyclic ring consists of a monocyclic

heterocyclic ring fused to a cycloalkyl group or the monocyclic heterocyclic ring fused to a phenyl group or the monocyclic heterocyclic ring fused to another monocyclic heterocyclic ring. The heterocycle is connected to the parent molecular moiety through any carbon or nitrogen atom contained within the heterocycle while maintaining proper valence. Representative examples of heterocycle include, but are not limited to, azetidinyl, azepanyl, aziridinyl, diazepanyl, 1,3-dioxanyl, 1,3-dioxolanyl, 1,3-dithiolanyl, 1,3-dithianyl, imidazolinyl, imidazolidinyl, isothiazolinyl, isothiazolidinyl, isoxazolinyl, isoxazolidinyl, morpholinyl, oxadiazolinyl, oxadiazolidinyl, oxazolinyl, oxazolidinyl, piperazinyl, piperidinyl, pyrazolinyl, pyrazolidinyl, pyrrolinyl, pyrrolidinyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydrothienyl, thiadiazolinyl, thiadiazolidinyl, thiazolinyl, thiazolidinyl, thiomorpholinyl, 1,1-dioxidothiomorpholinyl (thiomorpholine sulfone), thiopyranyl, and trithianyl.

The heterocycles of this invention are substituted with 0, 1, 2,or 3 substituents independently selected from alkenyl, alkoxy, alkoxyalkyl, alkoxycarbonyl, alkyl, alkylcarbonyl, alkylcarbonyloxy, alkylthio, alkylthioalkyl, alkynyl, carboxy, cyano, formyl, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, mercapto, nitro, -NR_ER_F, and (NR_ER_F)carbonyl.

The term "heterocyclealkyl" as used herein, means a heterocycle, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein.

The term "hydroxy" as used herein, means an -OH group.

The term "hydroxyalkyl" as used herein, means at least one hydroxy group, as defined herein, is appended to the parent molecular moiety through an alkyl group, as defined herein. Representative examples of hydroxyalkyl include, but are not limited to, hydroxymethyl, 2-hydroxyethyl, 3-hydroxypropyl, 2,3-dihydroxypentyl, and 2-ethyl-4-hydroxyheptyl.

The term "mercapto" as used herein, means a -SH group.

The term "nitro" as used herein, means a -NO_2 group.

The term "nonaromatic" as used herein, means that a 4 membered nonaromatic ring contains zero double bonds, a 5 membered nonaromatic ring contains zero or one double bond, a 6, 7, or 8 membered nonaromatic ring contains zero, one, or two double bonds.

The term "NR_AR_B" as used herein, means two groups, R_A and R_B, which are appended to the parent molecular moiety through a nitrogen atom. R_A and R_B are each independently

hydrogen, alkyl, and alkylcarbonyl. Representative examples of NR_AR_B include, but are not limited to, amino, methylamino, acetylamino, and acetylmethylamino.

The term "(NR_AR_B)carbonyl" as used herein, means a NR_AR_B group, as defined herein, appended to the parent molecular moiety through a carbonyl group, as defined herein. Representative examples of (NR_AR_B)carbonyl include, but are not limited to, aminocarbonyl, (methylamino)carbonyl, (dimethylamino)carbonyl, and (ethylmethylamino)carbonyl.

The term "NR_CR_D" as used herein, means two groups, R_C and R_D, which are appended to the parent molecular moiety through a nitrogen atom. R_C and R_D are each independently hydrogen, alkyl, and alkylcarbonyl. Representative examples of NR_CR_D include, but are not limited to, amino, methylamino, acetylamino, and acetylmethylamino.

The term "(NR_CR_D)carbonyl" as used herein, means a NR_CR_D group, as defined herein, appended to the parent molecular moiety through a carbonyl group, as defined herein. Representative examples of (NR_CR_D)carbonyl include, but are not limited to, aminocarbonyl, (methylamino)carbonyl, (dimethylamino)carbonyl, and (ethylmethylamino)carbonyl.

The term "(NR_CR_D)carbonylalkyl" as used herein, means a (NR_CR_D)carbonyl group, as defined herein, appended to the parent molecular moiety through an alkyl group, as defined herein.

The term "(NR_CR_D)sulfonyl" as used herein, means a NR_CR_D group, as defined herein, appended to the parent molecular moiety through a sulfonyl group, as defined herein. Representative examples of (NR_CR_D)sulfonyl include, but are not limited to, aminosulfonyl, (methylamino)sulfonyl, (dimethylamino)sulfonyl, and (ethylmethylamino)sulfonyl.

The term "NR_ER_F" as used herein, means two groups, R_E and R_F, which are appended to the parent molecular moiety through a nitrogen atom. R_E and R_F are each independently hydrogen, alkyl, and alkylcarbonyl. Representative examples of NR_ER_F include, but are not limited to, amino, methylamino, acetylamino, and acetylmethylamino.

The term "(NR_ER_F)carbonyl" as used herein, means a NR_ER_F group, as defined herein, appended to the parent molecular moiety through a carbonyl group, as defined herein. Representative examples of (NR_ER_F)carbonyl include, but are not limited to, aminocarbonyl, (methylamino)carbonyl, (dimethylamino)carbonyl, and (ethylmethylamino)carbonyl.

The term "oxo" as used herein, means a =O moiety.

Compounds of the present invention can exist as stereoisomers, wherein asymmetric or chiral centers are present. Stereoisomers are designated (R) or (S) depending on the

configuration of substituents around the chiral carbon atom. The terms (R) and (S) used herein are configurations as defined in IUPAC 1974 Recommendations for Section E, Fundamental Stereochemistry, Pure Appl. Chem., (1976), 45: 13-30, hereby incorporated by reference. The present invention contemplates various stereoisomers and mixtures thereof and are specifically included within the scope of this invention. Stereoisomers include enantiomers, diastereomers, and mixtures of enantiomers or diastereomers. Individual stereoisomers of compounds of the present invention may be prepared synthetically from commercially available starting materials which contain asymmetric or chiral centers or by preparation of racemic mixtures followed by resolution well-known to those of ordinary skill in the art. These methods of resolution are exemplified by (1) attachment of a mixture of enantiomers to a chiral auxiliary, separation of the resulting mixture of diastereomers by recrystallization or chromatography and liberation of the optically pure product from the auxiliary or (2) direct separation of the mixture of optical enantiomers on chiral chromatographic columns.

Compounds of the present invention were named by ACD/ChemSketch version 5.06 (developed by Advanced Chemistry Development, Inc., Toronto, ON, Canada) or were given names which appeared to be consistent with ACD nomenclature.

Determination of Biological Activity

Inhibition of PARP

Nicotinamide[2,5',8-3H]adenine dinucleotide and strepavidin SPA beads were purchased from Amersham Biosiences (UK) Recombinant Human Poly(ADP-Ribose) Polymerase (PARP) purified from E.coli and 6-Biotin-17-NAD^+, were purchase from Trevigen, Gaithersburg, MD. NAD^+, Histone, aminobenzamide, 3-amino benzamide and Calf Thymus DNA (dcDNA) were purchased from Sigma, St. Louis, MO. Stem loop oligonucleotide containing MCAT sequence was obtained from Qiagen. The oligos were dissoloved to 1mM in annealing buffer containing 10mM Tris HCl pH 7.5, 1mM EDTA, and 50mM NaCl, incubated for 5min at 95°C, and followed by annealing at 45°C for 45 minutes. Histone H1 (95% electrophoretically pure) was purchased from Roche, Indianapolis, IN. Biotinylated histone H1 was prepared by treating the protein with Sulfo-NHS-LC-Biotin from Pierce Rockford, IL. The biotinylation reaction was conducted by slowly and intermittently adding 3 equivalents of 10mM Sulfo-NHS-LC-Biotin to 100μM Histone H1 in

phosphate-buffered saline, pH 7.5, at 4°C with gentle vortexing over 1min followed by subsequent 4°C incubation for 1hr. Streptavidin coated (FlashPlate Plus) microplates were purchased from Perkin Elmer, Boston, MA.

PARP1 assay was conducted in PARP assay buffer containing 50 mM Tris pH 8.0, 1mM DTT, 4 mM $MgCl_2$. PARP reactions contained 1.5 μM [^{3}H]-NAD^+ (1.6uCi/mmol), 200 nM biotinylated histone H1, 200 nM slDNA, and 1nM PARP enzyme. Auto reactions utilizing SPA bead-based detection were carried out in 100μl volumes in white 96 well plates. Reactions were initiated by adding 50 μl of 2X NAD^+ substrate mixture to 50μl of 2X enzyme mixture containing PARP and DNA. These reactions were terminated by the addition of 150 μl of 1.5 mM benzamide (~1000-fold over its IC50). 170 μl of the stopped reaction mixtures were transferred to streptavidin Flash Plates, incubated for 1hr, and counted using a TopCount microplate scintillation counter. The K_i data was determined from inhibition curves at various substrate concentrations and are shown in Table 1 for representative compounds of the present invention and for non-quaternary compounds. The Table 1 data indicates that quaternary compounds of the present invention have a higher affinity for the PARP enzyme compared to non-quaternary compounds. Table 2 shows K_i data for compounds of the present invention, however, the corresponding non-quaternary compound was not made and thus there is only data in this table for the compounds of the present invention (the K_i values in Table 2 correspond to Examples 45-73).

Table 1

Inhibition of PARP

Compound	PARP Inhibition K_i (nM)
2-(2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide	4.3
2-[(2R)-pyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	8
2-[(2R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	5.4
2-[(2S)-pyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	28.4
2-[(2S)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	5.1

2-[(2S)-1-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	30.8
2-[(2R)-1-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	7.3
2-(1,2-dimethylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide	6.2
2-[(2S)-1-ethylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	49
2-(1-ethyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide	6
2-[(2S)-1-propylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	129
2-[(2R)-1-propylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	146
2-(2-methyl-1-propylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide	18.7
2-[(2R)-1-isopropylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	12.8
2-[(2S)-1-isopropylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	19.3
2-(1-isopropyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide	17.5
2-[(2S)-1-cyclobutylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	338
2-[(2R)-1-cyclobutylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	142
2-(1-cyclobutyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide	31.3
2-pyrrolidin-3-yl-1H-benzimidazole-4-carboxamide	3.9
2-(3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	3.9

2-(1-propylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	8.1
2-(3-methyl-1-propylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	4.2
2-[1-(cyclopropylmethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	5.2
2-[1-(cyclopropylmethyl)-3-methylpyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	5
2-(1-isobutylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	7.4
2-(1-isobutyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	3.8
2-(1-isopropylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	9.2
2-(1-isopropyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	4.4
2-(1-cyclobutylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	6.8
2-(1-cyclobutyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	4
2-(1-cyclopentylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	5.5
2-(1-cyclopentyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	3.4
2-(1-cyclohexylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	7
2-(1-cyclohexyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	5.8
2-(1-tetrahydro-2H-pyran-4-ylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide	8.2
2-(3-methyl-1-tetrahydro-2H-pyran-4-ylpyrrolidin-3-yl)-	7.2

1H-benzimidazole-4-carboxamide	
2-[1-(pyridin-4-ylmethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	14.2
2-[3-methyl-1-(pyridin-4-ylmethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	8.9
2-[1-(2-phenylethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	9.1
2-[3-methyl-1-(2-phenylethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	10.5
2-[1-(1-methyl-3-phenylpropyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	13.2
2-[3-methyl-1-(1-methyl-3-phenylpropyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide	12
2-azetidin-2-yl-1H-benzimidazole-4-carboxamide	34
2-(2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	14.1
2-(1-isopropylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	118
2-(1-isopropyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	41.6
2-(1-cyclobutylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	80
2-(1-cyclobutyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	33.3
2-(1-cyclopentylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	176
2-(1-cyclopentyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	31.1
2-(1-cyclohexylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	245
2-(1-cyclohexyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide	27.7

2-azetidin-3-yl-1H-benzimidazole-4-carboxamide	6
2-(3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	4.4
2-(1-propylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	14.1
2-(3-methyl-1-propylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	6.9
2-[1-(cyclopropylmethyl)azetidin-3-yl]-1H-benzimidazole-4-carboxamide	19
2-[1-(cyclopropylmethyl)-3-methylazetidin-3-yl]-1H-benzimidazole-4-carboxamide	8
2-(1-isobutylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	14.4
2-(1-isobutyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	5.6
2-(1-cyclobutylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	16.4
2-(1-cyclobutyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	6.1
2-(1-cyclopentylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	14
2-(1-cyclopentyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	4
2-(1-cyclohexylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	16
2-(1-cyclohexyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	5.6
2-(1-tetrahydro-2H-pyran-4-ylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	45.6
2-(3-methyl-1-tetrahydro-2H-pyran-4-ylazetidin-3-yl)-1H-benzimidazole-4-carboxamide	12.7
2-{1-[(dimethylamino)sulfonyl]azetidin-3-yl}-1H-benzimidazole-4-carboxamide	16

2-{1-[(dimethylamino)sulfonyl]-3-methylazetidin-3-yl}-1H-benzimidazole-4-carboxamide	7
2-[(2S)-piperidin-2-yl]-1H-benzimidazole-4-carboxamide	46.1
2-[(2R)-piperidin-2-yl]-1H-benzimidazole-4-carboxamide	47.4
2-[piperidin-2-yl]-1H-benzimidazole-4-carboxamide	32.2
2-(2-methylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide	4.6
2-(1-propylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide	120
2-(2-methyl-1-propylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide	18.7
2-{1-[(dimethylamino)sulfonyl]piperidin-4-yl}-1H-benzimidazole-4-carboxamide	31.1
2-{1-[(dimethylamino)sulfonyl]-4-methylpiperidin-4-yl}-1H-benzimidazole-4-carboxamide	8.8
2-(1-cyclobutylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	6.3
2-(1-cyclobutyl-4-methylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	9.2
2-(1-isopropylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	6
2-(1-isopropyl-4-methylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	8
2-(1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	8.6
2-(4-methyl-1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	13.5
2-azepan-4-yl-1H-benzimidazole-4-carboxamide	5.7
2-(4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide	3.3
2-(1-cyclopentylazepan-4-yl)-1H-benzimidazole-4-carboxamide	3.9

2-(1-cyclopentyl-4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide	7.3
2-(1-cyclohexylazepan-4-yl)-1H-benzimidazole-4-carboxamide	4.8
2-(1-cyclohexyl-4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide	11.9
2-[(2*R*)-2-methyl-5-oxopyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide	29
2-[(2*R*)-5-oxopyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide	16

Table 2

Inhibition of PARP

K_i (nM)

8.6	10.9	1.8	3.1	172	6.7	3.4	4.3
8.4	44.9	4175	14.9	26.4	24.4	11.1	8.1
5.1	422	9.2	5.5	52	24.8	2.4	4.5
4656	341	9.6	9.7				

Cellular PARP assay:

C41 cells were treated with a compound of the present invention for 30 minutes in 96 well plate. PARP was then activated by damaging DNA with 1 mM H_2O_2 for 10 minutes. The cells were then washed with ice-cold PBS once and fixed with pre-chilled methanol:acetone (7:3) at -20°C for 10 minutes. After air-drying, the plates were rehydrated with PBS and blocked 5% non-fat dry milk in PBS-tween (0.05%) (blocking solution) for 30 minutes at room temperature. The cells were incubated with anti-PAR antibody 10H (1:50) in Blocking solution at 37°C for 60 minutes followed by washing with PBS-Tween20 5 times, and incubation with goat anti-mouse fluorescein 5(6)-isothiocyanate-coupled antibody (1:50) and 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in blocking solution at 37°C for 60 minutes. After washing with PBS-Tween20 5 times, the analysis was performed using an

fmax Fluorescence Microplate Reader (Molecular Devices, Sunnyvalle, CA), set at the excitation wavelength of 490 nm and emission wavelength of 528 nm fluorescein 5(6)-isothiocyanate (FITC) or the excitation wavelength of 355 nm and emission wavelength of 460 nm (DAPI). The PARP activity (FITC signal) was normalized with cell numbers (DAPI).

The cellular assay measures the formation of poly ADP-ribose by PARP within cells and demonstrates that compounds of the present invention penetrate cell membranes and inhibit PARP in intact cells. The EC_{50s} for representative compounds of the present invention are provided in Table 3.

Table 3

Cellular Activity

EC_{50} (nM)				
5.5	9.3	6.3	2.2	26
0.8	1.1	1.3	2.2	2.4
5.0	32.6	1.0	2.3	1.9
14	12.6	29.0	137	4.8
1.6	4.3	16.1	2.8	6.1
13.3	21.0	2.0	12.5	12.7
5.2	3.2	3.5	3.5	2.8
31	3.9	7.9	590	10.9
2.7	1.2	1.5	53	8.8
5.8	6.7	9.8	15	1
2	13.5	2	13	2.4
7.4	5.2	3.2	8	13
17	1.2	2		

Deaths Following Lipopolysaccharide (LPS) challenge

Female BALB/c mice were dosed orally, twice a day with vehicle (0.2% HPMC), or drug at 30 mg/kg/day or 100 mg/kg/day. The mice were injected intravenous with 20 mg/kg LPS at 30 minutes after the first treatment dose. They were monitored for survival for 72 hours or until 80-90% lethality was observed. Table 4 provides lethality data for a

representative compound of the present invention and a non-quaternary compound, 2-(1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide.

Table 4

compound	0 (mg/kg)	30 (mg/kg)	100 (mg/kg)
2-[(2S)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	9/10	8/10	4/10*
2-(1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	8/10	8/10	9/10

*Indicates statistical significace, $p < 0.05$.

Percent Decrease in Inflammatory Cell Influx or IL-1 Levels in the Peritoneum Following Zymosan Challenge

Compounds were administered orally before an intraperitoneal zymosan injection (2 mg/animal). Four hours post zymosan injection, the peritoneal cavity was lavaged, and the lavage fluid was measured for cell influx and IL-1 levels. Table 4 provides the percent decrease in cell influx and IL-1 levels relative to control for representative compounds of the present invention and a non-quaternary compound, 2-(1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide. The data indicates the representative compounds of the present invention reduce or prevent inflammation.

Table 4

	0 mg/kg		30 mg/kg		100 mg/kg	
compound	Cell Influx	IL-1 level	Cell Influx	IL-1 level	Cell Influx	IL-1 level
2-[(2R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	0	0	38%	55%*	64%*	60%*
2-[(2S)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide	0	0	39%	54%*	66%*	65%*
2-(1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide	0	0	0	32%	23%	47%*

*Indicates statistical significace, $p < 0.05$.

As PARP inhibitors, the compounds of the present invention have numerous therapeutic applications related to, ischemia reperfusion injury, inflammatory diseases, degenerative diseases, protection from adverse effects of cytotoxic compounds, and potentiation of cytotoxic cancer therapy. In particular, compounds of the present invention potentiate radiation and chemotherapy by increasing apoptosis of cancer cells, limiting tumor growth, decreasing metastasis, and prolonging the survival of tumor-bearing mammals. Compounds of Fomula (I) can treat leukemia, colon cancer, glioblastomas, lymphomas, melanomas, carcinomas of the breast, and cervical carcinomas.

Other therapeutic applications include, but are not limited to, retroviral infection, arthritis, gout, inflammatory bowel disease, CNS inflammation, multiple sclerosis, allergic encephalitis, sepsis, septic shock, hemmorhagic shock, pulmonary fibrosis, uveitis, diabetes, Parkinsons disease, myocardial infarction, stroke, other neural trauma, organ transplantation, reperfusion of the eye, reperfusion of the kidney, reperfusion of the gut, reperfusion of skeletal muscle, liver toxicity following acetominophen overdose, cardiac and kidney toxicities from doxorubicin and platinum based antineoplastic agents, and skin damage secondary to sulfur mustards. (G. Chen et al. Cancer Chemo. Pharmacol. 22 (1988), 303; C. Thiemermann et al., Proc. Natl. Acad. Sci. USA 94 (1997), 679-683 D. Weltin et al. Int. J. Immunopharmacol. 17 (1995), 265- 271; H. Kröger et al. Inflammation 20 (1996), 203-215; W. Ehrlich et al. Rheumatol. Int. 15 (1995), 171-172; C. Szabo et al., Proc. Natl. Acad. Sci. USA 95 (1998), 3867-3872; S. Cuzzocrea et al. Eur. J. Pharmacol. 342 (1998), 67-76; V. Burkhart et al., Nature Medicine (1999), 5314-19).

When used in the above or other treatments, a therapeutically effective amount of one of the compounds of the present invention can be employed as a zwitterion or as a pharmaceutically acceptable salt. By a "therapeutically effective amount" of the compound of the invention is meant a sufficient amount of the compound to treat or prevent a disease or disorder ameliorated by a PARP inhibitor at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound

employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

By "pharmaceutically acceptable salt" is meant those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well-known in the art. The salts can be prepared in situ during the final isolation and purification of the compounds of the present invention or separately by reacting the free base of a compound of the present invention with a suitable acid. Representative acids include, but are not limited to acetatic, citric, aspartic, benzoic, benzenesulfonic, butyric, fumaric, hydrochloric, hydrobromic, hydroiodic, lactic, maleic, methanesulfonic, pamoic, pectinic, pivalic, propionic, succinic, tartaric, phosphic, glutamic, and p-toluenesulfonic. Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl and diamyl sulfates; long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides; arylalkyl halides like benzyl and phenethyl bromides and others. Water or oil-soluble or dispersible products are thereby obtained.

A compound of the present invention may be administered as a pharmaceutical composition containing a compound of the present invention in combination with one or more pharmaceutically acceptable excipients. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The compositions can be administered parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), rectally, or bucally. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions for parenteral injection comprise pharmaceutically-acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity may be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions can also contain adjuvants such as preservative, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

Compounds of the present invention may also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically-acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to a compound of the present invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. See, for example, Prescott, Ed., Methods in Cell Biology, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 *et seq*.

Total daily dose of the compositions of the invention to be administered to a human or other mammal host in single or divided doses may be in amounts, for example, from 0.0001 to 300 mg/kg body weight daily and more usually 1 to 300 mg/kg body weight. The dose, from 0.0001 to 300 mg/kg body, may be given twice a day.

Abbreviations which have been used in the descriptions of the examples that follow are: DBU for 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF for N,N-dimethylformamide; DMSO

for dimethylsulfoxide; Et_2O for diethyl ether; EtOAc for ethyl acetate; EtOH for ethanol; HPLC for high pressure liquid chromatography; LDA for lithium diisopropylamide; MeOH for methanol; psi for pounds per square inch; TFA for trifluoroacetic acid; THF for tetrahydrofuran, and TMS for trimethylsilane.

The following Examples are intended as an illustration of and not a limitation upon the scope of the invention as defined in the appended claims. The compounds of this invention can be prepared by a variety of synthetic routes.

Example 1

2-(2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

Example 1A

1-benzyl 2-methyl 2-methylpyrrolidine-1,2-dicarboxylate

A solution of 1-benzyl 2-methyl pyrrolidine-1,2-dicarboxylate (15.0 g, 57 mmol) and iodomethane (7.11 ml, 114 mmol) in THF (100 mL) was treated with $NaN(TMS)_2$ (1.0 M solution in THF, 114 mL, 114 mmol) at –75 °C under nitrogen. The temperature of the cooling bath was then slowly raised to –20 °C within 1 h and the mixture was stirred at the same temperature for another 3 h. After quenching with water, the mixture was acidified with 2 N HCl (~100 mL) and was partitioned between water (400 mL) and EtOAc (400 mL). The organic phase was washed with brine and concentrated. The residue was purified by flash column chromatography (silica gel, EtOAc/hexane) to give Example 1A (15.15 g, Yield: 96%). MS (DCI/NH_3) m/z 278 $(M+H)^+$.

Example 1B

1-[(benzyloxy)carbonyl]-2-methylpyrrolidine-2-carboxylic acid

A solution of Example 1A (15.15 g, 54.63 mmol) in a mixture of THF (100 mL) and water (50 mL) was treated with $LiOH \cdot H_2O$ (4.58 g, 109.26 mmol) in water (50 mL). Methanol was added until a transparent solution formed (60 mL). This solution was heated at 60 °C for overnight and the organic solvents were removed under vacuum. The residual aqueous solution was acidified with 2 N HCl to pH 2 and was partitioned between ethyl acetate and water. The organic phase was washed with water, dried ($MgSO_4$), filtered and

concentrated to give Example 1B as a white solid (13.72 g, 95.4% yield). MS (DCI/NH_3) m/z 264 $(M+H)^+$.

Example 1C

benzyl 2-({[2-amino-3-(aminocarbonyl)phenyl]amino}carbonyl)-2-methylpyrrolidine-1-carboxylate

A solution of Example 1B (13.7 g, 52 mmol) in a mixture of pyridine (60 mL) and DMF (60 mL) was treated with 1,1'-carbonyldiimidazole (9.27 g, 57.2 mmol) at 45 °C for 2 h. 2,3-Diamino-benzamide dihydrochloride (11.66 g, 52 mmol), which was synthesized as described in previous patent application WO0026192, was added and the mixture was stirred at rt overnight. After concentration under vacuum, the residue was partitioned between ethyl acetate and diluted sodium bicarbonate aqueous solution. The slightly yellow solid material was collected by filtration, washed with water and ethyl acetate, and dried to give Example 1C (16.26 g). Extraction of the aqueous phase with ethyl acetate followed by concentration, filtration and water-EtOAc wash, provided additional 1.03 g of Example 1C. Combined yield: 84%. MS (APCI) m/z 397 $(M+H)^+$.

Example 1D

benzyl 2-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-2-methylpyrrolidine-1-carboxylate

A suspension of Example 1C (17.28 g, 43.6 mmol) in acetic acid (180 mL) was heated under reflux for 2 h. After cooling, the solution was concentrated and the residual oil was partitioned between ethyl acetate and sodium bicarbonate aqueous solution. The organic phase was washed with water and concentrated. The residue was purified by flash column chromatography (silica gel, 3–15 % CH_3OH in 2:1 EtOAc/hexane) to provide Example 1D (16.42 g, Yield: 99%).

MS (APCI) m/z 379 $(M+H)^+$.

Example 1E

2-(2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

A solution of Example 1D (15.0 g, 40 mmol) in methanol (250 ml) was treated with 10% Pd/C (2.8 g) under 60 psi of hydrogen for overnight. Solid material was filtered off and the filtrate was concentrated. The residual solid was recrystallized in methanol to give 7.768 g of Example 1E as free base. The bis-HCl salt was prepared by dissolving the free base in

warm methanol and treating with 2 equivalents of HCl in ether (10.09 g). MS (APCI) m/z 245 $(M+H)^+$; ^1H NMR (500 MHz, D_2O): δ 1.92 (s, 3 H), 2.00-2.09 (m, 1 H), 2.21-2.29 (m, 1 H), 2.35-2.41 (m, 1 H), 2.52-2.57 (m, 1 H), 3.54-3.65 (m, 2 H), 7.31 (t, J=7.93 Hz, 1 H), 7.68 (dd, J=8.24, 0.92 Hz, 1 H), 7.72 (dd, J=7.63, 0.92 Hz, 1 H); Anal. Calcd for $C_{13}H_{16}N_4O$•2 HCl: C, 49.22; H, 5.72; N, 17.66. Found: C, 49.30; H, 5.60; N, 17.39.

Example 3

2-[(2R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide

Example 3A

benzyl (2R)-2-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-2-methylpyrrolidine-1-carboxylate

Example 1D (1.05 g, 2.8 mmol) was resolved on chiral HPLC (Chiralcel OD, 80/10/10 hexane/EtOH/MeOH). The faster eluting peak was collected and concentrated to provide Example 3A (99.4% e.e., 500 mg). MS (APCI) m/z 379 $(M+H)^+$.

Example 3B

2-[(2R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide

A solution of Example 3A (500 mg, 1.32 mmol) in methanol (10 ml) was treated with 10% Pd/C (150 mg) under hydrogen for overnight (balloon). Solid material was filtered off and the filtrate was concentrated. The residual solid was further purified by HPLC (Zorbax C-18, CH_3CN/H_2O/0.1%TFA) and was converted to bis-HCl salt to provide Example 4 as white solid (254 mg). Co-crystallization of the free base with 1 equivalent of L-tartaric acid in methanol gave a single crystal that was suitable for X-ray study. The X-ray structure with L-tartaric acid was assigned the R-configuration. MS (APCI) m/z 245 $(M+H)^+$; ^1H NMR (500 MHz, D_2O): δ 2.00 (s, 3 H), 2.10-2.19 (m, 1 H), 2.30-2.39 (m, 1 H), 2.45-2.51 (m, 1 H), 2.61-2.66 (m, 1 H), 3.64-3.73 (m, 2 H), 7.40 (t, J=7.95 Hz, 1 H), 7.77 (d, J=8.11 Hz, 1 H), 7.80 (d, J=7.49 Hz, 1 H); Anal. Calcd for $C_{13}H_{16}N_4O$•2 HCl: C, 49.22; H, 5.72; N, 17.66. Found: C, 49.10; H, 5.52; N, 17.61.

Example 4 (A-861696)

2-[(2S)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide

Example 4 was prepared as in Example 3 by chiral separation of Example 1D followed by hydrogenation. MS (APCI) m/z 245 $(M+H)^+$; ^1H NMR (500 MHz, D_2O): δ 1.99 (s, 3 H), 2.09-2.19 (m, 1 H), 2.30-2.38 (m, 1 H), 2.44-2.50 (m, 1 H), 2.61-2.66 (m, 1 H), 3.63-3.73 (m, 2 H), 7.40 (t, J=7.95 Hz, 1 H), 7.77 (dd, J=8.11, 0.94 Hz, 1 H), 7.81 (dd, J=7.80, 0.94 Hz, 1 H); Anal. Calcd for $C_{13}H_{16}N_4O$•2 HCl: C, 49.22; H, 5.72; N, 17.66. Found: C, 49.27; H, 5.60; N, 17.61.

Example 5

2-(1,2-dimethylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

A solution of the free base of Example 1E (300 mg, 1.22 mmol) in methanol (20 mL) was treated with formaldehyde (37 wt% in water, 228 μL, 3.07 mmol) at room temperature for overnight. Sodium cyanoborohydride (193 mg, 3.07 mmol) was then added and the solution was stirred at rt for 3 h. After concentration under reduced pressure, the residue was dissolved in a mixture of trifluoroacetic acid and water and was purfied by HPLC (Zorbax C-8, 0.1% TFA/CH_3CN/H_2O). The title compound as the TFA salt was converted to its HCl salt by dissolving in methanol and treating with HCl in ether (317 mg, 91%). MS (APCI) m/z 259 $(M+H)^+$; ^1H NMR (400 MHz, D_2O): δ 1.94 (s, 3 H), 2.25-2.43 (m, 2 H), 2.49-2.56 (m, 1 H), 2.61-2.68 (m, 1 H), 2.91 (br s, 3 H), 3.49-3.61 (m, 1 H), 3.79-3.99 (m, 1 H), 7.40 (t, J=7.98 Hz, 1 H), 7.76 (d, J=8.29 Hz, 1 H), 7.82 (d, J=7.67 Hz, 1 H); Anal. Calcd for $C_{14}H_{18}N_4O$•1.7 HCl: C, 52.50; H, 6.20; N, 17.49. Found: C, 52.37; H, 6.10; N, 17.42.

Example 6

2-(1-ethyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 5, substituting acetadehyde for formaldehyde. MS (APCI) m/z 273 $(M+H)^+$; ^1H NMR (400 MHz, D_2O): δ 1.26-1.36 (m, 3 H), 1.93 (br s, 3 H), 2.32-2.44 (m, 2 H), 2.45-2.56 (m, 2 H), 3.19-3.27 (m, 1 H), 3.41-3.52 (m, 1 H), 3.64-3.72 (m, 1 H), 3.98-4.09 (m, 1 H), 7.43 (t, J=7.83 Hz, 1 H), 7.80 (d, J=7.98 Hz, 1 H), 7.86 (d, J=7.36 Hz, 1 H); Anal. Calcd for $C_{15}H_{20}N_4O$•2 HCl: C, 52.18; H, 6.42; N, 16.23. Found: C, 52.47; H, 6.44; N, 16.69.

Example 7

2-(2-methyl-1-propylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 5, substituting propionaldehyde for formaldehyde. MS (APCI) m/z 287 $(M+H)^+$; ^1H NMR (400 MHz, D_2O): δ 0.94 (t, J=7.36 Hz, 3 H), 1.64-1.81 (m, 3 H), 1.94 (s, 3 H), 2.29-2.45 (m, 2 H), 2.48-2.57 (m, 2 H), 3.10-3.19 (m, 1 H), 3.47-3.62 (m, 1 H), 3.91-4.06 (m, 1 H), 7.42 (t, J=7.98 Hz, 1 H), 7.79 (d, J=7.98 Hz, 1 H), 7.85 (d, J=7.67 Hz, 1 H); Anal. Calcd for $C_{16}H_{22}N_4O$•2.5 HCl: C, 50.90; H, 6.54; N, 14.84. Found: C, 50.86; H, 6.80; N, 14.67.

Example 8

2-(1-isopropyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 5, substituting acetone for formaldehyde. MS (APCI) m/z 287 $(M+H)^+$; ^1H NMR (400 MHz, D_2O): δ 0.89 (d, J=4.91 Hz, 3 H), 1.42 (br s, 3 H), 2.01 (br s, 3 H), 2.34 (m, 2 H), 2.43-2.53 (m, 1 H), 2.55-2.65 (m, 1 H), 3.54-3.63 (m, 1 H), 3.71 (m, 1 H), 3.97-4.07 (m, 1 H), 7.43 (t, J=7.67 Hz, 1 H), 7.81 (d, J=7.98 Hz, 1 H), 7.87 (d, J=7.67 Hz, 1 H); Anal. Calcd for $C_{16}H_{22}N_4O$•2.7 HCl: C, 49.94; H, 6.47; N, 14.56. Found: C, 50.00; H, 6.30; N, 13.69.

Example 9

2-(1-cyclobutyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 5, substituting cyclobutanone for formaldehyde. MS (APCI) m/z 299 $(M+H)^+$; ^1H NMR (400 MHz, D_2O): δ 1.60-1.70 (m, 3 H), 1.78-1.84 (m, 1 H), 1.97 (br s, 3 H), 2.08-2.16 (m, 1 H), 2.24-2.38 (m, 3 H), 2.45 (ddd, J=13.50, 6.75, 6.75 Hz, 1 H), 2.85 (q, J=8.90 Hz, 1 H), 3.44-3.53 (m, 1 H), 3.69-3.85 (m, 2 H), 7.43 (t, J=7.98 Hz, 1 H), 7.79 (d, J=7.98 Hz, 1 H), 7.86 (d, J=7.67 Hz, 1 H); Anal. Calcd for $C_{17}H_{22}N_4O$•2.8 HCl: C, 50.99; H, 6.24; N, 13.99. Found: C, 51.00; H, 6.40; N, 13.52.

Example 10

2-(3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

Example 10A

1-benzyl 3-methyl 3-methylpyrrolidine-1,3-dicarboxylate

A solution of 1-benzyl 3-methyl pyrrolidine-1,3-dicarboxylate (4.0 g, 15.2 mmol) and iodomethane (2.0 ml) in THF (50 mL) was treated with $NaN(TMS)_2$ in THF (1.0 M, 32 mL, 32 mmol) at –70 °C under nitrogen. The temperature of the cooling bath was slowly raised to –20 °C within 1 h and the mixture was stirred at the same temperature for additional 2 h. After quenching with water, the mixture was partitioned between water and EtOAc. The organic phase was washed with water and concentrated. The residue was purified by flash column chromatogaphy to give Example 10A (4.1 g, 97%). MS (DCI/NH_3) m/z 278 $(M+H)^+$.

Example 10B

1-[(benzyloxy)carbonyl]-3-methylpyrrolidine-3-carboxylic acid

A solution of Example 10A (4.1 g, 14.8 mmol) in a mixture of THF (20 mL) and water (30 mL) was treated with $LiOH \cdot H_2O$ (0.93 g, 22.2 mmol) in water (10 mL). Methanol was added until a transparent solution formed (20 mL). This solution was heated at 60 °C for overnight and the organic solvents were removed under vacuum. The residual aqueous solution was acidified with 2N HCl to pH 2 and was partitioned between ethyl acetate and water. The organic phase was washed with water, dried ($MgSO_4$), filtered and concentrated to give Example 10B as a white solid (3.8 g, 97% yield). MS (DCI/NH_3) m/z 264 $(M+H)^+$.

Example 10C

benzyl 3-({[2-amino-3-(aminocarbonyl)phenyl]amino}carbonyl)-3-methylpyrrolidine-1-carboxylate

A solution of Example 10B (1.0 g, 3.8 mmol) in a mixture of pyridine (10 mL) and DMF (10 mL) was treated with 1,1'-carbonyldiimidazole (0.74 g, 4.6 mmol) at 45 °C for 2 h. 2,3-Diamino-benzamide dihydrochloride (0.9 g, 3.8 mmol) was added and the mixture was stirred at rt overnight. After concentration under vacuum, the residue was partitioned between ethyl acetate and diluted sodium bicarbonate aqueous solution. The formed slightly yellow solid material was collected by filtration, washed with water and ethyl acetate, and dried to give Example 10C (1.2 g). Yield: 80%. MS (APCI) m/z 397 $(M+H)^+$.

Example 10D

benzyl 3-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-3-methylpyrrolidine-1-carboxylate

A suspension of Example 10C (1.2 g, 3.0 mmol) in acetic acid (50 mL) was heated under reflux for 2 h. After cooling, the solution was concentrated and the residual oil was partitioned between ethyl acetate and sodium bicarbonate aqueous solution. The organic phase was washed with water and concentrated. The residue was purified by flash column chromatography to provide Example 10D (1.1 g, Yield: 99%). MS (APCI) m/z 379 $(M+H)^+$.

Example 10E

2-(3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

A solution of Example 10D (1.1 g, 2.9 mmol) in methanol (50 ml) was treated with 10% Pd/C (100 mg) under hydrogen overnight. Solid material was filtered off and the filtrate was concentrated. The residual solid was re-crystallized in methanol to give 0.5 g of Example 10E. Yield: 71%. MS (APCI) m/z 245 $(M+H)^+$; ^{1}H NMR (500 MHz, CD_3OD): δ 1.73 (s, 3 H), 2.29-2.36 (m, 1 H), 2.69-2.76 (m, 1 H), 3.40-3.48 (m, 2 H), 3.55-3.62 (m, 1 H), 4.21 (d, J=11.90 Hz, 1 H), 7.38 (t, J=7.78 Hz, 1 H), 7.73 (d, J=7.93 Hz, 1 H), 7.94 (d, J=6.71 Hz, 1 H); Anal. Calcd for $C_{13}H_{16}N_4O$•2.0 TFA: C, 45.29; H, 4.04; N, 13.20. Found: C, 45.14; H, 3.99; N, 12.55.

Example 11

2-(3-methyl-1-propylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

A solution of the free base of Example 10E (70 mg, 0.3 mmol) in methanol (5 mL) was treated with propionaldehyde (25 mg, 0.4 mmol) at room temperature overnight. Sodium triacetoxyborohydride (254 mg, 1.2 mmol) was then added and the solution was stirred at rt for 3 h. After concentration under vacuum, the residue was separated by HPLC (Zorbax C-8, 0.1% TFA/CH_3CN/H_2O) to give 55 mg of desired product. Yield: 35%. MS (APCI) m/z 287 $(M+H)^+$; ^{1}H NMR (500 MHz, CD_3OD): δ 0.89 (t, J=7.33 Hz, 2 H), 1.60 (s, 3 H), 1.63-1.77 (m, 2 H), 2.12-2.40 (m, 1 H), 2.59-2.73 (m, 1 H), 3.03-3.40 (m, 5 H), 3.69 (s, 1 H), 3.96-4.50 (m, 1 H), 7.21 (t, 1 H), 7.56 (s, 1 H), 7.76 (s, 1 H); Anal. Calcd for $C_{16}H_{22}N_4O$•2.0 TFA: C, 46.70; H, 4.70; N, 10.89. Found: C, 46.89; H, 4.68; N, 10.98.

Example 12

2-[1-(cyclopropylmethyl)-3-methylpyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11, substituting cyclopropyl acetadehyde for propionaldehyde. MS (APCI) m/z 299 $(M+H)^+$; ^{1}H

NMR (500 MHz, CD_3OD): δ 0.43-0.52 (m, 2 H), 0.77 (d, J=7.18 Hz, 2 H), 1.13-1.24 (m, 2 H), 1.76 (s, 3 H), 2.30-2.56 (m, J=21.53 Hz, 1 H), 2.77-2.89 (m, 1 H), 3.23 (s, 2 H), 3.30-3.59 (m, 1 H), 3.79-3.97 (m, 1 H), 4.15-4.73 (m, J=218.06 Hz, 1 H), 7.37 (t, J=7.96 Hz, 1 H), 7.73 (d, J=8.11 Hz, 1 H), 7.92 (d, J=7.80 Hz, 1 H); Anal. Calcd for $C_{17}H_{22}N_4O$•2 TFA: C, 47.91; H, 4.60; N, 10.64. Found: C, 47.88; H, 6.40; N, 10.23.

Example 13

2-(1-isobutyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11, substituting 2-methylpropanal for propionaldehyde. MS (APCI) m/z 301 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.08 (d, J=6.55 Hz, 6 H), 1.08-1.17 (m, 1 H), 1.78 (s, 3 H), 2.09-2.23 (m, 1 H), 2.27-2.54 (m, 1 H), 2.68-2.85 (m, 1 H), 3.12-3.24 (m, 2 H), 3.28-3.57 (m, 1 H), 3.72-3.95 (m, 1 H), 4.20-4.70 (m, 1 H), 7.34-7.40 (m, 1 H), 7.72 (d, J=8.11 Hz, 1 H), 7.93 (d, J=7.18 Hz, 1 H); Anal. Calcd for $C_{17}H_{24}N_4O$•2.5 TFA: C, 45.92; H, 4.55; N, 9.74. Found: C, 46.39; H, 4.67; N, 10.03.

Example 14

2-(1-isopropyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting acetone for propionaldehyde. MS (APCI) m/z 287 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.43 (d, J=5.93 Hz, 6 H), 1.78(s, 3 H), 2.29-2.48 (m, J=34.94 Hz, 1 H), 2.72-2.91 (m, 1 H), 3.33-3.66 (m, 3 H), 3.69-3.92 (m, 1 H), 4.17-4.57 (m, J=121.98 Hz, 1 H), 7.37 (t, J=7.80 Hz, 1 H), 7.73 (d, J=7.80 Hz, 1 H), 7.92 (d, J=7.18 Hz, 1 H); Anal. Calcd for $C_{16}H_{22}N_4O$•2.4 TFA: C, 45.03; H, 4.53; N, 10.50. Found: C, 45.49; H, 4.50; N, 10.41.

Example 15

2-(1-cyclobutyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting cyclobutanone for propionaldehyde. MS (APCI) m/z 299 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.73 (s, 3 H), 1.82-2.01 (m, 2 H), 2.18-2.33 (m, 2 H), 2.38 (s, 3 H), 2.75-2.85 (m, 1 H), 3.14-3.26 (m, J=1.53 Hz, 1 H), 3.33-3.69 (m, 1 H), 3.69-3.84 (m, 1 H), 3.92-4.01 (m, 1 H), 4.04-4.54 (m, 1 H), 7.37 (t, J=7.78 Hz, 1 H), 7.72 (d, J=7.93 Hz, 1 H),

7.93 (d, J=7.32 Hz, 1 H); Anal. Calcd for $C_{17}H_{22}N_4O$•1.7 TFA: C, 50.21; H, 4.85; N, 11.71. Found: C, 51.16; H, 4.97; N, 11.62

Example 16

2-(1-cyclopentyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11, substituting cyclopentanone for propionaldehyde. MS (APCI) m/z 313 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.73 (s, 3 H), 1.61-1.94 (m, 7 H), 2.20 (s, 2 H), 2.29-2.54 (m, J=58.59 Hz, 1 H), 2.74-2.96 (m, 1 H), 3.28-3.63 (m, 3 H), 3.67-3.95 (m, 3 H), 4.16-4.63 (m, J=160.20 Hz, 1 H), 7.37 (t, J=7.93 Hz, 1 H), 7.73 (d, J=7.93 Hz, 1 H), 7.92 (d, J=6.71 Hz, 1 H); Anal. Calcd for $C_{18}H_{24}N_4O$•1.7 TFA: C, 50.22; H, 5.12; N, 11.38. Found: C, 51.48; H, 5.12; N, 11.01.

Example 17

2-(1-cyclohexyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11, substituting cyclohexanone for propionaldehyde. MS (APCI) m/z 327 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.73 (s, 3 H), 1.25 (t, J=12.36 Hz, 1 H), 1.30-1.57 (m, 4 H), 1.68-1.81 (m, 4 H), 1.84-2.02 (m, 2 H), 2.11-2.52 (m, 3 H), 2.80 (s, 1 H), 3.21-3.48 (m, 2 H), 3.49-3.78 (m, J=8.24 Hz, 1 H), 3.72-3.89 (m, 1 H), 4.24-4.59 (m, J=113.36, 11.14 Hz, 1 H), 7.37 (t, J=7.78 Hz, 1 H), 7.73 (d, J=7.93 Hz, 1 H), 7.93 (s, 1 H); Anal. Calcd for $C_{19}H_{25}N_4O$•1.7: C, 52.28; H, 5.18; N, 11.08. Found: C, 52.08; H, 5.32; N, 11.59.

Example 18

2-(3-methyl-1-tetrahydro-2H-pyran-4-ylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting tetrahydro-4H-pyran-4-one for propionaldehyde. MS (APCI) m/z 329 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.75 (s, 3 H), 1.74-1.89 (m, 2 H), 2.04-2.21 (m, J=11.54 Hz, 2 H), 2.31-2.48 (m, 1 H), 2.75-2.93 (m, 1 H), 3.39-3.50 (m, 3 H), 3.49-3.60 (m, 2 H), 3.61-3.90 (m, 1 H), 4.06 (d, 2 H), 4.26-4.59 (m, 1 H), 7.37 (t, J=7.80 Hz, 1 H), 7.73 (d, J=7.80 Hz, 1 H), 7.93 (d, J=7.49 Hz, 1 H); Anal. Calcd for $C_{18}H_{24}N_4O2$•1.7 TFA: C, 41.13; H, 5.28; N, 11.29. Found: C, 41.58; H, 5.30; N, 11.55.

Example 19

2-[3-methyl-1-(pyridin-4-ylmethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11, substituting isonicotinaldehyde for propionaldehyde. MS (APCI) m/z 336 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.78 (s, 3 H), 2.34-2.48 (m, 1 H), 2.66-2.79 (m, 1 H), 3.34-3.49 (m, 2 H), 3.49-3.60 (m, 1 H), 4.16 (d, 1 H), 4.60 (dd, 2 H), 7.47 (t, 1 H), 7.80 (d, J=7.32 Hz, 1 H), 7.96 (d, J=7.63 Hz, 1 H), 8.12 (d, J=6.41 Hz, 2 H), 8.86 (d, J=6.41 Hz, 2 H); Anal. Calcd for $C_{19}H_{21}N_5O$•2 TFA: C, 49.03; H, 4.11; N, 12.43. Found: C, 49.54; H, 4.08; N, 11.97.

Example 20

2-[3-methyl-1-(2-phenylethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting phenylacetaldehyde for propionaldehyde. MS (APCI) m/z 349 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.75 (s, 3 H), 2.44 (s, 1 H), 2.75-2.89 (m, 1 H), 3.02-3.17 (m, 2 H), 3.50-3.64 (m, 3 H), 3.78 (s, 2 H), 4.37-4.80 (m, 1 H), 7.23-7.42 (m, 6 H), 7.72 (d, J=7.98 Hz, 1 H), 7.93 (d, J=7.67 Hz, 1 H).

Example 21

2-[3-methyl-1-(1-methyl-3-phenylpropyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting 4-phenylbutan-2-one for propionaldehyde. MS (APCI) m/z 377 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.50 (d, J=6.44 Hz, 3 H), 1.72 (d, J=2.76 Hz, 3 H), 1.83-2.00 (m, 1 H), 2.13-2.26 (m, 1 H), 2.27-2.45 (m, 1 H), 2.60-2.71 (m, 1 H), 2.73-2.91 (m, 2 H), 3.35-3.48 (m, 2 H), 3.49-3.86 (m, 2 H), 4.16-4.56 (m, 1 H), 7.15-7.33 (m, 5 H), 7.36 (t, J=7.83 Hz, 1 H), 7.72 (d, J=7.98 Hz, 1 H), 7.92 (d, J=7.06 Hz, 1 H).

Example 22

2-(2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide

Example 22A

dibenzyl azetidine-1,2-dicarboxylate

A suspension of benzyl azetidine-2-carboxylate (4.0 g, 21 mmol) and potassium carbonate (5 g, 36 mmol) in a mixture of 1,4-dioxane (25 ml) and water (30 ml) was treated with benzyl chloroformate (3 ml, 21 mmol) at room temperature for 6 hours. Piperazine (5 drops) was added and the mixture was stirred for additional 0.5 hour. The organic volatiles were removed in vacuo and the residue was partitioned between ethyl acetate and 2 N HCl solution. The organic layer was washed with brine and dried over $MgSO_4$. Removal of solvents gave Example 22A (6.8 g, Yield: 96%). MS (DCI/NH_3) m/z 278 $(M+H)^+$.

Example 22B

dibenzyl 2-methylazetidine-1,2-dicarboxylate

A solution of Example 22A (325 mg, 1 mmol) and iodomethane (0.12 ml, 2.0 mmol) in THF (5 mL) was treated with $NaN(TMS)_2$ in THF (1.0 M, 2 mL, 2.0 mmol) at -70 °C under nitrogen. The temperature of the cooling bath was slowly raised to –20 °C within 1 h and the mixture was stirred at the same temperature for additional 2 h. After quenching with water, the mixture was partitioned between water and EtOAc. The organic phase was washed with water and concentrated. The residue was purified on flash column chromatograph to give Example 22B. (250 mg, 77% yield). MS (DCI/NH_3) m/z 340 $(M+H)^+$.

Example 22C

1-[(benzyloxy)carbonyl]-2-methylazetidine-2-carboxylic acid

A solution of Example 22B (339 mg, 1.0 mmol) in a mixture of THF (5 mL) and water (3 mL) was treated with $LiOH \cdot H_2O$ (84 mg, 2.0 mmol) in water (3 mL). Methanol was added until a transparent solution formed (1 mL). This solution was heated at 60 °C overnight and the organic solvents were removed under vacuum. The residual aqueous solution was acidified with 2 N HCl to pH 2 and was partitioned between ethyl acetate and water. The organic phase was washed with water, dried ($MgSO_4$), filtered and concentrated to give Example 22C (310 mg, 88% yield). MS (DCI/NH_3) m/z 250 $(M+H)^+$.

Example 22D

benzyl 2-({[2-amino-3-(aminocarbonyl)phenyl]amino}carbonyl)-2-methylazetidine-1-carboxylate

A solution of Example 22C (1.67 g, 6.55 mmol) in a mixture of pyridine (15 mL) and DMF (15 mL) was treated with 1,1'-carbonyldiimidazole (1.27 g, 7.86 mmol) at 45 °C for 2 h. 2,3-Diamino-benzamide dihydrochloride (1.47 g, 6.55 mmol) was added and the mixture was stirred at rt overnight. After concentration under vacuum, the residue was partitioned between ethyl acetate and diluted sodium bicarbonate aqueous solution. The solid material was collected by filtration, washed with water and ethyl acetate, and dried to give Example 22D (1.88 g). Yield: 75%. MS (APCI) m/z 383 $(M+H)^+$.

Example 22E

benzyl 2-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-2-methylazetidine-1-carboxylate

A suspension of Example 22D (1.88 g, 4.9 mmol) in acetic acid (50 mL) was heated under reflux for 2 h. After cooling, the solution was concentrated and the residual oil was partitioned between ethyl acetate and sodium bicarbonate aqueous solution. The organic phase was washed with water and concentrated. The residue was purified by flash column chromatography to provide Example 22E (350 mg, Yield: 22%). MS (APCI) m/z 365 $(M+H)^+$.

Example 22F

2-(2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide

A solution of Example 22E (0.35 g, 1.0 mmol) in methanol (5 ml) was treated with 10% Pd/C (8 mg) under hydrogen overnight. The mixture was filtered and the filtrate was concentrated to provide a solid which was recrystallized from methanol to give 0.21 g of Example 22F. Yield: 93%. MS (APCI) m/z 231 $(M+H)^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.81 (s, 3 H), 2.36-2.44 (m, 2 H), 2.88-2.99 (m, 1 H), 3.00-3.12 (m, 1 H), 7.40 (t, J=7.67 Hz, 1 H), 7.77 (d, J=8.29 Hz, 1 H), 7.95 (d, J=7.67 Hz, 1 H).

Example 23

2-(1-isopropyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 22F for Example 10E and acetone for propionaldehyde. MS (APCI) m/z 305 $(M+H)^+$; ^{1}H NMR (500 MHz, CD_3OD): δ 1.30 (d, J=6.55, 6 H), 1.81 (s, 3 H), 2.30-

2.56 (m, 2 H), 2.92-3.06 (m, 1 H), 3.08-3.23 (m, 1 H), 3.33-3.50 (m, 1 H), 7.40 (t, J=7.80 Hz, 1 H), 7.77 (d, J=8.11 Hz, 1 H), 7.94 (d, 1 H).

Example 24

2-(1-cyclobutyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 22F for Example 10E and cyclobutanone for propionaldehyde. MS (APCI) m/z 317 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.79 (s, 3 H), 1.84-1.95 (m, 2 H), 2.09-2.21 (m, 2 H), 2.24-2.34 (m, 2 H), 2.35-2.46 (m, 2 H), 2.82-2.92 (m, 1 H), 2.99-3.08 (m, 1 H), 3.70-3.80 (m, 1 H), 7.40 (t, 1 H), 7.76 (d, J=6.86 Hz, 1 H), 7.94 (d, J=7.49 Hz, 1 H).

Example 25

2-(1-cyclopentyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 22F for Example 10E and cyclopentanone for propionaldehyde. MS (DCI) m/z 299 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.57-1.69 (m, 3 H), 1.75-1.82 (m, 2 H), 1.81 (s, 3 H), 2.03-2.12 (m, 2 H), 2.41-2.49 (m, 2 H), 2.95-3.01 (m, 1 H), 3.13-3.19 (m, 1 H), 3.30-3.32 (m, 1 H), 3.51-3.58 (m, 1 H), 7.42 (t, J=7.96 Hz, 1 H), 7.78 (d, J=8.11 Hz, 1 H), 7.95 (d, J=7.80 Hz, 1 H).

Example 26

2-(1-cyclohexyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 22F for Example 10E and cyclohexanone for propionaldehyde. MS (APCI) m/z 345 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.14-1.25 (m, 1 H), 1.26-1.39 (m, 4 H), 1.69 (d, J=12.79 Hz, 1 H), 1.81 (s, 3 H), 1.82-1.90 (m, 2 H), 2.05 (s, 2 H), 2.34-2.50 (m, 2 H), 2.94-3.09 (m, 2 H), 3.11-3.23 (m, 1 H), 7.41 (t, J=7.80 Hz, 1 H), 7.78 (d, J=8.11 Hz, 1 H), 7.95 (d, J=7.80 Hz, 1 H); Anal. Calcd for $C_{18}H_{24}N_4O$•2.8 TFA: C, 45.55; H, 4.32; N, 9.24. Found: C, 45.15; H, 4.82; N, 8.87.

Example 27

2-(3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

Example 27A

1-[(benzyloxy)carbonyl]azetidine-3-carboxylic acid

A suspension of azetidine-3-carboxylic acid (2.5 g, 24.75 mmol) and potassium carbonate (4.0 g) in a mixture of 1,4dioxane (25 ml) and water (50 ml) was treated with benzyl chloroformate (4.0 ml, 27.23 mmol) at room temperature for 6 hours. Piperazine (5 drops) was added and the mixture was stirred for additional 0.5 hour. The organic volatiles were removed and the residue was partitioned between ethyl acetate and 2 N HCl solution. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated to give Example 27A (4.8g, Yield: 83%). MS (DCI/NH_3) m/z 236 $(M+H)^+$.

Example 27B

1-benzyl 3-methyl azetidine-1,3-dicarboxylate

A solution of Example 27A (4.8 g, 20.3 mmol) in ether (100 ml) was treated with diazomethane (100 ml in ether, 60 mmol) at room temperature for 4 hours. Removal of the volatiles gave Example 27B (4.8g Yield: 98%). MS (DCI/NH_3) m/z 250 $(M+H)^+$.

Example 27C

1-benzyl 3-methyl 3-methylazetidine-1,3-dicarboxylate

A solution of Example 27B (250 mg, 1 mmol) and iodomethane (0.12 ml, 2.0 mmol) in THF (5 mL) was treated with $NaN(TMS)_2$ in THF (1.0 M, 2 mL, 2.0 mmol) at -70 °C under nitrogen. The temperature of the cooling bath was slowly raised to –20 °C within 1 h and the mixture was stirred at the same temperature for additional 2 h. After quenching with water, the mixture was partitioned between water and EtOAc. The organic phase was washed with water and concentrated. The residue was purified by flash chromatography to give Example 27C (220 mg, 85% yield). MS (DCI/NH_3) m/z 264 $(M+H)^+$.

Example 27D

1-[(benzyloxy)carbonyl]azetidine-3-carboxylic acid

A solution of Example 27C (2.6 g, 10.0 mmol) in a mixture of THF (20 mL) and water (10 mL) was treated with $LiOH \cdot H_2O$ (830 mg, 20.0 mmol) in water (5 mL). Methanol was added until a transparent solution formed (1 mL). This solution was heated at 60 °C

overnight and the organic solvents were removed under vacuum. The residual aqueous solution was acidified with 2 N HCl to pH 2 and was partitioned between ethyl acetate and water. The organic phase was washed with water, dried ($MgSO_4$), filtered and concentrated to give Example 27D (2.3 g, 90% yield). MS (DCI/NH_3) m/z 235 $(M+H)^+$.

Example 27E

benzyl 3-({[2-amino-3-(aminocarbonyl)phenyl]amino}carbonyl)-3-methylazetidine-1-carboxylate

A solution of Example 27D (250 mg, 1.0 mmol) in a mixture of pyridine (5 mL) and DMF (5 mL) was treated with 1,1'-carbonyldiimidazole (194 mg, 1.2 mmol) at 45 °C for 2 h. 2,3-Diamino-benzamide dihydrochloride (224 mg, 1.0 mmol) was added and the mixture was stirred at rt overnight. After concentration under vacuum, the residue was partitioned between ethyl acetate and diluted sodium bicarbonate aqueous solution. The formed slightly yellow solid material was collected by filtration, washed with water and ethyl acetate, and dried to give Example 27E (270 mg). Yield: 71%. MS (APCI) m/z 383 $(M+H)^+$.

Example 27F

benzyl 3-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-3-methylazetidine-1-carboxylate

A suspension of Example 27E (280 mg, 0.73 mmol) in acetic acid (10 mL) was heated under reflux for 2 h. After cooling, the solution was concentrated and the residual oil was partitioned between ethyl acetate and sodium bicarbonate aqueous solution. The organic phase was washed with water and concentrated. The residue was purified by flash column chromatography to provide Example 27F (250 mg, Yield: 96%). MS (APCI) m/z 365 $(M+H)^+$.

Example 27G

2-(3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

A solution of Example 27F (0.25 g, 0.7 mmol) in methanol (5 ml) was treated with 10% Pd/C (8 mg) under hydrogen overnight. The mixture was filtered and the filtrate was concentrated to provide a solid which was recrystallized from methanol to give 0.110 g of Example 27G. Yield: 69%. MS (APCI) m/z 231 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ

1.91 (br s, 3 H), 4.22 (d, J=11.54 Hz, 2 H), 4.69 (d, J=11.54 Hz, 2 H), 7.39 (t, J=7.80 Hz, 1 H), 7.74 (d, J=8.11 Hz, 1 H), 7.95 (d, J=7.49 Hz, 1 H).

Example 28

2-(3-methyl-1-propylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 27G for Example 10E. MS (APCI) m/z 273 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.02 (t, J=7.52 Hz, 3 H), 1.59-1.73 (m, 2 H), 1.92 (s, 3 H), 3.25-3.31 (m, 2 H), 4.28-4.46 (m, 2 H), 4.63-4.80 (m, 2 H), 7.39 (t, J=7.98 Hz, 1 H), 7.75 (d, J=7.06 Hz, 1 H), 7.95 (d, J=7.67 Hz, 1 H).

Example 29

2-[1-(cyclopropylmethyl)-3-methylazetidin-3-yl]-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 27G for Example 10E and cycolopropanecarbaldehyde for propionaldehyde. MS (APCI) m/z 285 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 0.40-0.48 (m, 2 H), 0.67-0.76 (m, 2 H), 1.02-1.14 (m, J=7.49 Hz, 1 H), 1.81-2.05 (m, 4 H), 3.24 (d, J=7.18 Hz, 2 H), 4.31-4.49 (m, 2 H), 4.68-4.94 (m, 1 H), 7.39 (t, 1 H), 7.75 (d, J=8.11 Hz, 1 H), 7.95 (d, J=7.49 Hz, 1 H); Anal. Calcd for $C_{16}H_{20}N_4O$•1.8 TFA: C, 49.14; H, 4.56; N, 12.06. Found: C, 48.80; H, 4.75; N, 11.83.

Example 30

2-(1-isobutyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 27G for Example 10E and 2-methylpropionaldehyde for propionaldehyde. MS (APCI) m/z 287 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.03 (d, J=6.75 Hz, 6 H), 1.92 (br s, 3 H), 1.96-2.10 (m, 1 H), 3.23 (d, J=7.36 Hz, 2 H), 4.34-4.52 (m, 2 H), 4.68-4.82 (m, 2 H), 7.39 (t, J=7.98 Hz, 1 H), 7.75 (d, J=7.98 Hz, 1 H), 7.95 (d, J=7.06 Hz, 1 H); Anal. Calcd for $C_{16}H_{22}N_4O$•2.4 TFA: C, 45.03; H, 4.53; N, 10.50. Found: C, 45.52; H, 4.72; N, 10.40.

Example 31

2-(1-cyclobutyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 27G for Example 10E and cyclobutanone for propionaldehyde. MS (APCI) m/z 285 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.83-2.01 (m, 4 H), 2.14-2.26 (m, 2 H), 2.28-2.42 (m, 2 H), 4.07-4.14 (m, 2 H), 4.30 (d, J=9.36 Hz, 2 H), 4.59-4.83 (m, 2 H), 7.38 (t, 1 H), 7.74 (d, 1 H), 7.95 (d, J=7.49 Hz, 1 H).

Example 32

2-(1-cyclopentyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 27G for Example 10E and cyclopentanone for propionaldehyde. MS (APCI) m/z 299 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.57-1.66 (m, 1 H), 1.67-1.76 (m, 2 H), 1.76-1.85 (m, 2 H), 1.86-2.00 (m, 3 H), 2.05-2.17 (m, 2 H), 3.90-3.97 (m, 1 H), 4.31-4.44 (m, 2 H), 4.63-4.79 (m, 2 H), 7.39 (t, J=7.95 Hz, 1 H), 7.75 (d, J=7.80 Hz, 1 H), 7.95 (d, J=7.80 Hz, 1 H).

Example 33

2-(1-cyclohexyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 27G for Example 10E and cyclohexanone for propionaldehyde. MS (APCI) m/z 313 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.22 (t, J=12.43 Hz, 3 H), 1.29-1.44 (m, 2 H), 1.73 (d, J=12.89 Hz, 1 H), 1.88 (br s, 3H), 1.79-2.02 (m, 2 H), 2.09 (d, J=10.74 Hz, 2 H), 3.23-3.34 (m, 1 H), 4.36 (s, 2 H), 4.71-4.84 (m, 2 H), 7.39 (t, J=7.82 Hz, 1 H), 7.75 (d, J=7.98 Hz, 1 H), 7.95 (d, J=7.67 Hz, 1 H); Anal. Calcd for $C_{18}H_{24}N_4O$•2.3 TFA: C, 44.04; H, 4.16; N, 8.56. Found: C, 44.96; H, 4.30; N, 8.56.

Example 34

2-(3-methyl-1-tetrahydro-2H-pyran-4-ylazetidin-3-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 27G for Example 10E and tetrahydro-4H-pyran-4-one for propionaldehyde. MS (APCI) m/z 315 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.46-1.61 (m, 2 H), 1.90 (br s, 3 H), 2.02 (dd, J=11.39, 2.96 Hz, 2 H), 3.42 (t, 2 H), 3.52-3.63 (m, 1 H),

4.06 (dd, J=11.70, 4.52 Hz, 2 H), 4.39 (d, J=10.61 Hz, 2 H), 4.79-4.83 (m, 2 H), 7.39 (t, 1 H), 7.75 (d, J=8.11 Hz, 1 H), 7.95 (d, J=6.55 Hz, 1 H); Anal. Calcd for $C_{17}H_{22}N_4O_2$•2.7 TFA: C, 43.43; H, 3.98; N, 9.21. Found: C, 43.05; H, 4.26; N, 8.98.

Example 35

2-{1-[(dimethylamino)sulfonyl]-3-methylazetidin-3-yl}-1H-benzimidazole-4-carboxamide

To a suspension of Example 27G (50 mg, 0.23 mmol) in methylene chloride (5 mL) was added dimethylsulfamoylchloride (50 μL, 0.46 mmol) and triethylamine (80 μL, 0.46 mmol) at room temperature. The reaction mixture was stirred overnight and the homogeneous solution was concentrated. Flash column chromatography of the residue (2-15 % CH_3OH in CH_2Cl_2) afforded Example 35 (42 mg, 54% yield). MS (APCI) m/z 338 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.89 (s, 3 H), 2.84 (s, 6 H), 4.01 (d, J=7.98 Hz, 2 H), 4.46 (d, J=7.98 Hz, 2 H), 7.50 (t, J=7.83 Hz, 1 H), 7.83 (d, J=7.36 Hz, 1 H), 7.98 (d, J=7.67 Hz, 1 H); Anal. Calcd for $C_{14}H_{19}N_5O_3S$•1.7 TFA: C, 39.46; H, 3.90; N, 13.53. Found: C, 39.79; H, 3.43; N, 14.02.

Example 36

2-(2-methylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide

Example 36A

1-benzyl 2-methyl piperidine-1,2-dicarboxylate

A solution of 1-[(benzyloxy)carbonyl]piperidine-2-carboxylic acid (5 g) and iodomethane (2.5 mL) in DMF (40 mL) was treated with potassium bicarbonate (3.8 g) and stirred at room temperature for 18 hrs. The reaction mixture was concentrated and the residual oil was partitioned between ethyl acetate and water. The organic phase was concentrated and the residue was purified by flash chromatography (silica gel, ethyl acetate/hexanes) to provide Example 36A (4.88 g, Yield: 93%). MS (DCI/NH_3) m/z 278 $(M+H)^+$.

Example 36B

1-benzyl 2-methyl 2-methylpiperidine-1,2-dicarboxylate

The title compound was prepared according to the procedure for Example 1A substituting Example 36A for 1-benzyl 2-methyl pyrrolidine-1,2-dicarboxylate. MS (DCI/NH_3) m/z 292 $(M+H)^+$.

Example 36C

1-[(benzyloxy)carbonyl]-2-methylpiperidine-2-carboxylic acid

The title compound was prepared according to the procedure for Example 1B substituting Example 36B for Example 1A. MS (DCI/NH_3) m/z 278 $(M+H)^+$

Example 36D

benzyl 2-({[2-amino-3-(aminocarbonyl)phenyl]amino}carbonyl)-2-methylpiperidine-1-carboxylate

The title compound was prepared according to the procedure for Example 1C substituting Example 36C for Example 1B. MS (DCI/NH_3) m/z 411 $(M+H)^+$.

Example 36E

benzyl 2-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-2-methylpiperidine-1-carboxylate

The title compound was prepared according to the procedure for Example 1D substituting Example 36D for Example 1C. MS (DCI/NH_3) m/z 393 $(M+H)^+$

Example 36F

2-(2-methylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 1E substituting Example 36E for Example 1D. MS (DCI/NH_3) m/z 245 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD) δ 1.53-1.63 (m, 1H), 1.83 (s, 3H), 1.84-1.90 (m, 2H), 1.91-1.99 (m, 1H), 2.14-2.26 (m, 1H), 2.45 (dd, J=14.88, 7.21 Hz, 1H), 3.37-3.51 (m, 2H), 7.44 (t, J=7.82 Hz, 1H), 7.77 (d, J=7.98 Hz, 1H), 8.01 (d, J=6.75 Hz, 1H).

Example 37

2-(2-methyl-1-propylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 36F for Example 10E. MS (DCI/NH_3) m/z 301 $(M+H)^+$; ^1H NMR (400

MHz, CD_3OD) δ 0.91 (t, J=7.36 Hz, 3H), 1.79-1.93 (m, 4H), 2.01 (s, 3H), 2.02-2.07 (m, 2H), 2.16-2.25 (m, 2H), 2.83-2.98 (m, 1H), 3.02-3.18 (m, 1H), 3.33-3.49 (m, 1H), 3.73-3.84 (m, 1H), 7.45 (t, J=7.83 Hz, 1H), 7.78 (d, J=8.29 Hz, 1H), 8.03 (d, J=7.67 Hz, 1H).

Example 38

2-{1-[(dimethylamino)sulfonyl]-4-methylpiperidin-4-yl}-1H-benzimidazole-4-carboxamide

Example 38A

1-benzyl 4-ethyl piperidine-1,4-dicarboxylate

A solution of ethyl piperidine-4-carboxylate (30 g) in 1:1 THF/water (300 mL) was treated with cesium carbonate (74.5 g) and benzyl choroformate (32.2 mL) and stirred at room temperature for 18 hrs. The reaction mixture was partitioned between ethyl acetate and water and the organics concentrated. The residue was purified by flash chromatography (silica gel, ethylacetate/hexanes) to provide Example 38A (50.87 g, Yield: 92%). MS (DCI/NH_3) m/z 292 $(M+H)^+$.

Example 38B

1-benzyl 4-ethyl 4-methylpiperidine-1,4-dicarboxylate

The title compound was prepared according to the procedure for Example 1A substituting Example 38A for 1-benzyl 2-methyl pyrrolidine-1,2-dicarboxylate (1.5 g, Yield: 41%). MS (DCI/NH_3) m/z 306 $(M+H)^+$.

Example 38C

1-[(benzyloxy)carbonyl]-4-methylpiperidine-4-carboxylic acid

The title compound was prepared according to the procedure for Example 1B substituting Example 38B for Example 1A (1.37 g, Yield: 99%). MS (DCI/NH_3) m/z 278 $(M+H)^+$

Example 38D

benzyl 4-({[2-amino-3-(aminocarbonyl)phenyl]amino}carbonyl)-4-methylpiperidine-1-carboxylate

The title compound was prepared according to the procedure for Example 1C substituting Example 38C for Example 1B. MS (DCI/NH_3) m/z 411 $(M+H)^+$.

Example 38E

benzyl 4-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-4-methylpiperidine-1-carboxylate

The title compound was prepared according to the procedure for Example 1D, substituting Example 38D for Example 1C (0.9 g, Yield: 88%). MS (DCI/NH_3) m/z 393 $(M+H)^+$.

Example 38F

2-(4-methylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 1E substituting Example 38E for Example 1D (0.6 g, 99%). MS (DCI/NH_3) m/z 259 $(M+H)^+$

Example 38G

2-{1-[(dimethylamino)sulfonyl]-4-methylpiperidin-4-yl}-1H-benzimidazole-4-carboxamide

To a solution of Example 38F (75 mg) in methylene chloride (5 mL) was added triethylamine (81 μL) and dimethylsulfamoyl chloride (38 μL) at room temperature. Methanol (1 mL) was added until a transparent solution formed. The solution was then stirred at rt for 16 h. The reaction mixture was concentrated. and the residue was purified by HPLC (Zorbax C-8, 0.1% TFA/CH_3CN/H_2O) to provide the title compound as TFA salt (52 mg, 49%). MS (DCI/NH_3) m/z 366 $(M+H)^+$; ^1H NMR (400 MHz, pyridine-d_5) δ 1.44 (s, 3H), 1.84-1.95 (m, 2H), 2.54-2.64 (m, 2H), 2.70-2.78 (m, 6H), 3.33-3.45 (m, 2H), 3.56-3.69 (m, 2H), 7.39 (t, J=7.67 Hz, 1H), 7.64 (d, J=7.67 Hz, 1H), 8.45 (s, 1H), 8.62 (d, J=7.67 Hz, 1H), 10.08 (s, 1H); Anal. Calcd for $C_{16}H_{23}N_5O_3S$•1.3 TFA: C, 43.49; H, 4.77; N, 13.63. Found: C, 43.31; H, 4.95; N, 13.42.

Example 39

2-(1-cyclobutyl-4-methylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11, substituting Example 38F for Example 10E and cyclobutanone for propionaldehyde (30 mg, Yield: 33%). MS (DCI/NH_3) m/z 313 $(M+H)^+$; ^1H NMR (400 MHz, pyridine-d_5) δ 1.47 (s,

3H), 1.49-1.55 (m, 1H), 1.59-1.73 (m, 1H), 1.99 (q, J=8.18 Hz, 2H), 2.38-2.65 (m, 4H), 2.73-2.98 (m, 4H), 3.18-3.41 (m, 3H), 7.41 (t, J=7.82 Hz, 1H), 7.69 (d, J=7.98 Hz, 1H), 8.47 (s, 1H), 8.61 (d, J=7.67 Hz, 1H), 9.89 (s, 1H); Anal. Calcd for $C_{18}H_{24}N_4O$•2.8 TFA: C, 44.87; H, 4.28; N, 8.87. Found: C, 45.04; H, 4.50; N, 9.01.

Example 40

2-(1-isopropyl-4-methylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 38F for Example 10E and acetone for propionaldehyde (43 mg, Yield: 49%). MS (DCI/NH_3) m/z 301 $(M+H)^+$; ^{1}H NMR (400 MHz, pyridine-d_5) δ 1.20 (d, J=6.44 Hz, 6H), 1.51 (s, 3H), 2.48-2.70 (m, 2H), 2.73-2.91 (m, 2H), 3.15-3.32 (m, 2H), 3.33-3.52 (m, 3H), 7.39 (t, J=7.82 Hz, 1H), 7.70 (d, J=7.98 Hz, 1H), 8.48 (s, 1H), 8.59 (d, J=7.67 Hz, 1H), 9.88 (s, 1H).

Example 41

2-(4-methyl-1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11 substituting Example 38F for Example 10E (46 mg, 53%). MS (DCI/NH_3) m/z 301 $(M+H)^+$; ^{1}H NMR (400 MHz, pyridine-d_5) δ 0.74 (t, J=7.36 Hz, 3H), 1.47 (s, 3H), 1.66-1.84 (m, 2H), 2.39-2.63 (m, 2H), 2.76-2.93 (m, 4H), 3.04-3.26 (m, 2H), 3.41-3.62 (m, 2H), 7.40 (t, J=7.67 Hz, 1H), 7.70 (d, J=7.67 Hz, 1H), 8.47 (s, 1H), 8.60 (d, J=7.06 Hz, 1H), 9.89 (s, 1H).

Example 42

2-(4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide

Example 42A

1-tert-butyl 4-ethyl 5-oxoazepane-1,4-dicarboxylate

tert-Butyl-4-oxo-1-piperidinecarboxylate (10 g, 50.19 mmol) was dissolved in Et_2O (100 ml) and cooled to –78 °C. Ethyl diazoacetate (7.3 ml, 70.26 mmol) and BF_3.EtO were sequentially added over 30 min. After stirring at the same temperature for 2 h, the reaction was quenched by careful addition of aqueous potassium bicarbonate, during which the cold bath was removed. After warming up to room temperature, the organic layer was washed

with water, dried over Na_2SO_4 and concentrated. Purification with flash chromatography provided the titled compound (13 g, Yield: 91%). MS (DCI/NH_3) m/z 286 $(M+H)^+$.

Example 42B

1-tert-butyl 4-ethyl 5-hydroxyazepane-1,4-dicarboxylate

A solution of Example 42A (7.0 g, 24.56 mmol) in MeOH (60 ml) was treated with $NaBH_4$ (933 mg, 24.56 mmol) in several portions at 0 °C. The reaction mixture was stirred for additional 2 h and was concentrated. The residue was purified by flash column chromatography (60% EtOAc in Hexane) to give the desired product (3.2g, Yield: 46%). MS (DCI/NH_3) m/z 288 $(M+H)^+$.

Example 42C

1-tert-butyl 4-ethyl 5-[(methylsulfonyl)oxy]azepane-1,4-dicarboxylate

A solution of Example 42B (250 mg, 1 mmol) and iodomethane (0.12 ml, 2.0 mmol) in THF (5 mL) was treated with $NaN(TMS)_2$ in THF (1.0 M, 2 mL, 2.0 mmol) at –78 °C under nitrogen. The temperature of the cooling bath was slowly raised to –20 °C within 1 h and the mixture was stirred at the same temperature for additional 2 h. After quenching with water, the mixture was partitioned between water and EtOAc. The organic phase was washed with water and concentrated. The residue was purified by flash column chromatography to give Example 42C (220 mg, 85% yield). MS (DCI/NH_3) m/z 264 $(M+H)^+$.

Example 42D

1-tert-butyl 4-ethyl 2,3,6,7-tetrahydro-1H-azepine-1,4-dicarboxylate

A solution of example 42C (0.77 g, 2.1 mmol) in 30 mL of benzene was treated with DBU (0.9 ml) at 60 °C for 1 hour. After cooling, the reaction mixture was concentrated and the residue was purified by flash column chromatography (30 % EtOAc in hexane) to give the title product (540 mg, 95% yield). MS (DCI/NH_3) m/z 270 $(M+H)^+$.

Example 42E

1-tert-butyl 4-ethyl azepane-1,4-dicarboxylate

A solution of example 42D (0.54 g, 2.0 mmol) in 20 ml of MeOH was treated with 10% Pd/C (50 mg) under hydrogen overnight. Solid material was filtered off and the filtrate

was concentrated. The residue was purified by flash column chromatography (20% EtOAc in hexane) to give the title product (310 mg, 55% yield). MS (DCI/NH_3) m/z 272 $(M+H)^+$.

Example 42F

1-tert-butyl 4-ethyl 4-methylazepane-1,4-dicarboxylate

A solution of Example 42E (1.7 g, 6.27 mmol) and iodomethane (0.8 ml, 12.55 mmol) in THF (15 mL) was treated with LDA (2.0 M solution in THF, 6.3 mL, 12.55 mmol) at –78 °C under nitrogen. The temperature of the cooling bath was slowly raised to –20 °C within 1 h and the mixture was stirred at the same temperature for additional 2 h. After quenching with water, the mixture was partitioned between water and EtOAc. The organic phase was washed with water and concentrated. The residue was purified by flash chromatography (20–40 % EtOAc in hexane) to give the title product (1.2 g, 67% yield). MS (DCI/NH_3) m/z 286 $(M+H)^+$.

Example 42G

ethyl 4-methylazepane-4-carboxylate

A solution of Example 42F (1.4 g, 5.6 mmol) in THF (50 mL) was treated with TFA (2.0 ml) at room temperature overnight. Removal of the volatiles provided Example 42G as TFA salt which was used in the next step without further purification. MS (DCI/NH_3) m/z 186 $(M+H)^+$.

Example 42H

1-benzyl 4-ethyl 4-methylazepane-1,4-dicarboxylate

A suspension of Example 42G (1.0 g, 5.6 mmol) and potassium carbonate (3.0 g) in a mixture of dioxan (25 ml) and water (50 ml) was treated with benzyl chloroformate (0.82 ml, 5.6 mmol) at room temperature for 6 hours. Piperazine (5 drops) was added and the mixture was stirred for additional 0.5 hour. The organic solvent was removed in vacuo and the residue was partitioned between ethyl acetate and 2 N HCl solution. The organic layer was washed with brine and concentrated to give the desired compound (1.48g, Yield: 85%). MS (DCI/NH_3) m/z 306 $(M+H)^+$.

Example 42I

1-[(benzyloxy)carbonyl]-4-methylazepane-4-carboxylic acid

A solution of Example 42H (1.6 g, 5.0 mmol) in a mixture of THF (20 mL) and water (10 mL) was treated with $LiOH \cdot H_2O$ (530 mg, 12.2 mmol) in water (5 mL). Methanol was added until a transparent solution formed (5 mL). This solution was heated at 60 °C for overnight and the organic solvents were removed under vacuum. The residual aqueous solution was acidified with 2 N HCl to pH 2 and was partitioned between ethyl acetate and water. The organic phase was washed with water, dried ($MgSO_4$), filtered and concentrated to give Example 42I (0.9 g, 62% yield). MS (DCI/NH_3) m/z 292 $(M+H)^+$.

Example 42J

benzyl 4-({[2-amino-3-(aminocarbonyl)phenyl]amino}carbonyl)-4-methylazepane-1-carboxylate

A solution of Example 42I (291 mg, 1.0 mmol) in a mixture of pyridine (5 mL) and DMF (5 mL) was treated with 1,1'-carbonyldiimidazole (194 mg, 1.2 mmol) at 45 °C for 2 h. 2,3-Diamino-benzamide dihydrochloride (224 mg, 1.0 mmol) was added and the mixture was stirred at rt overnight. After concentration under vacuum, the residue was partitioned between ethyl acetate and diluted sodium bicarbonate aqueous solution. The formed slightly yellow solid material was collected by filtration, washed with water and ethyl acetate, and dried to give Example 42J (288 mg). Yield: 68%. MS (APCI) m/z 425 $(M+H)^+$.

Example 42K

benzyl 4-[4-(aminocarbonyl)-1H-benzimidazol-2-yl]-4-methylazepane-1-carboxylate

A suspension of Example 42J (288 mg, 0.68 mmol) in acetic acid (10 mL) was heated under reflux for 2 h. After cooling, the solution was concentrated and the residue partitioned between ethyl acetate and aqueous sodium bicarbonate solution. The organic phase was washed with water and concentrated. The residue was purified by flash column chromatography to provide Example 42K (233 mg, Yield: 80%). MS (APCI) m/z 407 $(M+H)^+$.

Example 42L

2-(4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide

A solution of Example 42K (70 mg, 0.17 mmol) in methanol (5 ml) was treated with 10% Pd/C (8 mg) under hydrogen overnight. The mixture was filtered and the filtrate was concentrated. The residue was purified by HPLC (Zorbax C-18, 0.1 $TFA/CH_3CN/H_2O$) to

give the desired product (55 mg, 57% yield). MS (APCI) m/z 273 $(M+H)^+$. ^1H NMR (500 MHz, CD_3OD): δ 1.56 (s, 3 H), 1.88-1.97 (m, 1 H), 1.97-2.05 (m, 1 H), 2.05-2.13 (m, 1 H), 2.16-2.26 (m, 1 H), 2.57 (dd, J=14.97, 8.11 Hz, 1 H), 2.82 (dd, J=16.22, 6.86 Hz, 1 H), 3.22-3.29 (m, 1 H), 3.29-3.33 (m, 1 H), 3.34-3.49 (m, 2 H), 7.43 (t, J=7.80 Hz, 1 H), 7.78 (d, J=8.11 Hz, 1 H), 7.95 (d, J=6.86 Hz, 1 H);

Anal. Calcd for $C_{15}H_{20}N_4O$▪2.8 TFA: C, 42.41; H, 3.91; N, 9.89. Found: C, 41.90; H, 4.09; N, 9.41.

Example 43

2-(1-cyclopentyl-4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide

The title compound was prepared according to the procedure for Example 11, substituting Example 42L for Example 10E and cyclopantanone for propionaldehyde. MS (APCI) m/z 341$(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.50-1.53 (m, 3 H), 1.54-1.60 (m, 1 H), 1.62-1.78 (m, 4 H), 1.85 (s, 2 H), 1.90-2.07 (m, 3 H), 2.07-2.28 (m, 3 H), 2.30-2.67 (m, 1 H), 2.69-3.02 (m, 1 H), 3.11-3.28 (m, 1 H), 3.35-3.49 (m, 1 H), 3.50-3.79 (m, 2 H), 7.40 (t, 1 H), 7.74 (d, 1 H), 7.94 (d, J=6.86 Hz, 1 H).

Example 44

2-(1-Cyclohexyl-4-methyl-azepane-4-yl)-H-benzoimidazole-4-carboxylic acid amide

The title compound was prepared according to the procedure for Example 11, substituting Example 42L for Example 10E and cyclohexanone for propionaldehyde. MS (APCI) m/z 355 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.15 – 1.77 (m, 8 H), 1.55 (s, 3H), 1.87 – 2.22 (m, 8H), 2.30 – 2.82 (m, 1H), 2.46 – 2.96 (m, 1H), 3.22 – 3.50 (m, 2H), 3.38 – 3.60 (m, 1H), 7.39 (t, 1H), 7.75 (d, J=8.11 Hz, 1H), 7.94 (d, J=7.49 Hz, 1H).

Example 45

2-[1-(2-fluorobenzyl)-3-methylpyrrolidin-3-yl]-1*H*-benzimidazole-4-carboxamide

A solution of Example 10 (50 mg, 0.18 mmol) in methanol (10 mL) was treated with 2-fluorobenzaldehyde (45 mg, 0.36 mmol) at rt overnight. Sodium triacetoxyborohydride (84 mg, 0.40 mmol) was then added and the solution was stirred at rt for 3 h. After concentration, the residue was separated by HPLC (Zorbax C-18, 0.1% TFA/CH_3CN/H_2O) to provide the title compound as TFA salt (26 mg). MS (DCI) m/z 353 $(M+H)^+$; ^1H NMR (500

MHz, CD_3OD): δ 1.74 (s, 3 H); 2.43 (m, 1 H), 2.82 (m, 1 H); 3.62 (m, 2 H); 3.70 (m, 1 H); 4.44 (d, *J*=12.21 Hz, 1 H); 4.62 (s, 2 H); 7.36 (m, 3 H); 7.52 (m, 1 H); 7.63 (m, 1 H); 7.71 (d, *J*=7.32 Hz, 1 H); 7.92 (d, *J*=7.63 Hz, 1 H).

Example 46

6-chloro-2-(3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide

Example 46A

2-Amino-5-chloro-3-nitro-benzamide

A solution of 2-amino-3-nitro-benzamide (4.0 g, 22.08 mmol), which was synthesized as described in previous patent application WO0026192, in anhydrous acetonitrile (1250 mL) was treated with N-chlorosuccinimide (3.1 g, 23.18 mmol) at 60 °C for overnight. After cooling to room temperature, the formed orange crystalline material was collected by filtration, washed with acetonitrile and dried to give 2.95 g of Example 46A. The mother liquor was concentrated and the residue was recrystallized in acetonitrile (300 mL). The formed orange crystalline material was collected by filtration, washed with acetonitrile, and dried to provide Example 46A (800 mg, total yield: 79%). MS (DCI/NH_3) m/z 216 $(M+H)^+$.

Example 46B

2,3-Diamino-5-chloro-benzamide dihydrochloride

A solution of Example 46A (650 mg, 3.0 mmol) in a mixture of THF (100 mL) and ethanol (100 mL) was treated with Raney nickel (50% in water, 300 mg) under hydrogen at room temperature for 3 hours. Solid material was filtered off. The filtrate was treated with HCl in ether (1.0 M, 6 mL) and concentrated to give Example 46B (780 mg, 100%). MS (DCI/NH_3) m/z 186 $(M+H)^+$.

Example 46C

6-chloro-2-(3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide

A solution of Example 10B (500 mg, 1.9 mmol) in methylene chloride (10 ml) was treated with oxalyl chloride (0.17 ml, 1.9 mmol) and 2 drops of DMF at rt for 1 hour. The volatiles were removed and the residue was dissolved in methylene chloride (20 ml). This acyl chloride solution was then added into a solution of Example 46B (353 mg, 1.9 mmol)

and triethylamine (1 ml) in THF (10 mL). The reaction mixture was stirred at rt overnight and concentrated. The residue was treated with 10 ml of acetic acid at 80 °C overnight. After concentration, the residue was separated by flash chromatography (silica gel, EtOAc) to give Example 46C (690 mg, 88%).

Example 46D

6-chloro-2-(3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide

A solution of Example 46C (690 mg) in 20 ml of TFA was heated under reflux for 6 hours. After cooling, the volatiles were removed and the residue was purfied by HPLC (Zorbax C-18, 0.1% TFA/CH_3CN/H_2O) to provide Example 46D as TFA salt (340 mg). The HCl salt was prepared by dissolving the TFA salt in a mixture of methylene chloride and methanol and treating with 1M HCl solution in ether. Removal of the volatiles provided the title compound as HCl salt. MS (DCI) m/z 279 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.72 (s, 3 H); 2.35 (m, 1 H); 2.73 (m, 1 H); 3.35 (d, *J*=11.66 Hz, 1 H); 3.48 (m, 1 H); 3.61 (m, 1 H); 4.21 (d, *J*=11.66 Hz, 1 H); 7.66 (d, *J*=2.15 Hz, 1 H); 7.80 (d, *J*=1.84 Hz, 1 H); Anal. Calcd for $C_{13}H_{15}ClN_4O$•2.0 TFA: C, 40.29; H, 3.38; N, 11.06. Found: C, 40.72; H, 3.28; N, 11.10.

Example 47

6-chloro-2-(1,3-dimethylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide

A solution of Example 46D as HCl salt (80 mg, 0.22 mmol) in methanol (5 mL) was treated with triethylamine (92 μL, 0.66 mmol) and formaldehyde (37 wt% in water, 80 μL, 1.08 mmol) at room temperature for 1 hour. Sodium cyanoborohydride (67 mg, 1.08 mmol) was then added and the solution was heated at 50 °C for 5 hours. After cooling, the reaction mixture was concentrated and the residue was separated by HPLC (Zorbax, C-18, 250x2.54 column, Mobile phase A: 0.1% TFA in H_2O; B: 0.1% TFA in CH_3CN; 0-100% gradient) to provide Example 47 as TFA salt. This material was dissolved in 3 mL of 1:1 mixture of methylene chloride and methanol and treated with HCl in ether (1.0 M, 10 mL). Removal of the volatiles afforded Example 47 as HCl salt (70 mg, 83%). MS (APCI) m/z 293 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.82 (s, 3 H), 2.50 (m, 0.5 H), 2.60 (m, 0.5 H), 2.83 - 2.98 (m, 1.5 H), 3.07 (s, 1.5 H), 3.31 (s, 1.5 H), 3.40 - 3.52 (m, 2 H), 3.85 - 4.10 (m, 2 H), 4.51 (d, *J*=12.21 Hz, 0.5 H), 7.88 (s, 0.5 H), 7.92 (s, 0.5 H), 7.99 (s, 0.5 H), 8.03 (s, 0.5 H); Anal.

Calcd for $C_{14}H_{17}ClN_4O$•2.5 HCl: C, 43.80; H, 5.12; N, 14.59. Found: C, 43.73; H, 5.44; N, 14.27.

Example 48

6-chloro-2-(1-isopropyl-3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting acetone for formaldehyde. Yield: 50%. MS (APCI) m/z 321 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.38 - 1.47 (m, 6 H), 1.73 (s, 3 H), 2.26 - 2.33 (m, 0.5 H), 2.38 - 2.43 (m, 0.5 H), 2.73 - 2.84 (m, 1 H), 3.34 - 3.40 (m, 1 H), 3.52 - 3.62 (m, 2 H), 3.71 - 3.82 (m, 2 H), 4.25 (d, *J*=12.21 Hz, 0.5 H), 4.47 (d, *J*=11.60 Hz, 0.5 H), 7.70 (s, 1 H), 7.86 (s, 0.5 H), 7.88 (s, 0.5 H); Anal. Calcd for $C_{16}H_{21}ClN_4O$•2.15 TFA: C, 43.08; H, 4.12; N, 9.90. Found: C, 43.04; H, 4.13; N, 9.82.

Example 49

2-(2-methylpyrrolidin-2-yl)-6-(trifluoromethyl)-1*H*-benzimidazole-4-carboxamide

Example 49A

2-(4-Bromo-6-trifluoromethyl-1*H*-benzoimidazol-2-yl)-2-methyl-pyrrolidine-1-carboxylic acid benzyl ester

A solution of Example 1B (1.0 g, 3.8 mmol) in a mixture of pyridine (15 mL) and DMF (15 mL) was treated with 1,1'-carbonyldiimidazole (739 mg, 4.6 mmol) at 40 °C for 30 minutes. 2,3-Diamino-1-bromo-5-trifluoromethylbenzene (969 mg, 3.8 mmol) was added and the mixture was stirred at rt overnight. After concentration under vacuum, the residue was suspended in 20 ml of acetic acid. This mixture was heated at 80 °C overnight. After cooling, the acetic acid was removed by rotavapor and the residue was separated by flash chromatography (silica gel, EtOAc) to give Example 49A (500 mg, 30%). MS (DCI/NH_3) m/z 483 $(M+H)^+$.

Example 49B

2-(4-Cyano-6-trifluoromethyl-1*H*-benzoimidazol-2-yl)-2-methyl-pyrrolidine-1-carboxylic acid benzyl ester

A suspension of Example 49A (482 mg, 1.0 mmol), zinc cyanide (293 mg, 1.2 mmol)

and tetrakis(triphenylphosphine)palladium (0) (231 mg, 0.2 mmol) in anhydrous DMF (15 ml) was heated under nitrogen at 90 °C overnight. After cooling, the reaction mixture was partitioned between ethyl acetate and brine. The organic phase was washed with brine, water and concentrated. The residue was separated by flash chromatography (silica gel, Ethyl acetate) to provide Example 49B (320 mg, 75%). MS (DCI/NH_3) m/z 429 $(M+H)^+$.

Example 49C

2-(2-methylpyrrolidin-2-yl)-6-(trifluoromethyl)-1*H*-benzimidazole-4-carboxamide

A solution of Example 49B (50 mg, 0.12 mmol) in 38% HBr in acetic acid (10 ml) was aged at room temperature overnight. The volatiles were removed and the residue was separated by HPLC (Zorbax, C-18, 250x2.54 column, Mobile phase A: 0.1% TFA in H_2O; B: 0.1% TFA in CH_3CN; 0-100% gradient) to provide Example 49C as TFA salt (24 mg). MS (DCI): m/z 313 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.97 (s, 3 H); 2.12 (m, 1 H); 2.33 (m, 1 H); 2.43 (m, 1 H); 2.63 (m, 1 H); 3.65 (m, 2 H); 8.06 (s, 1 H); 8.24 (s, 1 H); Anal. Calcd for $C_{14}H_{15}F_3N_4O$•1.8 TFA: C, 40.85; H, 3.27; N, 10.83. Found: C, 40.76; H, 3.33; N, 10.99.

Example 50

2-(1,2-dimethylpyrrolidin-2-yl)-6-(trifluoromethyl)-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 49C for Example 46D. MS (DCI) m/z 327 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD) δ 1.97(s, 3 H); 2.36 (m, 2 H); 2.58 (m, 2 H) 2.99 (s, 3 H); 3.58 (m, 1 H); 3.90(m, 1 H); 8.08 (s, 1 H); 8.25 (s, 1 H); Anal. Calcd for $C_{15}H_{17}F_3N_4O$•1.8 TFA: C, 42.03; H, 3.56; N, 10.54. Found: C, 41.87; H, 3.44; N, 10.54.

Example 51

6-fluoro-2-(2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carboxamide

Example 51A

2-Bromo-4-fluoro-6-nitro-phenylamine

To a solution of 4-fluoro-2-nitroaniline (40.0 g, 0.256 mol) in a mixture of dichloromethane (900 mL) and acetic acid (300 mL) was added bromine (39.4 mL, 0.768

mol) at 0 °C. The reaction mixture was stirred at this temperature for 1 h and at room temperature for 18 h. After concentration, the residue was partitioned between ethyl acetate and aqueous sodium bicarbonate solution. The organic phase was washed with sodium bisulphite solution (300 ml), water and concentrated. The residual solid was recrystallized from hexanes/dichloromethane (10:1) to provide Example 51A (48 g, 79%). MS (DCI/NH_3) m/z 236 $(M+H)^+$.

Example 51B

2-Amino-5-fluoro-3-nitro-benzonitrile

A suspension of Example 51A (35.0 g, 0.15 mol), zinc cyanide (34.98 g, 0.3 mol) and tetrakis(triphenyphosphine)palladium (0) (12.05 g, 10 mmol) in anhydrous DMF (420 mL) was heated under nitrogen at 95 °C for 22 h. After cooling, insoluble material was filtered off and the filtrate was partitioned between ethyl acetate and brine. The organic phase was washed with water and concentrated. The residual solid was recrystallized from methanol to provide Example 51B (24 g, 89%). MS (DCI/NH_3) m/z 182 $(M+H)^+$.

Example 51C

2,3-Diamino-5-fluoro-benzonitrile

A solution of Example 51B (1.4 g, 7.72 mmol) in a mixture of tetrahydrofuran (60 mL) and ethanol (60 mL) was treated with Raney nickel (50% in water, 0.8 g) under hydrogen for 4 hours. The solid material was filtered off and the filtrate was concentrated to provide Example 51C (1.17 g, 100%). MS (DCI/NH_3) m/z 152 $(M+H)^+$.

Example 51D

2-(4-Cyano-6-fluoro-1H-benzoimidazol-2-yl)-2-methyl-pyrrolidine-1-carboxylic acid benzyl ester

A solution of Example 1B (574 mg, 2.18 mmol) in methylene chloride (8 mL) was treated with oxalyl chloride (285 μL, 3.27 mmol) and one drop of DMF at room temperature for 1 hour. After concentration, the residue was dissolved in methylene chloride (8 mL) and the solution was added to a solution of Example 51C (329 mg, 2.18 mmol) and triethylamine (364 μL, 2.62 mmol) in THF (8 mL). This reaction mixture was stirred at room temperature overnight before it was concentrated. The residue was dissolved in 15 mL of acetic acid and

this solution was heated at 100 °C for 1 hour. After cooling, the acetic acid was removed by rotavapor and the residue was partitioned between ethyl acetate and aqueous sodium bicarbonate solution. The organic layer was washed with sodium bicarbonate solution, water and concentrated. The residue was purified by flash chromatography (silica gel, 20 – 70% gradient EtOAc in hexane) to give Example 51D (679 mg, 82%). MS (DCI/NH_3) m/z 379 $(M+H)^+$.

Example 51E

6-fluoro-2-(2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carboxamide

A solution of Example 51D (460 mg, 1.21 mmol) in acetic acid (3 mL) was treated with 30% HBr/acetic acid (6 mL) at room temperature for 2 h. Water was added and the mixture was extracted with hexanes (2 x 50 mL). The clear aqueous solution was concentrated and the residue was purified by HPLC (Zorbax, C-18, 250x2.54 column, Mobile phase A: 0.1% TFA in water; B: 0.1% TFA in Acetonitrile, 0-100% gradient) to provide Example 51 as TFA salt. This product was dissolved in a mixture of methylene chloride and methanol and treated with 1M HCl solution in ether. Removal of the volatiles provided Example 51E as HCl salt (327 mg, 75%). MS (DCI/NH_3) m/z 263 $(M+H)^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.98 (s, 3 H), 2.09 - 2.19 (m, 1 H), 2.29 - 2.38 (m, 1 H), 2.42 - 2.48 (m, 1 H), 2.55 - 2.64 (m, 1 H), 3.61 - 3.74 (m, 2 H), 7.33 (dd, *J*= 8.24, 2.44 Hz, 1H), 7.37 (dd, *J*=8.24, 2.45 Hz, 1 H); Anal. Calcd for $C_{13}H_{15}FN_4O$•2.6 HCl: C, 43.73; H, 4.97; N, 15.69. Found: C, 43.68; H, 5.30; N, 15.81.

Example 52

6-chloro-2-(2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carboxamide

The title compound as HCl salt was prepared according to the procedures for Examples 46C and 46D, substituting Example 1B for Example 10B used in Example 46C. MS (APCI/NH_3) m/z 277 $(M+H)^-$; ^{1}H NMR (500 MHz, CD_3OD): δ 1.94 (s, 3 H), 2.05 - 2.13 (m, 1 H), 2.26 - 2.34 (m, 1 H), 2.36 - 2.43 (m, 1 H), 2.54 - 2.60 (m, 1 H), 3.55 - 3.62 (m, 1 H), 3.62 - 3.69 (m, 1 H), 7.77 (d, *J*=1.83 Hz, 1 H), 7.94 (d, *J*=2.14 Hz, 1 H), Anal. Calcd for $C_{13}H_{15}ClN_4O$•2.55 HCl: C, 42.21; H, 4.77; N, 15.15. Found: C, 42.65; H, 5.48; N, 14.51.

Example 53

6-chloro-2-[(2*R*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedures for Examples 46C and 46D, substituting (R)-2-methyl-pyrrolidine-1,2-dicarboxylic acid 1-benzyl ester (prepared according to the procedure as described in Overberger, C. G.; Jon, Y. S. *J. Polymer Science* **1977**, *15*, 1413 – 1421) for Example 10B used in Example 46C. MS (DCI) m/z 279 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.95 (s, 3 H); 2.10 (m, 1 H); 2.28 (m, 1 H); 2.40 (m, 1 H); 2.60 (m, 1 H); 3.65 (m, 2 H); 7.73 (s, 1 H); 7.88 (s, 1 H); Anal. Calcd for $C_{13}H_{15}ClN_4O$•1.5 TFA: C, 42.73; H, 3.59; N, 12.45. Found: C, 42.94; H, 3.69; N, 12.60.

Example 54

6-chloro-2-[(2*S*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedures for Examples 46C and 46D, substituting (S)-2-methyl-pyrrolidine-1,2-dicarboxylic acid 1-benzyl ester (prepared according to the procedure as described in Overberger, C. G.; Jon, Y. S. *J. Polymer Science* **1977**, *15*, 1413 – 1421) for Example 10B used in Example 46C. MS (DCI) m/z 279 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.94 (s, 3 H); 2.10 (m, 1 H); 2.30 (m, 1 H); 2.42 (m, 1 H); 2.58 (m, 1 H); 3.65 (m, 2 H); 7.75 (s, 1 H); 7.90 (s, 1 H); Anal. Calcd for $C_{13}H_{15}ClN_4O$•1.6 TFA: C, 43.70; H, 3.67; N, 12.78. Found: C, 43.82; H, 3.78; N, 12.98.

Example 55

6-fluoro-2-[(2*S*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedures for Examples 51D and 51E, substituting (S)-2-methyl-pyrrolidine-1,2-dicarboxylic acid 1-benzyl ester (prepared according to the procedure as described in Overberger, C. G.; Jon, Y. S. *J. Polymer Science* **1977**, *15*, 1413 – 1421) for Example 1B used in Example 51D. MS (DCI/NH_3) m/z 263 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.93 (s, 3 H), 2.03 - 2.15 (m, 1 H), 2.25 - 2.32 (m, 1 H), 2.35 - 2.42 (m, 1 H), 2.53 - 2.62 (m, 1 H), 3.54 - 3.60 (m, 1 H), 3.62 - 3.69 (m, 1 H), 7.49 (dd, *J*=8.29, 2.46 Hz, 1 H), 7.72 (dd, *J*=10.59, 2.30 Hz, 1 H); Anal. Calcd for $C_{13}H_{15}FN_4O$•1.5 TFA: C, 44.35; H, 3.72; N, 12.92. Found: C, 44.93; H, 3.78; N, 13.21.

Example 56

6-fluoro-2-[(2*R*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

Example 56A

(R)-2-Methyl-pyrrolidine-1,2-dicarboxylic acid 1-benzyl ester

A solution of Example 74D (20 g) in dichloromethane (150 mL) was treated with TFA (80 mL) at 0 °C. The ice-bath was removed and the mixture was stirred at ambient temperature for 3 h. Acetonitrile was added and the reaction mixture was concentrated. The residue was dissolved in a mixture of tetrahydrofuran (150 mL) and water (150 mL). Cs_2CO_3 (170.5 g) and benzyl chloroformate (14.7 mL) was then added. The reaction mixture was stirred at ambient temperature for 16 hours and was concentrated. The residue was diluted with 0.5 N NaOH solution, and was extracted with 20% Ether in hexanes. The aqueous layer was acidified with 2N HCl solution to a pH 3 and the mixture was extracted with ethyl acetate. The combined organic phases were concentrated and the residue purified by flash chromatography (silica gel, 5%-90% gradient EtOAc in hexanes) to provide the title compound (22.7 g, 99%). MS (DCI/NH_3) m/z 264 $(M+H)^+$.

Example 56B

6-fluoro-2-[(2*R*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as HCl salt was prepared according to the procedures for Examples 51D and 51E, substituting Example 56A for Example 1B used in Example 51D. MS (DCI/NH_3) m/z 263 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.96 (s, 3H), 2.05 - 2.14 (m, 1H), 2.26 - 2.36 (m, 1H), 2.38 - 2.47 (m, 1H), 2.56 - 2.65 (m, 1H), 3.57 - 3.63 (m, 1H), 3.64 - 3.70 (m, 1H), 7.52 (dd, *J*=8.24, 2.44 Hz, 1H), 7.72 (dd, *J*=10.37, 2.44 Hz, 1H); Anal. Calcd for $C_{13}H_{15}FN_4O \cdot 2.5\ HCl \cdot 0.25\ H_2O$: C, 43.62; H, 5.07; N, 15.65. Found: C, 43.85; H, 5.47; N, 15.43.

Example 57

6-chloro-2-[(2*R*)-1,2-dimethylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 53 for Example 46D. MS (DCI) m/z 293 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.93 (s, 3 H); 2.23 (m, 1 H); 2.33 (m, 1 H); 2.54 (m, 2 H); 3.00 (s, 3 H); 3.54 (m, 1 H); 3.98 (m, 1 H); 7.77 (s, 1 H); 7.93 (s, 1 H); Anal. Calcd for $C_{14}H_{17}ClN_4O \cdot 1.4$

TFA: C, 44.56; H, 4.10; N, 12.38. Found: C, 44.46; H, 4.20; N, 12.59

Example 58

6-chloro-2-[(2*R*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 53 for Example 46D and acetone for formaldehyde. MS (DCI) m/z 321 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.09 (br s, 3 H); 1.45 (br s, 3 H); 2.02 (s, 3 H); 2.36 (m, 2 H); 2.54 (m, 2 H); 3.62 (m, 1 H); 3.81 (m, 1 H); 3.98 (m, 1 H); 7.77 (s, 1 H); 7.93 (s, 1 H); Anal. Calcd for $C_{16}H_{21}ClN_4O$•1.7 TFA: C, 45.23; H, 4.41; N, 10.88. Found: C, 45.55; H, 4.32; N, 11.00

Example 59

6-chloro-2-[(2*R*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 53 for Example 46D and cyclopentanone for formaldehyde. MS (DCI) m/z 347 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.12 (m, 1 H); 1.59 (m, 3 H); 1.75 (m, 1 H); 1.77 (m, 1 H); 2.03 (s, 3 H); 2.16 (m, 1 H); 2.36 (m, 2 H); 2.49 (m, 2 H); 2.70 (m, 1 H); 3.63 (m, 1 H); 3.81 (m, 1 H); 3.98 (m, 1 H); 7.78 (s, 1 H); 7.94 (s, 1 H); Anal. Calcd for $C_{18}H_{23}ClN_4O$•1.8 TFA: C, 42.76; H, 3.50; N, 11.87. Found: C, 42.65; H, 3.33; N, 11.78.

Example 60

6-chloro-2-[(2*S*)-1,2-dimethylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 54 for Example 46D. MS (DCI) m/z 293 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.95 (s, 3 H); 2.27 (m, 2 H); 2.54 (m, 2 H); 2.99 (s, 3 H); 3.57 (m, 1 H); 3.90 (m, 1 H); 7.75 (d, *J*=1.84 Hz, 1 H), 7.90 (d, *J*=1.84 Hz, 1 H); Anal. Calcd for $C_{14}H_{17}ClN_4O$•1.4 TFA: C, 44.56; H, 4.07; N, 12.38. Found: C, 44.66; H, 4.10; N, 12.66.

Example 61

6-chloro-2-[(2*S*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 54 for Example 46D and acetone for formaldehyde. MS (DCI) m/z

321 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.08 (br s, 3 H); 1.44 (br s, 3 H); 2.02 (s, 3 H); 2.34 (m, 2 H); 2.54 (m, 2 H); 3.63 (m, 1 H); 3.81 (m, 1 H); 3.97 (m, 1 H); 7.78 (s, 1 H); 7.94 (s, 1 H); Anal. Calcd for $C_{16}H_{21}ClN_4O$•1.7 TFA: C, 45.23; H, 4.41; N, 10.88. Found: C, 45.51; H, 4.30; N, 11.01.

Example 62

6-chloro-2-[(2*S*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 54 for Example 46D and cyclopentanone for formaldehyde. MS (DCI) m/z 347 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.14 (m, 1 H); 1.59 (m, 3 H); 1.74 (m, 1 H); 1.88 (m, 1 H); 2.03 (s, 3 H); 2.16 (m, 1 H); 2.35 (m, 2 H); 2.50 (m, 2 H); 2.68 (m, 1 H); 3.64(m, 1 H); 3.80 (m, 1 H); 3.98 (m, 1 H); 7.78 (s, 1 H); 7.92 (s, 1 H); Anal. Calcd for $C_{18}H_{23}ClN_4O$•1.9 TFA: C, 46.43; H, 4.42; N, 9.94. Found: C, 46.19; H, 4.39; N, 10.33.

Example 63

2-[(2*S*)-1,2-dimethylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 55 for Example 46D. MS (APCI) m/z 277 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.93 (s, 3 H); 2.35 (m, 2 H); 2.53 (m, 2 H); 2.98 (s, 3 H); 3.55 (m, 1 H); 3.88 (m, 1 H); 7.49 (d, *J*=8.00 Hz, 1 H) 7.71 (d, *J*=8.00 Hz, 1 H).

Example 64

6-fluoro-2-[(2*S*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 55 for Example 46D and acetone for formaldehyde. MS (DCI) m/z 305 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.09 (br s, 3 H); 1.44 (br s, 3 H); 2.01 (s, 3 H); 2.35 (m, 2 H); 2.48 (m, 2 H); 3.62 (m, 1 H); 3.81 (m, 1 H); 3.97 (m, 1 H); 7.51 (dd, *J*=7.98, 2.45 Hz, 1 H); 7.75 (dd, *J*=10.43, 2.46 Hz, 1 H).

Example 65

2-[(2*S*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example

47, substituting Example 55 for Example 46D and cyclopentanone for formaldehyde. MS (DCI) m/z 331 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD) δ 1.13 (m, 1 H); 1.60 (m, 3 H); 1.80 (m, 2 H); 2.01 (s, 3 H); 2.15 (m, 1 H); 2.33 (m, 2 H); 2.47 (m, 2 H); 2.68 (m, 1 H); 3.62 (m, 1 H); 3.79 (m, 1 H); 3.97 (m, 1 H); 7.51 (dd, *J*=7.98, 2.45 Hz, 1 H); 7.74 (dd, *J*=10.43, 2.45 Hz, 1 H).

Example 66

2-[(2*R*)-1,2-dimethylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 56B for Example 46D. MS (DCI) m/z 277 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.96 (s, 3 H); 2.33 (m, 2 H); 2.53 (m, 2 H); 3.00 (s, 3 H); 3.59 (m, 1 H); 3.90 (m, 1 H); 7.51 (dd, *J*=7.98, 2.45 Hz, 1 H); 7.74 (dd, *J*=10.43, 2.45 Hz, 1 H).

Example 67

6-fluoro-2-[(2*R*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 56B for Example 46D and acetone for formaldehyde. MS (DCI) m/z 305 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.07 (br s, 3 H); 1.43 (br s, 3 H); 2.00 (s, 3 H); 2.35 (m, 2 H); 2.49 (m, 2 H); 3.61 (m, 1 H); 3.79 (m, 1 H); 3.94 (m, 1 H); 7.50 (dd, *J*=7.98, 2.15 Hz, 1 H); 7.73 (dd, *J*=10.43, 2.45 Hz, 1 H).

Example 68

2-[(2*R*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide

The title compound as TFA salt was prepared according to the procedure for Example 47, substituting Example 56B for Example 46D and cyclopentanone for formaldehyde. MS (DCI) m/z 331 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD) δ 1.14 (m, 1 H); 1.58 (m, 3 H); 1.83 (m, 2 H); 2.03 (s, 3 H); 2.15 (m, 1 H); 2.35 (m, 2 H); 2.50 (m, 2 H); 2.70 (m, 1 H); 3.64 (m, 1 H); 3.79 (m, 1 H); 3.98 (m, 1 H); 7.51 (dd, *J*=7.98, 2.45 Hz, 1 H); 7.73 (dd, *J*=10.43, 2.45 Hz, 1 H).

Example 69

2-[(2*R*)-1-ethyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

A solution of Example 3B as bis-HCl salt (50 mg, 0.15 mmol) in methanol (3 mL) was treated with triethylamine (63 μL, 0.45 mmol) and acetaldehyde (32 wt% in water, 80 μL, 0.75 mmol) at room temperature for 1 hour. Sodium cyanoborohydride (47 mg, 0.75 mmol) was then added and the solution was stirred at room temperature overnight and at 50 °C for 5 hours. After cooling, the reaction mixture was concentrated and the residue was separated by HPLC (Zorbax, C-18, 250x2.54 column, Mobile phase A: 0.1% TFA in H_2O; B: 0.1% TFA in CH_3CN; 0-100% gradient) to provide Example 69 as TFA salt. This material was dissolved in 3 mL of 1:1 mixture of methylene chloride and methanol and treated with HCl in ether (1.0 M, 10 mL). Removal of the volatiles afforded Example 69 as HCl salt (57 mg, 96%). MS (APCI/NH_3) m/z 273 $(M+H)^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.42 (t, *J*=6.90 Hz, 3 H), 1.97 (s, 3 H), 2.39 (m, 2 H), 2.55 (m, 2 H), 3.22 - 3.33 (m, 1 H), 3.55 (m, 2 H), 4.05 (m, 1 H), 7.48 (t, *J*= 7.98 Hz, 1 H), 7.84 (d, *J*=7.98 Hz, 1 H), 8.03 (d, *J*=7.67 Hz, 1 H); Anal. Calcd for $C_{15}H_{20}N_4O$•2.9 HCl: C, 47.65; H, 6.10; N, 14.82. Found: C, 47.72; H, 6.58; N, 14.42.

Example 70

2-[(2*S*)-1-ethyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as HCl salt was prepared according to the procedure for Example 69, substituting Example 4 for Example 3B. Yield: 85%. MS (DCI/NH_3) m/z 273 $(M+H)^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.42 (t, *J*=6.90 Hz, 3 H), 1.94 (s, 3 H), 2.40 (m, 2 H), 2.53 (m, 2 H), 3.23 (m, 1 H), 3.52 (m, 2 H), 4.06 (m, 1 H), 7.46 (t, *J*=7.83 Hz, 1 H), 7.82 (d, *J*=7.98 Hz, 1 H), 8.02 (d, *J*=7.67 Hz, 1 H); Anal. Calcd for $C_{15}H_{20}N_4O$•2.75 HCl: C, 48.35; H, 6.15; N, 15.04. Found: C, 48.45; H, 6.76; N, 14.58.

Example 71

6-chloro-2-(1-ethyl-3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide

The title compound as HCl salt was prepared according to the procedure for Example 69, substituting Example 46D for Example 3B. Yield: 95%. MS (DCI/NH_3) m/z 307 $(M+H)^+$; ^{1}H NMR (500 MHz, CD_3OD): δ 1.38 - 1.45 (m, 3 H), 1.82 (s, 3 H), 2.44 - 2.53 (m, 0.5 H), 2.54 - 2.62 (m, 0.5 H), 2.86 - 2.95 (m, 1 H), 3.37 - 3.54 (m, 3 H), 3.85 - 4.10 (m, 2.5 H), 4.51 (d, *J*=12.21 Hz, 0.5 H), 7.91 (d, *J*=10.37 Hz, 1 H), 8.01 (d, *J*=8.85 Hz, 1 H); Anal. Calcd for $C_{15}H_{19}ClN_4O$•2.5 HCl: C, 45.27; H, 5.45; N, 14.08. Found: C, 45.45; H, 5.67; N,

13.78.

Example 72

2-[(2*R*)-1,2-dimethylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

The title compound as HCl salt was prepared according to the procedure for Example 47, substituting Example 3B for Example 46D. Yield: 69%. MS (DCI/NH_3) m/z 259 $(M+H)^+$; ^1H NMR (500 MHz, CD_3OD): δ 1.97 (s, 3 H), 2.24 (m, 1 H), 2.32 - 2.41 (m, 2 H), 2.51 - 2.66 (m, 2 H), 2.99 (s, 3 H), 3.57 (m, 1 H), 3.91 (m, 1 H), 7.47 (t, *J*=7.78 Hz, 1 H), 7.83 (d, *J*=7.93 Hz, 1 H), 8.02 (d, *J*=7.63 Hz, 1 H); Anal. Calcd for $C_{14}H_{18}N_4O$•3 HCl: C, 45.73; H, 5.76; N, 15.24. Found: C, 45.49; H,6.37; N, 14.86.

Example 73

2-[(2*R*)-2-methyl-5-oxopyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

Example 73A

(R)-2-Methyl-5-oxo-pyrrolidine-1,2-dicarboxylic acid 1-tert-butyl ester

To a solution of Example 74D (348 mg, 1.52 mmol) in a mixture of acetonitrile (3 mL), carbon tetrachloride (3 mL) and water (4.6 mL) was added sodium periodate (1.3 g, 6.08 mmol) and ruthenium (III) chloride hydrate (64 mg, 0.30 mmol). This mixture was stirred vigorously at room temperature for 4 days. Solid material was filtered off and the filtrate was partitioned between ethyl acetate and brine. The organic phase was concentrated and the residue was separated by flash chromatography (silica gel, 0-15% gradient methanol in 2:1 EtOAc/hexane) to give the title compound (122 mg, 32%). MS (DCI/NH_3) m/z 244 $(M+H)^+$.

Example 73B

2-[(2*R*)-2-methyl-5-oxopyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide

A solution of Example 73A (120 mg, 0.49 mmol) in a mixture of pyridine (3 mL) and DMF (3 mL) was treated with 1,1'-carbonyldiimidazole (88 mg, 0.54 mmol) at 45 °C for 2 h. 2,3-Diamino-benzamide dihydrochloride (110 mg, 0.49 mmol, synthesized as described in previous patent application WO0026192), was added and the mixture was stirred at rt overnight. After concentration under vacuum, the residue was dissolved in acetic acid (6 mL)

and heated at 80 °C for 3 hour. After cooled, the reaction mixture was concentrated. The residue was separated by flash chromatography (silica gel, 0-15% gradient MeOH in CH_2Cl_2) to give the crude product. This material was further purified by HPLC (Zorbax, C-18, 250x2.54 column, Mobile phase A: 0.1% TFA in H_2O; B: 0.1% TFA in CH_3CN; 0-100% gradient) to provide Example 73B as TFA salt (80 mg, 36%). MS (DCI/NH_3) m/z 259 $(M+H)^+$; ^1H NMR (400 MHz, CD_3OD): δ 1.88 (s, 3 H), 2.41 - 2.48 (m, 1 H), 2.50 - 2.55 (m, 2 H), 2.58 - 2.66 (m, 1 H), 7.48(t, *J*= 7.67 Hz, 1 H), 7.81(d, *J*= 7.98 Hz, 1 H), 7.97 (d, *J*=7.67 Hz, 1 H); Anal. Calcd for $C_{13}H_{14}N_4O_2$•1.75 TFA: C, 43.29; H, 3.47; N, 12.24. Found: C, 43.29; H,3.85; N, 12.38.

Example 74

(R)-2-methyl-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester

Example 74A

L-Alanine benzyl ester hydrochloride (24.0 g), acetonitrile (96 mL), 1-bromo-3-chloropropane (70.6 g) and *N,N*-diisopropylethylamine (43.2 g) were charged to a reactor. The reaction mixture was warmed to 30 °C for 74 hours. The reaction mixture was cooled to 20 °C and quenched with 2N citric acid (112 g). The aqueous phase was extracted twice with heptane (72 g each). The pH of the aqueous phase was adjusted to pH 5.8-6.0 with 4N NaOH solution. The product was extracted from the aqueous phase with methyl tert-butyl ether (twice with 122 mL then once with 100 mL). The combined organic phases were washed with saturated sodium bicarbonate solution (76 mL) and 25% brine (48 mL). The organic phase was dried by passing it through a bed of sodium sulfate and distilling it to approximately half of the original volume, and was used without further purification (assay yield was 20.9 g, 73%).

Example 74B

Example 74A (10.2 g, as solution in 81 mL methyl tert-butyl ether) was charged to a reactor containing di-*tert*-butyldicarbonate (10.0 g). This mixture was stirred at 25 °C overnight. *N,N*-dimethylethylenediamine (1.15 g) was then charged to react with the excess di-*tert*-butyldicarbonate. After mixing at 25 °C overnight a sample was taken for NMR analysis. The reaction mixture was then washed twice with 1N H_3PO_4 solution (27 g each),

then with 5% $NaHCO_3$ (28 g), water (27 g), and brine (36 g). The product solution was dried with Na_2SO_4, and then concentrated. Following a chase distillation with toluene the product solution for 13.9 g (96% yield). The solution was used without further purification.

Example 74C

Example 74B (60 wt% solution in toluene, 50.0 g, 30.0 g assay) was diluted with DMF (240 mL) was added and the solution was cooled to <-20 °C. Lithium bis(trimethylsilyl)amide (25 wt% in THF, 70 g) was added continuously over ~3 hours, such that the internal temperature was maintained. The reaction was quenched into 10 wt% aq. NH_4Cl (250 g). The resulting mixture was extracted twice with heptane (225 mL each). The combined heptane layers were washed with 10% NaCl solution (206 g) then 20% NaCl solution (201 g). The heptane layer was distilled, then isopropyl acetate was added (175 mL) and distilled. More isopropyl acetate (175 mL) was added and the solution was filtered, then more isopropyl acetate (0.7 kg / kg SM) was used as a rinse. Finally, the isopropyl acetate was distilled to ~40 g, and used without further purification for an assay of 27.4 g (102%). ^1H NMR (400 MHz, $CDCl_3$), as a ~2:1 mixture of rotamers δ ppm 1.35 (s, 6 H) 1.41 (s, 3 H) 1.54 (s, 2 H) 1.60 (s, 1 H) 1.77 - 1.97 (m, 3 H) 2.08 - 2.22 (m, 1 H) 3.39 - 3.64 (m, 2 H) 5.02 - 5.24 (m, 2 H) 7.26 - 7.38 (m, 5 H)

Example 74D

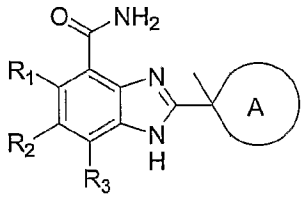
(R)-2-methyl-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester

A pressure reactor was charged with 5% palladium on carbon (2.56 g) and purged with nitrogen. Example 74C (~60 wt% solution in isopropyl acetate, 83.1 g assay) was added, along with denatured EtOH (335 g). The reactor was pressurized with hydrogen (40 psig). The hydrogenolysis was continued while maintaining a reaction temperature under 40°C. The catalyst was filtered off to afford 97% assay yield, 93.9% ee product. The solvents were distilled under vacuum and chased with isopropyl acetate (240 g). The resulting solution was further chased with heptanes (200 g), then additional heptanes (500 g) were added and heated to reflux until all solids dissolved. After cooling to 20 °C, the solids were collected by filtration and washed with heptane (80 g) and dried to yield 54.8 g (88% yield) of Example 74D. ^1H NMR (400 MHz, $CDCl_3$, mixture of rotamers) δ ppm 1.42 (s) and 1.47 (s), (9 H); 1.52 (s) and 1.61 (s) (2 H); 1.73 - 2.05 (m, 3 H) 2.19 - 2.38 (m) and 2.46 -

2.67 (m) (1H); 3.26 - 3.71 (m, 2 H).

WHAT IS CLAIMED IS

1. A compound of Formula (I)



(I),

or a therapeutically acceptable salt thereof, wherein

R_1, R_2, and R_3 are independently selected from the group consisting of hydrogen, alkenyl, alkoxy, alkoxycarbonyl, alkyl, alkynyl, cyano, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, nitro, NR_AR_B, and (NR_AR_B)carbonyl;

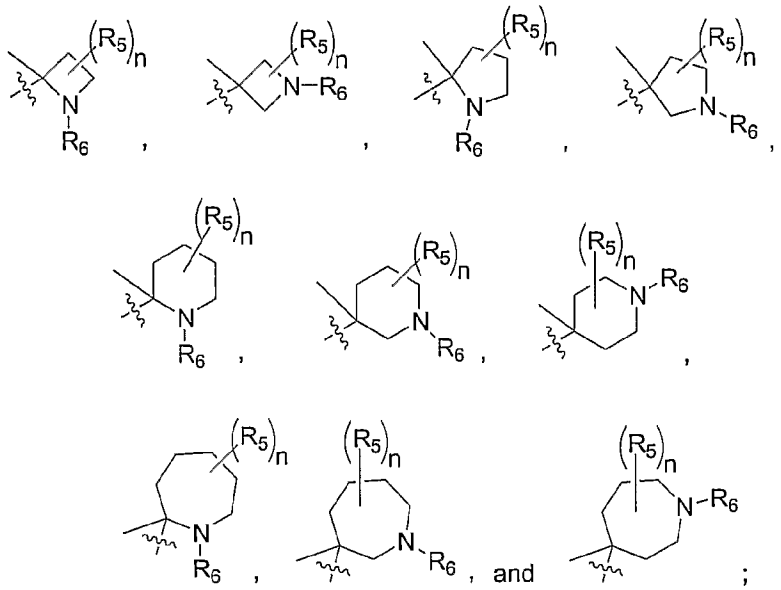
A is a nonaromatic 4, 5, 6, 7, or 8-membered ring that contains 1 or 2 nitrogen atoms and, optionally, one sulfur or oxygen atom, wherein the nonaromatic ring is optionally substituted with 1, 2, or 3 substituents selected from the group consisting of alkenyl, alkoxy, alkoxyalkyl, alkoxycarbonyl, alkoxycarbonylalkyl, alkyl, alkynyl, aryl, arylalkyl, cycloalkyl, cycloalkylalkyl, cyano, haloalkoxy, haloalkyl, halogen, heterocycle, heterocyclealkyl, heteroaryl, heteroarylalkyl, hydroxy, hydroxyalkyl, nitro, NR_CR_D, (NR_CR_D)alkyl, (NR_CR_D)carbonyl, (NR_CR_D)carbonylalkyl, (NR_CR_D)sulfonyl, and oxo; and

R_A, R_B, R_C, and R_D are independently selected from the group consisting of hydrogen, alkyl, and alkycarbonyl.

2. The compound according to claim 1 wherein

R_1, R_2, and R_3 are independently selected from the group consisting of hydrogen, alkenyl, alkoxy, alkoxycarbonyl, alkyl, alkynyl, cyano, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, nitro, NR_AR_B, and (NR_AR_B)carbonyl;

A is selected from the group consisting of



R_5 is independently selected from the group consisting of alkenyl, alkoxy, alkoxycarbonyl, alkyl, alkynyl, haloalkoxy, haloalkyl, halogen, hydroxy, hydroxyalkyl, NR_CR_D, and (NR_CR_D)carbonyl;

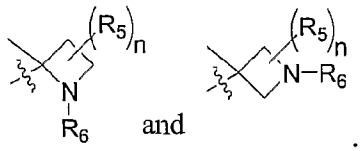
n is 0, 1, 2, or 3;

R_6 is selected from the group consisting of hydrogen, alkenyl, alkoxyalkyl, alkoxycarbonyl, alkoxycarbonylalkyl, alkyl, alkynyl, aryl, arylalkyl, cycloalkyl, cycloalkylalkyl, heterocycle, heterocyclealkyl, heteroaryl, heteroarylalkyl, hydroxyalkyl, (NR_CR_D)alkyl, (NR_CR_D)carbonyl, (NR_CR_D)carbonylalkyl, and (NR_CR_D)sulfonyl;

R_A and R_B are independently selected from the group consisting of hydrogen, alkyl, and alkycarbonyl; and

R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

3. The compound according to claim 2 wherein A is selected from the group consisting of



4. The compound according to claim 3 wherein

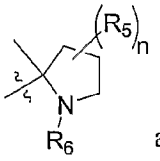
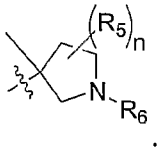
R_1, R_2, R_3, and R_5 are hydrogen;

n is 0;

R_6 is selected from the group consisting of hydrogen, alkyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heterocycle, heteroarylalkyl, and (NR_CR_D)sulfonyl; and

R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

5. The compound according to claim 2 wherein A is selected from the group consisting of


and

.

6. The compound according to claim 5 wherein

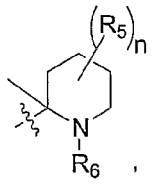
R_1, R_2, R_3, and R_5 are hydrogen;

n is 0;

R_6 is selected from the group consisting of hydrogen, alkyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heterocycle, heteroarylalkyl, and (NR_CR_D)sulfonyl; and

R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

7. The compound according to claim 2 wherein A is selected from the group consisting of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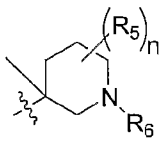
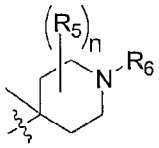

, and

.

8. The compound according to claim 7 wherein

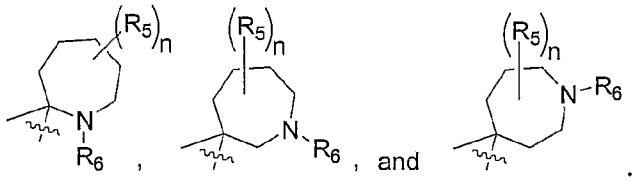
R_1, R_2, R_3, and R_5 are hydrogen;

n is 0;

R_6 is selected from the group consisting of hydrogen, alkyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heterocycle, heteroarylalkyl, and (NR_CR_D)sulfonyl; and

R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

9. The compound according to claim 2 wherein A is selected from the group consisting of



10. The compound according to claim 9 wherein

R_1, R_2, R_3, and R_5 are hydrogen;

n is 0;

R_6 is selected from the group consisting of hydrogen, alkyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heterocycle, heteroarylalkyl, and (NR_CR_D)sulfonyl; and

R_C and R_D are independently selected from the group consisting of hydrogen and alkyl.

11. A compound selected from the group consisting of

2-(2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide

2-[(2R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide;

2-[(2S)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide;

2-(1,2-dimethylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(1-ethyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(2-methyl-1-propylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(1-isopropyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclobutyl-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(3-methyl-1-propylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-[1-(cyclopropylmethyl)-3-methylpyrrolidin-3-yl]-1H-benzimidazole-4-

carboxamide;

2-(1-isobutyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(1-isopropyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclobutyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclopentyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclohexyl-3-methylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(3-methyl-1-tetrahydro-2H-pyran-4-ylpyrrolidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-[3-methyl-1-(pyridin-4-ylmethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide;

2-[3-methyl-1-(2-phenylethyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide;

2-[3-methyl-1-(1-methyl-3-phenylpropyl)pyrrolidin-3-yl]-1H-benzimidazole-4-carboxamide;

2-(2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(1-isopropyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclobutyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclopentyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclohexyl-2-methylazetidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(3-methyl-1-propylazetidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-[1-(cyclopropylmethyl)-3-methylazetidin-3-yl]-1H-benzimidazole-4-carboxamide;

2-(1-isobutyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclobutyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclopentyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclohexyl-3-methylazetidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-(3-methyl-1-tetrahydro-2H-pyran-4-ylazetidin-3-yl)-1H-benzimidazole-4-carboxamide;

2-{1-[(dimethylamino)sulfonyl]-3-methylazetidin-3-yl}-1H-benzimidazole-4-carboxamide;

2-(2-methylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-(2-methyl-1-propylpiperidin-2-yl)-1H-benzimidazole-4-carboxamide;

2-{1-[(dimethylamino)sulfonyl]-4-methylpiperidin-4-yl}-1H-benzimidazole-4-carboxamide;

2-(1-cyclobutyl-4-methylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide;

2-(1-isopropyl-4-methylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide;

2-(4-methyl-1-propylpiperidin-4-yl)-1H-benzimidazole-4-carboxamide;

2-(4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide;

2-(1-cyclopentyl-4-methylazepan-4-yl)-1H-benzimidazole-4-carboxamide; and

2-(1-Cyclohexyl-4-methyl-azepane-4-yl)-H-benzoimidazole-4-carboxylic acid amide.

12. A pharmaceutical composition comprising a compound of Formula (I), or a therapeutically acceptable salt thereof, in combination with a therapeutically acceptable carrier.

13. A method of inhibiting poly(ADP-ribose)polymerase (PARP) in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

14. A method of treating inflammation in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

15. A method of treating sepsis in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

16. A method of treating septic shock in a mammal in recognized need of such treatment comprising administering to the mammal a therapeutically acceptable amount of a compound of Formula (I) or a therapeutically acceptable salt thereof.

17. A compound selected from the group consisting of

2-[1-(2-fluorobenzyl)-3-methylpyrrolidin-3-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-(3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-(1,3-dimethylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-(1-isopropyl-3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide;

2-(2-methylpyrrolidin-2-yl)-6-(trifluoromethyl)-1*H*-benzimidazole-4-carboxamide;

2-(1,2-dimethylpyrrolidin-2-yl)-6-(trifluoromethyl)-1*H*-benzimidazole-4-carboxamide;

6-fluoro-2-(2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carboxamide;

6-fluoro-2-(2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-(2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*R*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*S*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-fluoro-2-[(2*S*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-fluoro-2-[(2*R*)-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*R*)-1,2-dimethylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*R*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*R*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*S*)-1,2-dimethylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*S*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-[(2*S*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

2-[(2*S*)-1,2-dimethylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide;

6-fluoro-2-[(2*S*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

2-[(2*S*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide;

2-[(2*R*)-1,2-dimethylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide;

6-fluoro-2-[(2*R*)-1-isopropyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

2-[(2*R*)-1-cyclopentyl-2-methylpyrrolidin-2-yl]-6-fluoro-1*H*-benzimidazole-4-carboxamide;

2-[(2*R*)-1-ethyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

2-[(2*S*)-1-ethyl-2-methylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide;

6-chloro-2-(1-ethyl-3-methylpyrrolidin-3-yl)-1*H*-benzimidazole-4-carboxamide;

2-[(2*R*)-1,2-dimethylpyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide; and

2-[(2*R*)-2-methyl-5-oxopyrrolidin-2-yl]-1*H*-benzimidazole-4-carboxamide.

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(54) **Title**: HUMAN SERUM ALBUMIN LINKERS AND CONJUGATES THEREOF

(57) **Abstract**: The present invention relates to a human serum albumin (HSA) linker and binding, diagnostic, and therapeutic agents conjugated thereto. Also disclosed are methods and kits for the diagnostic and therapeutic application of an HSA linker conjugate.

HUMAN SERUM ALBUMIN LINKERS AND CONJUGATES THEREOF

FIELD OF THE INVENTION

The invention provides a human serum albumin (HSA) linker and binding, diagnostic, and therapeutic conjugates thereof. In one embodiment, the HSA linker includes two amino acid substitutions. The invention further provides methods for the manufacture and administration of diagnostic and therapeutic HSA linker conjugates.

BACKGROUND OF THE INVENTION

The serum albumins belong to a family of proteins that includes alpha-fetoprotein and human group-specific component, also known as vitamin-D binding protein. The serum albumins are the major soluble proteins of the circulatory system and contribute to many vital physiological processes. Serum albumin generally comprises about 50% of the total blood component by dry weight. The albumins and their related blood proteins also play an important role in the transport, distribution, and metabolism of many endogenous and exogenous ligands in the human body, including a variety of chemically diverse molecules, such as fatty acids, amino acids, steroids, calcium, metals such as copper and zinc, and various pharmaceutical agents. The albumin family of molecules are generally thought to facilitate transfer of many of these ligands across organ-circulatory interfaces, such as the liver, intestines, kidneys, and the brain. The albumins are thus involved in a wide range of circulatory and metabolic functions.

Human serum albumin (HSA) is a protein of about 66,500 kD and is comprised of 585 amino acids including at least 17 disulphide bridges. As with many of the members of the albumin family, human serum albumin plays an important role in human physiology and is located in virtually every human tissue and bodily secretion. As indicated above, HSA has theability to bind and transport a wide spectrum of ligands throughout the circulatory system, including the long-chain fatty acids, which are otherwise insoluble in circulating plasma.

SUMMARY OF THE INVENTION

In a first aspect, the invention features an agent that includes a human serum albumin (HSA) linker having an amino acid sequence set forth in any one of SEQ ID NOS:6-10 and first and second

binding moieties selected from antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, receptors (e.g., cell surface receptors), ligands, aptamers, and biologically-active fragments thereof, in which the first binding moiety is bonded to the amino terminus of the HSA linker and the second binding moiety is bonded to the carboxy terminus of the HSA linker. In one embodiment, the first binding moiety specifically binds ErbB3 and the second binding moiety specifically binds ErbB2. In other embodiments, the HSA linker has the amino acid sequences set forth in SEQ ID NOS:9 or 10.

In a second aspect, the invention features an agent that includes an HSA linker that has an amino acid sequence set forth in any one SEQ ID NOS:11-15 and at least first and second binding moieties, which can be selected from antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, receptors (e.g., cell surface receptors), ligands, aptamers, and biologically-active fragments thereof, wherein the first binding moiety is bonded to the amino terminus and the second binding moiety is bonded to the carboxy terminus of the sequence. In an embodiment, three or more binding moieties (e.g., 4, 5, 6, 7, 8, 9, 10, or more) can be included in the agent; these additional binding moities can be added to the agent, e.g., in tandem (e.g., 2, 3, 4, or 5 or more in tandem) with the first or second binding moiety. In one embodiment, the first binding moiety specifically binds ErbB3 and the second binding moiety specifically binds ErbB2. In other embodiments, the HSA linker has the amino acid sequences set forth in SEQ ID NOS:14 or 15.

In a third aspect, the invention provides an HSA linker that has an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO:1, and a serine residue at position 34 and a glutamine residue at position 503 of the amino acid sequence set forth in SEQ ID NO:1. In one embodiment, the amino acid sequence has at least 95% sequence identity to the sequence set forth in SEQ ID NO:1. In another embodiment, the HSA linker has the amino acid sequence set forth in SEQ ID NO:1.

In a fourth aspect, the invention features an agent that includes an HSA linker having at least 90% amino acid sequence identity to the sequence set forth in SEQ ID NO:1 and at least a first binding moiety. In one embodiment, the agent includes a first polypeptide connector that binds the first binding moiety to the HSA linker.

In a fifth aspect, the invention features an agent that includes an HSA linker having an amino acid sequence set forth in any one of SEQ ID NOS:11-15, or a fragment or variant of any one of these sequences, and at least a first binding moiety.

In either the fourth or fifth aspect of the invention, the agent further includes a first polypeptide connector (e.g., AAS, AAQ, or AAAL) that binds the first binding moiety to the amino or carboxy terminus of the HSA linker. In an embodiment, the first connector covalently binds the first binding moiety to the HSA linker.

In either the fourth or fifth aspect of the invention, the agent further includes at least a second binding moiety. In an embodiment, the agent further includes a second polypeptide connector (e.g., AAS, AAQ, or AAAL) that binds the second binding moiety to the HSA linker. In other embodiments, the second connector binds the second binding moiety to the amino or carboxy terminus of the HSA linker. In a further embodiment, the second connector covalently binds the second binding moiety to the HSA linker. In other embodiments, the agent further includes three or more binding moieties which are included in tandem with the first or second binding moiety; the three or more binding moieties can further include a connector sequence that joins the three or more binding moieties to the first or second binding moiety and to each other.

In either the fourth or fifth aspect of the invention, the agent includes a first polypeptide connector that covalently binds a first binding moiety to the amino terminus of the HSA linker and a second polypeptide connector that covalently binds a second binding moiety to the carboxy terminus of the HSA linker. In one embodiment, the first connector has the amino acid sequence AAS or AAQ and the second connector has the amino acid sequence set forth in SEQ ID NO:5.

In either the fourth or fifth aspect of the invention, the first or second binding moiety (or third or more binding moiety) is an antibody, single-chain Fv molecule, bispecific single chain Fv ($(scFv')_2$) molecule, domain antibody, diabody, triabody, hormone, Fab fragment, $F(ab')_2$ molecule, tandem scFv (taFv) fragment, receptor (e.g., cell surface receptor), ligand, aptamer, or biologically-active fragment thereof. In other embodiments, the agents of the invention include combinations of these different types of binding moities. In one embodiment, at least the first or second binding moiety is a human or humanized single-chain Fv molecule.

In an embodiment of any one of the first, second, third, fourth, or fifth aspects of the invention, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to a protein selected from the group consisting of an insulin-

like growth factor 1 receptor (IGF1R), IGF2R, insulin-like growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met; also known as hepatocyte growth factor receptor (HGFR)), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, an integrin (e.g., an α4 integrin or a β-1 integrin), P-selectin, sphingosine-1-phosphate receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-related apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), $Lewis^Y$, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and fragments thereof. In a further embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to erythroblastic leukemia viral oncogene homolog (ErbB) receptor (e.g., ErbB1 receptor; ErbB2 receptor; ErbB3 receptor; and ErbB4 receptor). In another embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to alpha-fetoprotein (AFP) or an interferon, or a biologically-active fragment thereof. In a further embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, panitumumab, or anakinra.

In any one of the first, second, third, fourth, or fifth aspects of the invention, the agent is conjoined to a diagnostic, a therapeutic agent, or both. In one embodiment, the diagnostic agent is a detectable label, such as a radioactive, fluorescent, or heavy metal label. In another embodiment, the therapeutic agent is a cytotoxic agent, cytostatic, or immunomodulatory agent. Cytotoxic agents include alkylating agents, antibiotics, antineoplastic agents, antiproliferative agents, antimetabolites,

tubulin inhibitors, topoisomerase I or II inhibitors, hormonal agonists or antagonists, immunomodulators, DNA minor groove binders, and radioactive agents, or any agent capable of binding to and killing a tumor cell or inhibiting tumor cell proliferation. Antineoplastic agents include cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, caleachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and their derivatives.

In any one of the first, second, third, fourth, or fifth aspects of the invention, the agent is admixed with a pharmaceutically acceptable carrier, excipient, or diluent. In one embodiment, the agent exhibits an *in vivo* half-life of between 6 hours and 7 days. In another embodiment, the agent exhibits an *in vivo* half-life greater than 8 hours.

In a sixth aspect, the invention features a method for treating a mammal having a disease or disorder by administering any one of the HSA agents described in the first five aspects. In one embodiment, the disease or disorder is associated with cellular signaling through a cell surface receptor. In another embodiment, the mammal is a human. In a further embodiment, the disease or disorder is a proliferative or autoimmune disease. Proliferative diseases include melanoma, clear cell sarcoma, head and neck cancer, bladder cancer, breast cancer, colon cancer, ovarian cancer, endometrial cancer, gastric cancer, pancreatic cancer, renal cancer, prostate cancer, salivary gland cancer, lung cancer, liver cancer, skin cancer, and brain cancer. Autoimmune diseases include multiple sclerosis, psoriasis, myasthenia gravis, uveitis, systemic lupus erythematosus, and rheumatoid arthritis. In one embodiment, the HSA agent is administered in combination with one or more therapeutic agents, such as an antineoplastic agent.

In a seventh aspect, the invention features a method for making an HSA linker agent by bonding at least a first binding moiety to the amino terminus and a second binding moiety to the carboxy terminus of the amino acid sequence set forth in any one of SEQ ID NOS:1, 3, or 6-15, or a sequence having at least 90%, 95%, 97%, or 100% sequence identity to a sequence set forth in any one of SEQ ID NOS:1, 3, or 6-15. In one embodiment, the first or second binding moiety is covalently joined to the amino or carboxy terminus of an HSA linker. In other embodiments, a third or additional binding moiety (e.g., a fourth, fifth, sixth, seventh, eighth, ninth, or tenth binding moiety) is covalently joined in tandem with the first or second binding moiety to the amino or

carboxy terminus of the HSA linker. In another embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is an antibody, single-chain Fv molecule, bispecific single chain Fv ($(scFv')_2$) molecule, domain antibody, diabody, triabody, hormone, Fab fragment, $F(ab')_2$ molecule, tandem scFv (taFv) fragment, receptor (e.g., cell surface receptor), ligand, or aptamer. In another embodiment, the first or second binding moiety (or, if present, the third or further binding moiety) is a human or humanized single-chain Fv molecule. In yet another embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to insulin-like growth factor 1 receptor (IGF1R), IGF2R, insulin-like growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met; also known as hepatocyte growth factor receptor (HGFR)), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, an integrin (e.g., an α4 integrin or a β-1 integrin), P-selectin, sphingosine-1-phosphate receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-related apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), $Lewis^Y$, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and fragments thereof. In a further embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to erythroblastic leukemia viral oncogene homolog (ErbB) receptor (e.g., ErbB1 receptor; ErbB2 receptor; ErbB3 receptor; and ErbB4 receptor). In another embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to alpha-fetoprotein (AFP) or an interferon, or a biologically-active fragment thereof. In a further embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is

natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, panitumumab, or anakinra. In another embodiment, the agent is conjoined to a diagnostic or therapeutic agent. In one embodiment, the diagnostic agent is a detectable label, such as a radioactive, bioluminescent, fluorescent, heavy metal, or epitope tag. In another embodiment, the therapeutic agent is a cytotoxic agent, cytostatic, or immunomodulatory agent. Cytotoxic agents include alkylating agents, antibiotics, antineoplastic agents, antiproliferative agents, antimetabolites, tubulin inhibitors, topoisomerase I and II inhibitors, hormonal agonists or antagonists, immunomodulators, DNA minor groove binders, and radioactive agents, or any agent capable of binding to and killing a tumor cell or inhibiting tumor cell proliferation. Antineoplastic agents include cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, caleachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and their derivatives. In a further embodiment, the agent is admixed with a pharmaceutically acceptable carrier, excipient, or diluent.

In a eighth aspect, the invention features a method for making an HSA agent by substituting one or more surface-exposed amino acid residues in the amino acid sequences set forth in any one of SEQ ID NOS:1, 3, and 6-15 with a substitute amino acid capable of chemical modification that allows conjugation of a diagnostic or therapeutic agent. In one embodiment, the substitute amino acid is cysteine and the surface exposed amino acid residues are serine or threonine. In another embodiment, the chemical modification results in a covalent bond between the substitute amino acid and the diagnostic or therapeutic agent. In a further embodiment, the surface-exposed amino acid residues is threonine at position 496, serine at position 58, threonine at position 76, threonine at position 79, threonine at position 83, threonine at position 125, threonine at position 236, serine at position 270, serine at position 273, serine at position 304, serine at position 435, threonine at position 478, threonine at position 506, or threonine at position 508.

In a ninth aspect, the invention features a method for making an HSA agent by substituting one or more of the residues in the amino acid sequences set forth in any one of SEQ ID NOS:1, 3, and 6-15 with an asparagine, serine, or threonine, thereby incorporating a glycosylation site within the HSA agent.

In a tenth aspect, the invention features a method for making an HSA agent by substituting one or more of the asparagine, serine, or threonine residues in the amino acid sequences set forth in any one of SEQ ID NOS:1, 3, and 6-15 with any amino acid other than asparagine, serine, or threonine, thereby removing a glycosylation site from the HSA agent.

In an eleventh aspect, the invention features an agent that has at least 90% sequence identity to one of the amino acid sequences set forth in SEQ ID NOS:16-25. In one embodiment, the agent has at least 95% sequence identity to one of the amino acid sequences set forth in SEQ ID NOS:16-25. In another embodiment, the agent has one of the amino acid sequences set forth in SEQ ID NOS:16-25. In a further embodiment, the agent is conjoined to a diagnostic or therapeutic agent. Diagnostic agents include detectable labels, such as a radioactive, bioluminescent, fluorescent, or heavy metal labels, or epitope tags. Fluorescent molecules that can serve as detectable labels include green fluorescent protein (GFP), enhanced GFP (eGFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), red fluorescent protein (RFP), and dsRed. In one embodiment, the bioluminescent molecule is luciferase. In another embodiment, the epitope tag is c-myc, hemagglutinin, or a histidine tag. In a further embodiment, the therapeutic agent is a cytotoxic polypeptide such as cytochrome c, caspase 1-10, granzyme A or B, tumor necrosis factor-alpha (TNF-α), TNF-β, Fas, Fas ligand, Fas-associated death doman-like IL-1β converting enzyme (FLICE), TRAIL/APO2L, TWEAK/APO3L, Bax, Bid, Bik, Bad, Bak, RICK, vascular apoptosis inducing proteins 1 and 2 (VAP1 and VAP2), pierisin, apoptosis-inducing protein (AIP), IL-1α propiece polypeptide, apoptin, apoptin-associated protein 1 (AAP-1), endostatin, angiostatin, and biologically-active fragments thereof. An agent of the invention can be combined with one or more therapeutic agents such as cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, caleachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and derivatives thereof.

In an embodiment of any aspect of the invention, the first and second binding moieties (and, if present, one or more of the third or further binding moiety) specifically bind the same target molecule. In another embodiment of any aspect of the invention, the first and second binding moieties (and, if present, one or more of the third or further binding moiety) specifically bind different target molecules. In a further embodiment of any aspect of the invention, the first

and second binding moieties (and, if present, one or more of the third or further binding moiety) specifically bind different epitopes on the same target molecule.

In a twelfth aspect, the invention features a polypeptide linker that has amino acid residues 25-44 and 494-513 of the amino acid sequence set forth in SEQ ID NO:1. In one embodiment, the polypeptide linker has amino acid residues 25-70 and 450-513 of the amino acid sequence set forth in SEQ ID NO:1. In another embodiment, the polypeptide linker has amino acid residues 15-100 and 400-520 of the amino acid sequence set forth in SEQ ID NO:1. In a further embodiment, the polypeptide linker has amino acid residues 10-200 and 300-575 of the amino acid sequence set forth in SEQ ID NO:1. In another embodiment, the polypeptide linker has amino acid residues 5-250 and 275-580 of the amino acid sequence set forth in SEQ ID NO:1.

In the twelfth aspect of the invention, the polypeptide linker includes at least a first binding moiety. In one embodiment, the polypeptide linker includes at least a first polypeptide connector that binds the first binding moiety to the amino or carboxy terminus of the polypeptide linker. In another embodiment, the first polypeptide connector covalently binds the first binding moiety to the polypeptide linker. In a further embodiment, the polypeptide linker includes a second binding moiety. In one embodiment, the polypeptide linker includes a second polypeptide connector that binds the second binding moiety to the polypeptide linker. In other embodiments, the second connector binds the second binding moiety to the amino or carboxy terminus of the polypeptide linker. In a further embodiment, the second connector covalently binds the second binding moiety to the polypeptide linker. In other embodiments, the polypeptide linker includes a third, fourth, fifth, sixth, seventh, eighth, ninth, or tenth binding moiety. In other embodiments, these additional binding moieties are present in tandem with one or both of the first or second binding moiety. In yet other embodiments, a polypeptide connector (e.g., AAS, AAQ, or AAAL) separates one or more of these additional binding moities from each other, the first or second binding moiety, or the polypeptide linker.

In the twelfth aspect of the invention, the polypeptide linker includes a first polypeptide connector that covalently binds a first binding moiety to the amino terminus of the polypeptide linker and a second polypeptide connector that covalently binds a second binding moiety to the carboxy terminus of the polypeptide linker. In one embodiment, the first connector has the amino acid

sequence AAS or AAQ and the second connector has the amino acid sequence set forth in SEQ ID NO:5.

In the twelfth aspect of the invention, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is an antibody, single-chain Fv molecule, bispecific single chain Fv ($(scFv')_2$) molecule, domain antibody, diabody, triabody, hormone, Fab fragment, $F(ab')_2$ molecule, tandem scFv (taFv) fragment, receptor (e.g., cell surface receptor), ligand, aptamer, or biologically-active fragment thereof. In one embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is a human or humanized single-chain Fv molecule.

In the twelfth aspect of the invention, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to a protein selected from the group consisting of an insulin-like growth factor 1 receptor (IGF1R), IGF2R, insulin-like growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met; also known as hepatocyte growth factor receptor (HGFR)), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, an integrin (e.g., an α4 integrin or a β-1 integrin), P-selectin, sphingosine-1-phosphate receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-related apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), $Lewis^Y$, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and fragments thereof. In a further embodiment, the first or second binding moiety is or specifically binds to erythroblastic leukemia viral oncogene homolog (ErbB) receptor (e.g., ErbB1 receptor; ErbB2 receptor; ErbB3 receptor; and ErbB4 receptor). In another embodiment, one or more of the

first or second binding moiety (or, if present, the third or further binding moiety) is or specifically binds to alpha-fetoprotein (AFP) or an interferon, or a biologically-active fragment thereof. In a further embodiment, one or more of the first or second binding moiety (or, if present, the third or further binding moiety) is natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, panitumumab, or anakinra.

In the twelfth aspect of the invention, the polypeptide linker is conjoined to a diagnostic, a therapeutic agent, or both. In one embodiment, the diagnostic agent is a detectable label, such as a radioactive, fluorescent, or heavy metal label. In another embodiment, the therapeutic agent is a cytotoxic agent, cytostatic, or immunomodulatory agent. Cytotoxic agents include alkylating agents, antibiotics, antineoplastic agents, antiproliferative agents, antimetabolites, tubulin inhibitors, topoisomerase I or II inhibitors, hormonal agonists or antagonists, immunomodulators, DNA minor groove binders, and radioactive agents, or any agent capable of binding to and killing a tumor cell or inhibiting tumor cell proliferation. Antineoplastic agents include cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, calcachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and their derivatives. In one embodiment, the polypeptide linker is admixed with a pharmaceutically acceptable carrier, excipient, or diluent. In another embodiment, the polypeptide linker exhibits an *in vivo* half-life of between 6 hours and 7 days. In a further embodiment, the polypeptide linker exhibits an *in vivo* half-life greater than 8 hours.

A thirteenth aspect of the invention features an agent of any of the prior aspects of the invention (one through twelve), in which the HSA polypeptide linker sequence is replaced by another polypeptide linker sequence. For example, the polypeptide linker sequence could be a mammalian, non-human serum albumin polypeptide sequence, such as, e.g., a bovine, murine, feline, and canine serum albumin (BSA) polypeptide sequence. In other embodiments this polypeptide linker sequence is between 5 and 1,000 amino acids in length, e.g., 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, or 900 amino acids in length, or any number of amino acids within this range. In other embodiments, the polypeptide linker sequence includes a single amino acid (including, but not limited to, e.g., glycine, alanine, serine, glutamine, leucine, and valine), or combinations of amino acids.

In another embodiment, the HSA polypeptide linker sequence is replaced by an alpha-fetoprotein polypeptide (AFP) polypeptide sequence, e.g., mammalian AFP polypeptide sequence, such as a human, murine, bovine, or canine AFP polypeptide sequence. In an embodiment, the AFP linker sequence of the agent can correspond to the full-length human AFP polypeptide sequence (a.a. 1-609; SEQ ID NO: 58), the mature human AFP polypeptide sequence lacking amino acids 1-18 of the signal sequence (a.a., 19-609 of SEQ ID NO: 58), or fragments thereof. In other embodiments, the AFP polypeptide linker sequence contains at least 5 to 8 contiguous amino acids, preferably at least 10, 20, or 50 contiguous amino acids, more preferably at least 100 contiguous amino acids, and most preferably at least 200, 300, 400, or more contiguous amino acids of SEQ ID NO: 58, or has at least 90% sequence identity (e.g., at least 95%, 97%, 99%, or more sequence identity) to a continguous polypeptide sequence of SEQ ID NO: 58 having one or more of these lengths. For example, an AFP polypeptide linker sequence having 90% sequence identity to a 34-mer human AFP peptide corresponding to amino acids 446-479 of SEQ ID NO: 58 (LSEDKLLACGEGAADIIIGHLCIRHEMTPVNPGV; SEQ ID NO: 59) may contain up to 3 amino acids altered from the 446-479 segment of SEQ ID NO: 58. One such example of sequence deviation in biologically active human AFP fragments is found in, e.g., U.S. Patent No. 5,707,963 (incorporated by reference herein), which discloses a 34-amino acid fragment of human AFP (SEQ ID NO: 59) with flexibility at two amino acid residues (amino acid 9 and 22 of SEQ ID NO: 59). Other examples of AFP polypeptide linker sequences include, e.g., amino acids 19-198 of SEQ ID NO: 58 (human AFP Domain I), amino acids 217-408 of SEQ ID NO: 58 (human AFP Domain II), amino acids 409-609 of SEQ ID NO: 58 (human AFP Domain III), amino acids 19-408 of SEQ ID NO: 58 (human AFP Domain I+II), amino acids 217-609 of SEQ ID NO: 58 (human AFP Domain II+III), and amino acids 285-609 of SEQ ID NO: 58 (human AFP Fragment I). In another embodiment, the human AFP polypeptide linker sequence is an 8-amino acid sequence that includes amino acids 489-496 (i.e., EMTPVNPG) of SEQ ID NO: 58.

A fourteenth aspect of the invention features kits that include any of the HSA linkers, HSA linker agents, or any other agents described in the first, second, third, fourth, fifth, eleventh, twelfth, and thirteenth aspects of the invention. The kits further include instructions to allow a practitioner (e.g., a physician, nurse, or patient) to administer the compositions and agents contained therein. In an embodiment, the kits include multiple packages of a single- or multi-dose pharmaceutical composition containing an effective amount of an agent of the invention, e.g,. an HSA linker of the

invention that includes, e.g., one or more binding moieties (e.g., antibodies or antibody fragments (e.g., scFv)), diagnostic agents (e.g., radionuclide or chelating agents), and/or therapeutic agents (e.g., cytotoxic or immunomodulatory agents). Optionally, instruments or devices necessary for administering the pharmaceutical composition(s) may be included in the kits. For instance, a kit of this invention may provide one or more pre-filled syringes containing an effective amount of an HSA linker of the invention, or any binding, diagnostic, and/or therapeutic agent conjugated thereto. Furthermore, the kits may also include additional components such as instructions or administration schedules for a patient suffering from a disease or condition (e.g., a cancer, autoimmune disease, or cardiovascular disease) to use the pharmaceutical composition(s) containing, e.g., an HSA linker of the invention, or any binding, diagnostic, and/or therapeutic agent conjugated thereto.

DEFINITIONS

The term "antibody" as used interchangeably herein, includes whole antibodies or immunoglobulins and any antigen-binding fragment or single chains thereof. Antibodies, as used herein, can be mammalian (e.g., human or mouse), humanized, chimeric, recombinant, synthetically produced, or naturally isolated. In most mammals, including humans, antibodies have at least two heavy (H) chains and two light (L) chains connected by disulfide bonds. Each heavy chain consists of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region consists of three domains, C_H1, C_H2, and C_H3 and a hinge region between C_H1 and C_H2. Each light chain consists of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region consists of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. Antibodies of the present invention include all known forms of antibodies and other protein scaffolds with antibody-like properties. For example, the antibody can be a human antibody, a humanized

antibody, a bispecific antibody, a chimeric antibody, or a protein scaffold with antibody-like properties, such as fibronectin or ankyrin repeats. The antibody also can be a Fab, Fab'2, scFv, SMIP, diabody, nanobody, aptamers, or a domain antibody. The antibody can have any of the following isotypes: IgG (e.g., IgG1, IgG2, IgG3, and IgG4), IgM, IgA (e.g., IgA1, IgA2, and IgAsec), IgD, or IgE. Antibodies that can be used as binding moieties, as defined herein, in combination with an HSA linker of the invention include, but are not limited to, natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, and panitumumab.

The term "antibody fragment," as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, ErbB2). The antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include but are not limited to: (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L, and C_H1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_H1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb including V_H and V_L domains; (vi) a dAb fragment (Ward *et al., Nature* 341:544-546 (1989)), which consists of a V_H domain; (vii) a dAb which consists of a V_H or a V_L domain; (viii) an isolated complementarity determining region (CDR); and (ix) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al., Science* 242:423-426 (1988) and Huston *et al., Proc. Natl. Acad. Sci. USA* 85:5879-5883 (1988)). These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. Antibody fragments can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins.

By "autoimmune disease" is meant a disease in which an immune system response is generated against self epitopes or antigens. Examples of autoimmune diseases include, but are not

limited to, alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune hemolytic anemia, autoimmune hepatitis, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac Sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatricial pemphigoid, CREST syndrome, cold agglutinin disease, Crohn's disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, Grave's disease, Guillain-Barré syndrome, Hashimoto's thyroiditis, hypothyroidism, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, insulin dependent diabetes, juvenile arthritis, lichen planus, lupus, Ménière's disease, mixed connective tissue disease, multiple sclerosis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, Raynaud's phenomenon, Reiter's syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjögren's syndrome, Stiff-Man syndrome, systemic lupus erythematosus (SLE), Takayasu arteritis, temporal arteritis/giant cell arteritis, ulcerative colitis, uveitis (e.g., birdshot retinochoroidopathy uveitis and sarcoid uveitis), vasculitis, vitiligo, Wegener's granulomatosis, and myasthenia gravis.

By "binding moiety" is meant any molecule that specifically binds to a target epitope, antigen, ligand, or receptor. Binding moieties include but are not limited to antibodies (e.g., monoclonal, polyclonal, recombinant, humanized, and chimeric antibodies), antibody fragments (e.g., Fab fragments, Fab'2, scFv antibodies, SMIP, domain antibodies, diabodies, minibodies, scFv-Fc, affibodies, nanobodies, and domain antibodies), receptors, ligands, aptamers, and other molecules having a known binding partner.

By "biologically-active" is meant that a molecule, including biological molecules, such as nucleic acids, peptides, polypeptides, and proteins, exerts a physical or chemical activity on itself or other molecule. For example, a "biologically-active" molecule may possess, e.g., enzymatic activity, protein binding activity (e.g., antibody interactions), or cytotoxic activities are "biologically-active."

The term "chimeric antibody" refers to an immunoglobulin or antibody whose variable regions derive from a first species and whose constant regions derive from a second species. Chimeric antibodies can be constructed, for example, by genetic engineering, from immunoglobulin gene segments belonging to different species (e.g., from a mouse and a human).

By "connector" or "polypeptide connector" is meant an amino acid sequence of 2 to 20 residues in length that is covalently attached to one or both of the amino or carboxy termini of an HSA linker of the invention, or is covalently attached to one or more residues of an HSA linker of the invention (e.g., a residue between the amino and carboxy terminal residues). In preferred embodiments, the polypeptide connector attached to the amino terminus of an HSA linker has the amino acid sequence "AAS" or "AAQ" and the connector attached to the carboxy terminus has the amino acid sequence "AAAL" (SEQ ID NO:5).

The terms "effective amount" or "amount effective to" or "therapeutically effective amount" means an amount of an agent of the invention (e.g., an HSA linker bonded with one or more binding moieties or diagnostic or therapeutic agents with or without a connector sequence) sufficient to produce a desired result, for example, killing a cancer cell, reducing tumor cell proliferation, reducing inflammation in a diseased tissue or organ, or labeling a specific population of cells in a tissue, organ, or organism (e.g., a human).

The term "human antibody," as used herein, is intended to include antibodies, or fragments thereof, having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences as described, for example, by Kabat *et al.*, (*Sequences of proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 (1991)). Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences (i.e., a humanized antibody or antibody fragment).

The term "humanized antibody" refers to any antibody or antibody fragment that includes at least one immunoglobulin domain having a variable region that includes a variable framework region substantially derived from a human immunoglobulin or antibody and complementarity determining regions (e.g., at least one CDR) substantially derived from a non-human immunoglobulin or antibody.

As used herein, an "inflammatory signaling inhibitor" or "ISI" is an agent that decreases the binding between a pro-inflammatory cytokine (e.g., TNF-alpha, TNF-beta, or IL-1) and its receptor (e.g., TNF receptor 1 or 2, or IL-1 receptor, respectively); decreases the binding of activating molecules to pro-inflammatory cell surface signaling molecules (e.g., CD20, CD25, CTLA-4, CD80/CD86, or CD28); or decreases the downstream activation of, or activity of, intracellular signaling molecules that are activated following the binding of pro-inflammatory cytokines to their receptors or the binding of activating molecules to pro-inflammatory cell surface signaling molecules (e.g., an agent that decreases the activation of, or activity of, signaling molecules in the p38 MAPK signaling pathway). Preferably, the decrease in binding between a pro-inflammatory cytokine and its receptor, the decrease in binding of an activating molecule to a pro-inflammatory cell surface signaling molecule, or the decrease in intracellular signaling which occurs following the binding of pro-inflammatory cytokines to their receptors or activating molecules to pro-inflammatory cell surface signaling molecules is by at least about 10%, preferably by 20%, 30%, 40%, or 50%, more preferably by 60%, 70%, 80%, 90% or more (up to 100%). An ISI may act by reducing the amount of pro-inflammatory cytokine (e.g., TNF-alpha, TNF-beta, or IL-1) freely available to bind the receptor. For example, an ISI may be a soluble pro-inflammatory cytokine receptor protein (e.g., a soluble TNF receptor fusion protein such as etanercept (ENBREL®) or lenercept), or a soluble pro-inflammatory cell surface signaling molecule (e.g., a soluble CTLA-4 (abatacept)), or an antibody directed against a pro-inflammatory cytokine or a pro-inflammatory cell surface signaling molecule (e.g., an anti-TNF antibody, such as adalimumab, certolizumab, infliximab, or golimumab; an anti-CD20 antibody, such as rituximab; or TRU-015 (TRUBION®)). In addition, an ISI may act by disrupting the ability of the endogenous wild-type pro-inflammatory cytokine or the pro-inflammatory cell surface signaling molecule to bind to its receptor (e.g., TNF receptor 1 or 2, IL-1 receptor, or CD11a (e.g., efalizumab (RAPTIVA®, Genentech))). Examples of dominant-negative TNF-alpha variants are XENP345 (a pegylated version of TNF variant A145R/I97T) and XproTM1595, and further variants disclosed in U.S. Patent Application Publication Nos. 20030166559 and 20050265962, herein incorporated by reference. An example of a dominant negative IL-1 variant is anakinra (KINERET®), which is a soluble form of IL-1 that binds to the IL-1 receptor without activating intracellular signaling pathways. Inflammatory signaling inhibitors, which can be used in the present invention, are also small molecules which inhibit or reduce the signaling pathways downstream of pro-inflammatory cytokine or pro-inflammatory cell surface

signaling molecules (e.g., DE 096). Examples of ISIs of this kind include inhibitors of p38 MAP kinase, e.g., 5-amino-2-carbonylthiopene derivatives (as described in WO 04/089929, herein incorporated); ARRY-797; BIRB 796 BS, (1-5-tert-butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-2(morpholin-4-yl-ethoxy)-naphtalen-1-yl]-urea); CHR-3620; CNI-1493; FR-167653 (Fujisawa Pharmaceutical, Osaka, Japan); ISIS 101757 (Isis Pharmaceuticals); ML3404; NPC31145; PDI69316; PHZ1112; RJW67657, (4-(4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl)-3-butyn-1-ol; SCIO-469; SB202190; SB203580, (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole); SB239063, trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl-methoxypyridimidin-4-yl)imidazole; SB242235; SD-282; SKF-86002; TAK 715; VX702; and VX745. Furthermore, an ISI may interfere with the processing of a pro-inflammatory cytokine (e.g., TNF-alpha and TNF-beta) from its membrane bound form to its soluble form. Inhibitors of TACE are ISIs of this class. Examples of inhibitors of TACE include BB-1101, BB-3103, BMS-561392, butynyloxyphenyl β-sulfone piperidine hydroxomates, CH4474, DPC333, DPH-067517, GM6001, GW3333, Ro 32-7315, TAPI-1, TAPI-2, and TMI 005. Additional examples of ISIs include short peptides derived from the *E. coli* heat shock proteins engineered for disease-specific immunomodulatory activity (e.g., dnaJP1).

By "integrin antagonist" is meant any agent that reduces or inhibits the biological activity of an integrin molecule (e.g., a reduction or inhibition of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or more relative to the biological activity in the absence of the integrin antagonist), such as the α4 subunit of an integrin molecule. The agent may act directly or indirectly on the α4 integrin subunit (NCBI Accession No. P13612; Takada *et al., EMBO J.* 8:1361-1368 (1989)) by inhibiting the activity or expression of the α4 integrin subunit, or may act on a target to which the intact integrin containing an α4 subunit binds. For example, an antibody or blocking peptide that binds to vascular cell adhesion molecule-1 (VCAM-1), thus preventing the binding of α4β1 integrin to VCAM-1 is considered an integrin antagonist for purposes of the present invention. Non-limiting exemplary integrin antagonists suitable for use with the present invention may include proteins, blocking peptides, antibodies, such as natalizumab (TYSABRI®), and small molecule inhibitors. Examples of α4 integrin antagonists include, but are not limited to, natalizumab (Elan/Biogen Idec; see, e.g., U.S. Patent Nos. 5,840,299; 6,033,665; 6,602,503; 5,168,062; 5,385,839; and 5,730,978; incorporated by reference herein), oMEPUPA-V (Biogen; U.S. Patent No. 6,495,525; incorporated by reference herein), alefacept, CDP-323 (Celltech); firategrast (SB-68399;

GlaxoSmithKline); TR-9109 (Pfizer); ISIS-107248 (Antisense Therapeutics); R-1295 (Roche); and TBC-4746 (Schering-Plough). Additional non-limiting examples of α4 integrin antagonists include the small molecules described in U.S. Patent Nos. 5,821,231; 5,869,448; 5,936,065; 6,265,572; 6,288,267; 6,365,619; 6,423,728; 6,426,348; 6,458,844; 6.479,666; 6,482,849; 6,596,752; 6,667,331; 6,668,527; 6,685,617; 6,903,128; and 7,015,216 (each herein incorporated by reference); in U.S. Patent Application Publication Nos. 2002/0049236; 2003/0004196; 2003/0018016; 2003/0078249; 2003/0083267; 2003/0100585; 2004/0039040; 2004/0053907; 2004/0087574; 2004/0102496; 2004/0132809; 2004/0229858; 2006/0014966; 2006/0030553; 2006/0166866; 2006/0166961; 2006/0241132; 2007/0054909; and 2007/0232601 (each herein incorporated by reference); in European Patent Nos. EP 0842943; EP 0842944; EP 0842945; EP 0903353; and EP 0918059; and in PCT Publication Nos. WO 95/15973; WO 96/06108; WO 96/40781; WO 98/04247; WO 98/04913; WO 98/42656; WO 98/53814; WO 98/53817; WO 98/53818; WO 98/54207; WO 98/58902; WO 99/06390; WO 99/06431; WO 99/06432; WO 99/06433; WO 99/06434; WO 99/06435; WO 99/06436; WO 99/06437; WO 99/10312; WO 99/10313; WO 99/20272; WO 99/23063; WO 99/24398; WO 99/25685; WO 99/26615; WO 99/26921; WO 99/26922; WO 99/26923; WO 99/35163; WO 99/36393; WO 99/37605; WO 99/37618; WO 99/43642; WO 01/42215; and WO 02/28830; all of which are incorporated by reference herein. Additional examples of α4 integrin antagonists include the phenylalanine derivatives described in: U.S. Patent Nos. 6,197,794; 6,229,011; 6,329,372; 6,388,084; 6,348,463; 6,362,204; 6,380,387; 6,445,550; 6,806,365; 6,835,738; 6,855,706; 6,872,719; 6,878,718; 6,911,451; 6,916,933; 7,105,520; 7,153,963; 7,160,874; 7,193,108; 7,250,516; and 7,291,645 (each herein incorporated by reference). Additional amino acid derivatives that are α4 integrin antagonists include those described in, e.g., U.S. Patent Application Publication Nos. 2004/0229859 and 2006/0211630 (herein incorporated by reference), and PCT Publication Nos. WO 01/36376; WO 01/47868; and WO 01/70670; all of which are incorporated by reference herein. Other examples of α4 integrin antagonists include the peptides, and the peptide and semi-peptide compounds described in, e.g., PCT Publication Nos. WO 94/15958; WO 95/15973; WO 96/00581; WO 96/06108; WO 96/22966 (Leu-Asp-Val tripeptide; Biogen, Inc.); WO 97/02289; WO 97/03094; and WO 97/49731. An additional example of an α4 integrin antagonist is the pegylated molecule described in U.S. Patent Application Publication No. 2007/066533 (herein incorporated by reference). Examples of antibodies that are α4 integrin antagonists include those described in, e.g.,

PCT Publication Nos. WO 93/13798; WO 93/15764; WO 94/16094; and WO 95/19790. Additional examples of α4 integrin antagonists are described herein.

By "interferon" is meant a mammalian (e.g., a human) interferon -alpha, -beta, -gamma, or –tau polypeptide, or biologically-active fragment thereof, e.g., IFN-α (e.g., IFN-α-1a; see U.S. Patent Application No. 20070274950, incorporated herein by reference in its entirety), IFN-α-1b, IFN-α-2a (see PCT Application No. WO 07/044083, herein incorporated by reference in its entirety), and IFN-α-2b), IFN-β (e.g., described in U.S. Patent No. 7,238,344, incorporated by reference in its entirety; IFN-b-1a (AVONEX® and REBIF®), as described in U.S. Patent No. 6,962,978, incorporated by reference in its entirety, and IFN-β-1b (BETASERON®, as described in U.S. Patent Nos. 4,588,585; 4,959,314; 4,737,462; and 4,450,103; incorporated by reference in their entirety), IFN-g, and IFN-t (as described in U.S. Patent No. 5,738,845 and U.S. Patent Application Publication Nos. 20040247565 and 20070243163; incorporated by reference in their entirety).

By "HSA linker conjugate" is meant a human serum albumin (HSA) linker protein of the invention in combination with one or more binding moieties, polypeptide connectors, diagnostic agents, or therapeutic agents.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Monoclonal antibodies can be prepared using any art recognized technique and those described herein such as, for example, a hybridoma method, as described by Kohler *et al.*, *Nature* 256:495 (1975), a transgenic animal (*e.g.*, Lonberg *et al., Nature* 368(6474):856-859 (1994)), recombinant DNA methods (*e.g.*, U.S. Pat. No. 4,816,567), or using phage, yeast, or synthetic scaffold antibody libraries using the techniques described in, for example, Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991).

By "pharmaceutically acceptable carrier" is meant a carrier which is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline.

Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in *Remington's Pharmaceutical Sciences*, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA incorporated herein by reference.

By "proliferative disease" or "cancer" is meant any condition characterized by abnormal or unregulated cell growth. Examples of proliferative diseases include, for example, solid tumors such as: sarcomas (e.g., clear cell sarcoma), carcinomas (e.g., renal cell carcinoma), and lymphomas; tumors of the breast, colon, rectum, lung, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, bilecyst, bile duct, small intestine, urinary system (including the kidney, bladder, and epithelium of the urinary tract), female genital system (including the uterine neck, uterus, ovary, chorioma, and gestational trophoblast), male genital system (including the prostate, seminal vesicle, and testicles), endocrine glands (including the thyroid gland, adrenal gland, and pituitary body), skin (including angioma, melanoma, sarcoma originating from bone or soft tissue, and Kaposi's sarcoma), brain and meninges (including astrocytoma, neuroastrocytoma, spongioblastoma, retinoblastoma, neuroma, neuroblastoma, neurinoma and neuroblastoma), nerves, eyes, hemopoietic system (including chloroleukemia, plasmacytoma and dermal T lymphoma/leukemia), and immune system (including lymphoma, e.g., Hodgkin's lymphoma and non-Hodgkin's lymphoma). An example of a non-solid tumor proliferative disease is leukemia (e.g., acute lymphoblastic leukemia).

The term "recombinant antibody," refers to an antibody prepared, expressed, created, or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal for immunoglobulin genes (e.g., human immunoglobulin genes) or a hybridoma prepared therefrom, (b) antibodies isolated from a host cell transformed to express the antibody, e.g., from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial antibody library (e.g., containing human antibody sequences) using phage, yeast, or synthetic scaffold display, and (d) antibodies prepared, expressed, created, or isolated by any other means that involve splicing of immunoglobulin gene sequences (e.g., human immunoglobulin genes) to other DNA sequences.

By "specifically bind" is meant the preferential association of a binding moiety (e.g., an antibody, antibody fragment, receptor, ligand, or small molecule portion of an agent of the invention) to a target molecule (e.g., a secreted target molecule, such as a cytokine, chemokine, hormone, receptor, or ligand) or to a cell or tissue bearing the target molecule (e.g., a cell surface antigen, such as a receptor or ligand) and not to non-target cells or tissues lacking the target molecule. It is

recognized that a certain degree of non-specific interaction may occur between a binding moiety and a non-target molecule (present alone or in combination with a cell or tissue). Nevertheless, specific binding may be distinguished as mediated through specific recognition of the target molecule. Specific binding results in a stronger association between the binding moiety (e.g., an antibody) and e.g., cells bearing the target molecule (e.g., an antigen) than between the binding moiety and e.g., cells lacking the target molecule. Specific binding typically results in greater than 2-fold, preferably greater than 5-fold, more preferably greater than 10-fold and most preferably greater than 100-fold increase in amount of bound binding moiety (per unit time) to e.g., a cell or tissue bearing the target molecule or marker as compared to a cell or tissue lacking that target molecule or marker. Binding moieties bind to the target molecule or marker with a dissociation constant of e.g., less than 10^{-6}M, more preferably less than 10^{-7}M, 10^{-8}M, 10^{-9}M, 10^{-10}M, 10^{-11}M, or 10^{-12}M, and most preferably less than 10^{-13}M, 10^{-14}M, or 10^{-15}M. Specific binding to a protein under such conditions requires a binding moiety that is selected for its specificity for that particular protein. A variety of assay formats are appropriate for selecting binding moieties (e.g., antibodies) capable of specifically binding to a particular target molecule. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow & Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The term "substantial identity" or "substantially identical," when used in the context of comparing a polynucleotide or polypeptide sequence to a reference sequence, means that the polynucleotide or polypeptide sequence is the same as the reference sequence or has a specified percentage of nucleotides or amino acid residues that are the same at the corresponding locations within the reference sequence when the two sequences are optimally aligned. For instance, an amino acid sequence that is "substantially identical" to a reference sequence has at least about 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher percentage identity (up to 100%) to the reference sequence (e.g., the HSA amino acid sequence as set forth in SEQ ID NO:1, or a fragment thereof), when compared and aligned for maximum correspondence over the full length of the reference sequence as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters, or by manual alignment and visual inspection (see, e.g., NCBI web site).

By "surface-exposed amino acid residue" or "surface-exposed" is meant an amino acid residue that is present on the exterior face of the folded and conformationally-correct tertiary structure of a HSA polypeptide. Such residues can be substituted with e.g., other, chemically-reactive, amino acids (e.g., cysteine) to allow for site-specific conjugation of diagnostic or therapeutic agents. Additionally, surface-exposed amino acid residues can be substituted to allow (e.g., by addition of serine, threonine, or asparagine residues, or glycosylation motifs) or prevent (e.g., by removal of serine, threonine, or asparagine residues, or glycosylation motifs) glycosylation. Surface-exposed amino acid residues include, but are not limited to, threonine at position 496, serine at position 58, threonine at position 76, threonine at position 79, threonine at position 83, threonine at position 125, threonine at position 236, serine at position 270, serine at position 273, serine at position 304, serine at position 435, threonine at position 478, threonine at position 506, and threonine at position 508 (amino acid numbering is relative to e.g., the sequence of the HSA linker set forth in SEQ ID NO:1). Other surface-exposed residues can be identified by the skilled artisan using the HSA crystal structure (Sugio *et al.*, "Crystal structure of human serum albumin at 2.5 A resolution," *Protein Eng.* 12:439-446 (1999)).

A "target molecule" or "target cell" is meant a molecule (e.g., a protein, epitope, antigen, receptor, or ligand) or cell to which a binding moiety (e.g., an antibody), or an HSA conjugate that contains one or more binding moieties (e.g., an HSA linker bonded to one or more antibodies or antibody fragments) can specifically bind. A target molecule can be present on the exterior of a target cell (e.g., a cell-surface or secreted protein) or in the interior of a target cell.

By "treating" is meant the reduction (e.g., by at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or even 100%) in the progression or severity of a human disease or disorder (e.g., an autoimmune or proliferative disease), or in the progression, severity, or frequency of one or more symptoms of the human disease or disorder in a human patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an illustration showing the schematic representation of an exemplary HSA linker conjugate. The connector between the amino terminal binding moiety and the HSA linker has a sequence of alanine, alanine and serine. The connector between the HSA linker and the carboxy terminal binding moiety has a sequence of alanine, alanine, alanine, leucine (SEQ ID NO:5).

Figure 2 is a graph showing that B2B3 variants inhibit HRG-induced pErbB3 in ZR75-1 breast cancer cells.

Figures 3A-D are graphs showing the inhibition of phosphorylated ErbB3 in BT474 breast cancer cells following 24 hour pre-treatment with the B2B3 variants A5-HSA-B1D2 (Figure 3A), H3-HSA-B1D2 (Figure 3B), H3-HSA-F5B6H2 (Figure 3C), and F4-HSA-F5B6H2 (Figure 3D).

Figures 4A-D are graphs showing the inhibition of phosphorylated AKT in BT474 breast cancer cells following 24 hour pre-treatment with the B2B3 variants A5-HSA-B1D2 (Figure 4A), H3-HSA-B1D2 (Figure 4B), H3-HSA-F5B6H2 (Figure 4C), and F4-HSA-F5B6H2 (Figure 4D).

Figures 5A-D are graphs showing that inhibition of phosphorylated ERK in BT474 breast cancer cells following 24 hour pre-treatment with the B2B3 variants A5-HSA-B1D2 (Figure 5A), H3-HSA-B1D2 (Figure 5B), H3-HSA-F5B6H2 (Figure 5C), and F4-HSA-F5B6H2 (Figure 5D).

Figure 6 is a graph showing that treatment of BT474 breast cancer cells with B2B3-1 variants causes G1 arrest and a decrease in the number of cells in S phase.

Figure 7 is a flow cytometry histogram showing that pre-incubation of BT-474-M3 cells with 1 μM B2B3-1 substantially blocks binding of HRG.

Figures 8A-D are graphs showing that B2B3-1 inhibits ErbB3 and AKT phosphorylation in B-T474-M3 and ZR75-30 cell lines. Breast cancer cell lines BT-474-M3 (Figures 8A and 8C) and ZR75-3 0 (Figures 8B and 8D) were pre-treated with a dose titration of B2B3-1 for 24 hours and then stimulated for 10 minutes with 5 nM of HRG 1β EGF domain. The phosphorylation status of ErbB3 and AKT was then examined using an ELISA assay.

Figure 9 is a photograph of a Western blot that shows the effect of B2B3-1 treatment on signaling proteins in BT474 breast cancer cells.

Figure 10 is a photograph of a Western blot that shows the immunoprecipitation of B2B3-1 treated BT474 breast cancer cells.

Figures 11A-C are graphs showing B2B3-1 treatment of BT-474 cell line causes G1 arrest and a decrease in the population of cells in S phase (Figure 11A), and inhibits colony formation in both BT-474 and SKBr3 cells compared to untreated cells (Figure 11B). In addition, B2B3-1 inhibits proliferation of BT-474-M3 cells in a cell impedance assay (Figure 11C).

Figure 12 is a graph showing that B2B3-1 does not stimulate ErbB3 phosphorylation in ZR75-1 cells.

Figures 13A-B are graphs showing that B2B3-1 binds specifically to ErbB3 (Figure 13A) and ErbB2 (Figure 13B).

Figure 14 is a graph showing that avidity binding of B2B3-1 to MALME-3 cells results in a significant increase in apparent binding affinity compared to ErbB2-only binding variant, SKO-3, and ErbB3-only binding variant, SKO-2.

Figures 15A-C are graphs showing the stability of B2B3-1 in mouse, Cynomolgus monkey, and human serum. 100 nM B2B3-1 was incubated in mouse (Figure 15A), Cynomolgus monkey (Figure 15B), or human (Figure 15C) serum at 37°C for a period of 120 hours. Samples were removed at 0, 24, 48, 72, 96 and 120 hours and the ability of B2B3-1 to bind both ErbB2 and ErbB3 was measured by ELISA (RLU = relative light units).

Figures 16 is a graph showing B2B3-1 dose response in a BT-474-M3 breast cancer xenograft model. The relationship of B2B3-1 dose on tumor response was assessed in the BT-474-M3 breast tumor line at the doses indicated. HSA was given at 52.5 mg/kg, which is an equimolar dose to the 90 mg/kg B2B3-1 dose.

Figures 17A-E are graphs showing that B2B3-1 is effective in multiple xenograft models in an ErbB2 dependent manner. Calu-3 (human lung adenocarcinoma; Figure 17A), SKOV-3 (human ovarian adenocarcinoma; Figure 17B), NCI-N87 (human gastric carcinoma; Figure 17C), ACHN (human kidney adenocarcinoma; Figure 17D), and MDA-MB-361 (human breast adenocarcinoma; Figure 17E) xenograft model are shown. Mice were treated with 30 mg/kg of B2B3-1 every 3 days or HAS control at an equimolar dose to B2B3-1.

Figures 18A-B are graphs showing that the over-expression of ErbB2 converts B2B3-1 non-responder ADRr breast cancer xenograft model into a responder. ErbB2 was over-expressed in wild type ADRr xenografts (Figure 18A) and ADRr-E2 xenografts (Figure 18B) using a retroviral expression system.

Figures 19A-B are graphs showing that B2B3-1 activity correlates positively with ErbB2 expression levels *in vitro* (Figure 19A) and *in vivo* (Figure 19B).

Figure 20A-B show that B2B3-1 treatment modifies tumor cell cycling. Figure 20A includes fluorescent micrographs showing that B2B3-1 treatment of BT474-M3 breast tumor cells for 6 hours results in translocation of cell cycle inhibitor $p27^{kip1}$ to the nucleus. Hoechst stain was used to identify the nucleus. Figure 20B is a Western blot of BT-474-M3 cells treated with B2B3-1 for 72

hours, which resulted in a decrease in the levels of the cell cycle regulator Cyclin D1. The cytoskeleton protein vinculin was probed as a protein loading control in this experiment.

Figures 21A-B are micrographs showing that B2B3-1 treatment of BT474 breast tumor xenografts results in translocation of $p27^{kip1}$ to the nucleus. BT474 breast tumor xenografts were treated with B2B3-1 (Figure 21A) at a dose of 30 mg/kg or an equimolar dose of HSA (Figure 21B) every 3 days for a total of 4 doses and stained for $p27^{kip1}$.

Figure 22A-B are fluorescent micrographs showing that B2B3-1 treatment results in a reduction of the proliferation marker Ki67 in BT474-M3 breast cancer xenograft. BT474-M3 breast tumor xenografts were treated with B2B3-1 (Figure 22A) at a dose of 30 mg/kg or an equimolar dose of HSA (Figure 22B) every 3 days for a total of 4 doses.

Figures 23A-B are fluorescent micrographs showing that B2B3-1 treatment results in a reduction of vessel density in BT474-M3 breast cancer xenograft tumors (as determined by a reduction in CD31 staining). BT474-M3 breast tumor xenografts were treated with B2B3-1 (Figure 23A) at a dose of 30 mg/kg or an equimolar dose of HSA (Figure 23B) every 3 days for a total of 4 doses.

Figures 24A-B are graphs showing that B2B3-1 inhibits phosphorylation of ErbB3 *in vivo*. Lysates from individual BT-474-M3 xenograft tumors treated with B2B3-1 (M1-M5) or control HSA (H1-H2) were subjected to SDS-PAGE and probed for pErbB3 and beta tubulin using Western blot analysis (Figure 24A). Normalization of the mean pErbB3 signal to the mean beta tubulin signal demonstrated that B2B3-1 treated tumors contained significantly less pErbB3 than HSA tumors (Figure 24B).

Figures 25A and B are graphs showing the *in vivo* activity of B2B3-1 in BT-474-M3 xenografts which have reduced PTEN activity. Cultured BT-474-M3 tumor cells were transfected with a control vector (Figure 25A) or with a retroviral vector encoding shPTEN (Figure 25B), which knocks out PTEN activity. BT-474-M3 breast cancer cells engineered to knock out PTEN activity were injected into the right flank of mice, while cells transfected with control vector were injected into the left flank of the same mouse. Mice were treated with 30mg/kg B2B3-1 every 3 days or 10mg/kg Herceptin every week and HSA was injected as a control at an equimolar dose to B2B3-1. B2B3-1 and Herceptin promoted a reduction in the size of tumors formed by control BT-474-M3 breast cancer cells (Figure 25A), whereas only B2B3-1

(and not Herceptin) promoted a reduction in the size of tumors formed by BT-474-M3 breast cancer cells lacking expression of PTEN (Figure 25B).

Figures 26A-B show that B2B3-1 inhibits phosphorylation of AKT in BT-474-M3 xenografts that have reduced PTEN activity. Tumors were lysed following the completion of treatment (q3dx11) and tested for PTEN, pErbB3, and pAKT expression levels by Western blot analysis (Figure 26A). Densitometry on the band intensity for pAKT normalized to total AKT and total protein demonstrate that B2B3-1 was able to inhibit phosphorylation of this protein, when Herceptin did not (Figure 26B).

Figures 27A-D are graphs showing the single dose pharmacokinetic properties of 5 (Figure 27A), 15 (Figure 27B), 30 (Figure 27C), and 45 (Figure 27D) mg/kg bolus dose of B2B3-1 in nu/nu mice. B2B3-1 serum concentrations are comparable measured by the HSA assay or ErbB2/ErbB3 assay, indicating that the antigen binding activity of B2B3-1 is retained in circulation.

Figure 28 is a graph showing the dose-exposure relationship for 5, 15, 30, and 45 mg/kg bolus doses of B2B3-1 in nude mice. Increases in dose result in a linear increase in overall exposure to B2B3-1.

Figure 29 is a graph showing the B2B3-1 serum concentrations measured in Cynomolgus monkeys dosed every three days for 4 doses with 4 mg/kg (n=2), 20 mg/kg (n=2) and 200 mg/kg (up to 336 hour n=4, for 384, 552 and 672 hour time points n=2).

Figure 30 is an illustration of the B2B3-1 expression plasmid pMP10k4H3-mHSA-B1D2.

Figure 31 is an illustration of the neomycin resistance plasmid pSV2-neo.

Figure 32 is an illustration of the hygromycin resistance plasmid pTK-Hyg.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a human serum albumin (HSA) linker which, when used to produce an agent of the invention (e.g., a binding, diagnostic, or therapeutic agent) exhibits, for example, an increased *in vivo* half-life of between 6 hours and 7 days and which does not induce significant humoral or cell-mediated immune responses when administered *in vivo* to a mammal (e.g., a human). In one aspect, the invention provides a mutated HSA linker that has two defined amino acid substitutions (i.e., the "C34S" and "N503Q" substitutions, as set forth in SEQ ID NO:1). In another aspect, the invention provides an HSA linker bonded to one or more binding moieties (e.g.,

antibodies, antibody fragments, receptor/ligands, or small molecules) for diagnostic or therapeutic applications in a mammal (e.g., a human) *in vivo* or for use *in vitro* in connection with mammalian cells, tissues, or organs. In a further aspect, the HSA linker may be coupled to one or more immunomodulatory agents, cytotoxic or cytostatic agents, detectable labels, or radioactive agents for diagnostic or therapeutic applications in a mammal (or in connection with a mammalian cell, tissue, or organ). An agent of the invention, which includes the HSA linker, can be optionally combined with one or more pharmaceutically acceptable carriers or excipients and can be formulated to be administered intravenously, intramuscularly, orally, by inhalation, parenterally, intraperitoneally, intraarterially, transdermally, sublingually, nasally, through use of suppositories, transbuccally, liposomally, adiposally, opthalmically, intraocularly, subcutaneously, intrathecally, topically, or locally. An agent of the invention which includes the HSA linker can be combined or coadministered with one or more biologically-active agents (e.g., biological or chemical agents, such as chemotherapeutics and antineoplastic agents). In a further aspect, the invention provides a kit, with instructions, for the conjugation of binding moieties (e.g., antibodies, antibody fragments, receptors or ligands), immunomodulatory agents, cytotoxic or cytostatic agents, detectable labels, or radioactive agents to the HSA linker of the invention to prepare agents of the invention that can be used for diagnostic or therapeutic applications.

A Mutated Human Serum Albumin (HSA) Linker

A mutated HSA linker of the invention contains two amino acid mutations, at positions 34 and 503, relative to the wild-type HSA amino acid sequence set forth in SEQ ID NO:3. The cysteine residue at position 34 (i.e., C34) can be mutated to any amino acid residue other than cysteine (e.g., serine, threonine, or alanine). Likewise, the asparagine residue at position 503 (i.e., N503) can be mutated to any amino acid residue other than asparagine (e.g., glutamine, serine, histidine, or alanine). In one embodiment, the HSA linker of the invention has the the amino acid and corresponding nucleotide sequence set forth in SEQ ID NOS:1 and 2, respectively. This mutated HSA linker of the invention contains two amino acid substitutions (i.e., serine for cysteine at amino acid residue 34 ("C34S") and glutamine for asparagine at amino acid residue 503 ("N503Q")). The HSA linker, when bonded to one or more binding moieties (e.g., antibodies, antibody fragments (e.g., single chain antibodies), or other targeting or biologically active agents (e.g., receptors and ligands)), confers several beneficial pharmacologic properties to those conjugates and to additional

diagnostic or therapeutic agents also conjoined (e.g., immunomodulatory agents, cytotoxic or cytostatic agents, detectable labels, or radioactive agents)) relative to the pharmacologic properties of these agents in the absence of the HSA linker of the invention. These benefits can include decreased immunogenicity (e.g., decreased host antibody neutralization of linker-antibody conjugates), increased detection of HSA linker conjugates (e.g., by mass spectroscopy) and increased pharmacologic half-life (e.g., a half-life greater than 6 hours, 8 hours, 12 hours, 24 hours, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days) when administered to a mammal (e.g., a human). Specifically, the substitution of serine for cysteine at amino acid residue 34 results in reduced oxidation and protein heterogeneity of the HSA linker. In wild-type HSA, the asparagine at amino acid residue 503 is sensitive to deamination, likely resulting in reduced pharmacologic half-life. The substitution of glutamine for asparagine at amino acid residue 503 can result in increased pharmacologic half-life of the HSA linker of the invention, and correspondingly, of conjugate agents that include the HSA linker when administered to a mammal (e.g., a human) or cells, tissues, or organs thereof.

In other embodiments, the mutated HSA linker of the invention includes domain I of HSA (SEQ ID NO:53; residues 1-197 of SEQ ID NO:1), domain III of HSA (SEQ ID NO:55; residues 381-585 of SEQ ID NO:1), combination of domains I and III of HSA, or a combination of domain I or III of HSA with domain II of HSA (SEQ ID NO:54; residues 189-385 of SEQ ID NO:1). For example, an HSA linker of the invention can include domains I and II, I and III, or II and III. In addition, the cysteine residue at position 34 (i.e., C34) of domain I (SEQ ID NO:53) can be mutated to any amino acid residue other than cysteine (e.g., serine, threonine, or alanine). Likewise, the asparagine residue at position 503 (i.e., N503) of domain III (SEQ ID NO:55) can be mutated to any amino acid residue other than asparagine (e.g., glutamine, serine, histidine, or alanine). These HSA linkers can be incorporated into an HSA linker conjugate of the invention, which includes one or more of a polypeptide connector, a binding moiety, and therapeutic or diagnostic agents, each of which is described in detail below.

Polypeptide Connectors

To facilitate the conjugation of binding moieties, as defined herein, to the HSA linker of the invention, short (e.g., 2-20 amino acids in length) polypeptide connectors that can be bonded (e.g, covalently (e.g., a peptidic bond), ionically, or hydrophobically bonded, or via a high-affinity

protein-protein binding interaction (e.g., biotin and avidin)) to the amino or carboxy termini of an HSA linker. These connectors provide flexible tethers to which any of the binding moieties described herein can be attached. A polypeptide connector may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acids in length. In one embodiment, the connector is a sequence of, e.g., glycine, alanine, serine, glutamine, leucine, or valine residues. Although not specifically enumerated herein, the connector of the invention can be solely glycine, alanine, serine, glutamine, leucine, or valine residues, or it may be any combination of these residues up to about 20 amino acids in length. In a preferred embodiment, the connector attached to the amino terminus of an HSA linker has the amino acid sequence "AAS" or "AAQ" and the connector attached to the carboxy terminus has the amino acid sequence "AAAL" (SEQ ID NO:5). The connector can be covalently bound to the amino or carboxy terminal residue of the HSA linker, to an amino acid residue within the HSA linker, or can be included between one or more binding moietics, if present.

HSA Linker Manufacture

The HSA linker of the invention, which may optionally include the peptide connectors described above, one or more of the binding moieties described below, polypeptide-based detectable labels, and other polypeptide-based therapeutic agents, can be produced recombinantly. For example, a nucleotide sequence encoding the HSA linker (and one or more of the optional elements) may be expressed (e.g., in a plasmid, viral vector, or transgenically) in a bacterial (e.g., *E. coli*), insect, yeast, or mammalian cell (e.g., a CHO cell), or a mammalian tissue, organ, or organism (e.g., a transgenic rodent, ungulate (e.g., a goat), or non-human primate). After expression of the HSA linker in the host cell, tissue, or organ, the skilled artisan may isolate and purify the HSA linker using standard protein purification methods (e.g., FPLC or affinity chromatography). A recombinant expression system for the production of an HSA linker in combination with two binding moieties is illustrated in Figure 1.

The HSA linker of the invention, which may include one or more of the optional elements described above, can be synthetically produced. For example, the HSA linker can be prepared by techniques generally established in the art of peptide synthesis, such as the solid-phase approach. Solid-phase synthesis involves the stepwise addition of amino acid residues to a growing peptide chain that is linked to an insoluble support or matrix, such as polystyrene. The C-terminus residue of the peptide is first anchored to a commercially available support with its amino group protected with

an N-protecting agent such as a t-butyloxycarbonyl group (tBoc) or a fluorenylmethoxycarbonyl (FMOC) group. The amino-protecting group is removed with suitable deprotecting agents such as TFA in the case of tBOC or piperidine for FMOC and the next amino acid residue (in N-protected form) is added with a coupling agent such as dicyclocarbodiimide (DCC). Upon formation of a peptide bond, the reagents are washed from the support. After addition of the final residue, the agent is cleaved from the support with a suitable reagent, such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF). If desired, the HSA linker, which may include one or more of the optional elements described above, can be manufactured in one, two, three, or more segments, which can then be ligated to form the whole HSA linker construct.

HSA Linker Binding Moieties

The HSA linker of the invention can also be prepared as a construct that includes one or more binding moieties, such as antibodies, antibody fragments (as defined herein, e.g., a single chain Fv (scFv)) or receptor/ligands (i.e., protein or glycoprotein ligands or receptors) that allow selective and specific binding of the HSA linker construct to a target cell, tissue, or organ. The binding moieties can be bonded to the HSA linker (e.g., via a covalent (e.g., a peptide bond), ionic, or hydrophobic bond, or via a high-affinity protein-protein binding interaction (e.g., biotin and avidin)). As is discussed above, an HSA linker of the invention that includes one or more polypeptide connectors or binding moieties can be recombinantly or synthetically produced.

One or more binding moieties can be bonded to an HSA linker of the invention. In one embodiment, two or more identical binding moieties (i.e., moieties having the same structure and binding affinities) are bonded to an HSA linker, one or more (e.g., in tandem) each at the amino and carboxy termini, the HSA linker thereby affording improved avidity of the binding moieties for their target antigen. Alternatively, two or more different binding moieties (e.g., an antibody, such as a scFv, with binding affinities for two or more different target molecules, or scFv with binding affinities for two or more different epitopes on the same target molecule) can be bonded to an HSA linker (e.g., a bispecific HSA linker conjugate) to allow multiple target antigens or epitopes to be bound by the HSA linker conjugate. In another embodiment, different species of binding moieties can also be bonded to an HSA linker to bestow, for example, two or more different binding specificities or agonistic/antagonistic biological properties on the linker conjugate. Useful combinations of binding moiety pairs for use in the preparation of bispecific HSA linker conjugates

are disclosed in, e.g., International Patent Application Publications WO 2006/091209 and WO 2005/117973, herein incorporated by reference. In other embodiments, more than two binding moieties (e.g., the same or different binding moieties) can be bonded to an HSA linker to form an agent of the invention.

The invention features an agent having at least first and second binding moieties, each of which can be bound at either the amino or carboxy terminus of the HSA linker protein, or to polypeptide connectors, as defined herein, present at either or both termini. Figure 1 illustrates an exemplary mutated HSA linker of the invention in which two binding moieties ("arm 1" and "arm 2") are bonded to the mutated HSA linker by the amino terminal polypeptide connector AAS and carboxy terminal polypeptide connector AAAL (SEQ ID NO:5). Binding moieties (e.g., an antibody or scFv) can also be bound to other loci (e.g., internal amino acid residues of the HSA linker), for example, covalently or ionically, e.g., using biotin-avidin interactions. Biotinylation of amine (e.g., lysine residues) and sulfhydryl (e.g., cysteine residues) amino acid side chains is known in the art and can be used to attach binding moieties to the HSA linker.

Binding moieties that can be included in an HSA linker conjugate of the invention include antibodies, antibody fragments, receptors, and ligands. Binding moieties bound to an HSA linker of the invention may be recombinant (e.g., human, murine, chimeric, or humanized), synthetic, or natural. The invention features complete antibodies, domain antibodies, diabodies, bi-specific antibodies, antibody fragments, Fab fragments, F(ab')2 molecules, single chain Fv (scFv) molecules, tandem scFv molecules, antibody fusion proteins, and aptamers.

Antibodies

Antibodies of the invention include the IgG, IgA, IgM, IgD, and IgE isotypes. Antibodies or antibody fragments of the invention, as used herein, contain one or more complementarity determining regions (CDR) or binding peptides that bind to target proteins, glycoproteins, or epitopes present on the exterior or in the interior of target cells.

Many of the antibodies, or fragments thereof, described herein can undergo non-critical amino-acid substitutions, additions or deletions in both the variable and constant regions without loss of binding specificity or effector functions, or intolerable reduction of binding affinity (e.g., below about 10^{-7} M). Usually, an antibody or antibody fragment incorporating such alterations exhibits substantial sequence identity to a reference antibody or antibody fragment from which it is derived.

Occasionally, a mutated antibody or antibody fragment can be selected having the same specificity and increased affinity compared with a reference antibody or antibody fragment from which it was derived. Phage-display technology offers powerful techniques for selecting such antibodies. See, e.g., Dower *et al.*, WO 91/17271 McCafferty *et al.*, WO 92/01047; and Huse, WO 92/06204, incorporated by reference herein.

The HSA linker of the invention can also be bonded to one or more fragments of an antibody that retain the ability to bind with specificity to a target antigen. Antibody fragments include separate variable heavy chains, variable light chains, Fab, Fab', $F(ab')_2$, Fabc, and scFv. Fragments can be produced by enzymatic or chemical separation of intact immunoglobulins. For example, a $F(ab')_2$ fragment can be obtained from an IgG molecule by proteolytic digestion with pepsin at pH 3.0-3.5 using standard methods such as those described in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Pubs., N.Y. (1988). Fab fragments may be obtained from $F(ab')_2$ fragments by limited reduction, or from whole antibody by digestion with papain in the presence of reducing agents. Fragments can also be produced by recombinant DNA techniques. Segments of nucleic acids encoding selected fragments are produced by digestion of full-length coding sequences with restriction enzymes, or by *de novo* synthesis. Often fragments are expressed in the form of phage-coat fusion proteins. This manner of expression is advantageous for affinity-sharpening of antibodies.

Humanized Antibodies

The invention further provides for humanized antibodies in combination with the HSA linker of the invention, in which one or more of the antibody CDRs are derived from a non-human antibody sequence, and one or more, but preferably all, of the CDRs bind specifically to an antigen (e.g., a protein, glycoprotein, or other suitable epitope).

A humanized antibody contains constant framework regions derived substantially from a human antibody (termed an acceptor antibody), as well as, in some instances, a majority of the variable region derived from a human antibody. One or more of the CDRs (all or a portion thereof, as well as discreet amino acids surrounding one or more of the CDRs) are provided from a non-human antibody, such as a mouse antibody. The constant region(s) of the antibody, may or may not be present.

The substitution of one or more mouse CDRs into a human variable domain framework is most likely to result in retention of their correct spatial orientation if the human variable domain framework adopts the same or a similar conformation as the mouse variable framework from which the CDRs originated. This is achieved by obtaining the human variable domains from human antibodies whose framework sequences exhibit a high degree of sequence and structural identity with the murine variable framework domains from which the CDRs were derived. The heavy and light chain variable framework regions can be derived from the same or different human antibody sequences. The human antibody sequences can be the sequences of naturally occurring human antibodies, consensus sequences of several human antibodies, or can be human germline variable domain sequences. See, e.g., Kettleborough *et al.*, *Protein Engineering* 4:773 (1991); Kolbinger *et al.*, *Protein Engineering* 6:971 (1993).

Suitable human antibody sequences are identified by alignments of the amino acid sequences of the mouse variable regions with the sequences of known human antibodies. The comparison is performed separately for heavy and light chains but the principles are similar for each.

Methods of preparing chimeric and humanized antibodies and antibody fragments are described in, e.g., U.S. Patent Nos. 4,816,567, 5,530,101, 5,622,701, 5,800,815, 5,874,540, 5,914,110, 5,928,904, 6,210,670, 6,677,436, and 7,067,313 and U.S. Patent Application Nos. 2002/0031508, 2004/0265311, and 2005/0226876. Preparation of antibody or fragments thereof is further described in, e.g., U.S. Patent Nos. 6,331,415, 6,818,216, and 7,067,313.

Receptors and Ligands

The invention provides for protein or glycoprotein receptors or ligands bound to an HSA linker of the invention. HSA linkers bonded with a receptor or ligand can be used, for example, to specifically target a secreted protein, a cell (e.g., a cancer cell), tissue, or organ. Furthermore, the specific binding of the HSA linker-receptor or ligand conjugate to cognate target receptors or ligands can cause agonistic or antagonistic biological activity in intracellular or intercellular signaling pathways. As with the other binding moieties described herein, receptors and ligands, or fragments thereof, can be conjoined to the amino and/or carboxy termini of an HSA linker of the invention, or to an amino acid residue within the HSA linker.

Exemplary receptors and ligands that can be joined to an HSA linker of the invention include, but are not limited to, insulin-like growth factor 1 receptor (IGF1R), IGF2R, insulin-like

growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met; also known as hepatocyte growth factor receptor (HGFR)), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, α4 integrin, P-selectin, sphingosine-1-phosphate receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-relatied apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, receptors alpha and beta, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), $Lewis^Y$, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and biologically-active fragments thereof.

Receptors and ligands can be expressed, isolated, or joined to an HSA linker of the invention using any of the methods described *supra* relating to antibodies or antibody fragments.

Diagnostic Agents

The HSA linker of the invention, or any binding moiety conjugated thereto (e.g., antibody, antibody fragment, receptor, or ligand), can be coupled to a chelating agent or to a detectable label to form a diagnostic agent of the invention. Also contemplated are HSA linker conjugates that include a detectable label, as described herein, as well as one or more of the therapeutic agents or binding moieties described herein.

The HSA linker and chelator components can be coupled to form a conjugate by reacting the free amino group of a threonine residue of the HSA linker with an appropriate functional group of the chelator, such as a carboxyl group or activated ester. For example, a conjugate may be formed by incorporating the chelator ethylenediaminetetraacetic acid (EDTA), which is common in the art of coordination chemistry, when functionalized with a carboxyl substituent on the ethylene chain.

Synthesis of EDTA derivatives of this type are reported in Arya *et al.* (*Bioconjugate Chemistry* 2:323 (1991)), which describes blocking each of the four coordinating carboxyl groups with a t-butyl group while the carboxyl substituent on the ethylene chain is free to react with the amino group of a peptide portion of the agent of the invention, thereby forming a conjugate.

An HSA linker or an HSA linker conjugate may incorporate a metal chelator component that is peptidic, i.e., compatible with solid-phase peptide synthesis. In this case, the chelator may be coupled to the HSA linker of the invention in the same manner as EDTA described above or, more conveniently, the chelator and HSA linker or HSA linker conjugate of the invention are synthesized *in toto* starting from the C-terminal residue of the HSA linker or HSA linker conjugate and ending with the N-terminal residue of the chelator.

An HSA linker or an HSA linker conjugate may further incorporate a linking group component that serves to couple the HSA linker of the invention to the chelator while not adversely affecting the biological properties of the HSA linker, the targeting function of the binding moiety portion(s) of the HSA linker conjugate, or the metal binding function of the chelator. Suitable linking groups include amino acid chains and alkyl chains functionalized with reactive groups for coupling to the HSA linker or the HSA linker conjugate and to the chelator. An amino acid chain is the preferred linking group when the chelator is peptidic so that the HSA linker or HSA linker conjugate can be synthesized *in toto* by solid-phase techniques. An alkyl chain-linking group may be incorporated in the HSA linker or HSA linker conjugate by reacting the amino group of a threonine residue of a peptide portion of an HSA linker of the invention with a first functional group on the alkyl chain, such as a carboxyl group or an activated ester. Subsequently the chelator is attached to the alkyl chain to complete the formation of the HSA linker or HSA linker conjugate by reacting a second functional group on the alkyl chain with an appropriate group on the chelator. The second functional group on the alkyl chain is selected from substituents that are reactive with a functional group on the chelator while not being reactive with a threonine residue of the mutated HSA linker protein. For example, when the chelator incorporates a functional group such as a carboxyl group or an activated ester, the second functional group of the alkyl chain-linking group can be an amino group. It will be appreciated that formation of the HSA linker or HSA linker conjugate may require protection and deprotection of the functional groups present in order to avoid formation of undesired products. Protection and deprotection are accomplished using protecting groups, reagents, and

protocols common in the art of organic synthesis. Particularly, protection and deprotection techniques employed in solid phase peptide synthesis described above may be used.

An alternative chemical linking group to an alkyl chain is polyethylene glycol (PEG), which is functionalized in the same manner as the alkyl chain described above for incorporation in the HSA linker or HSA linker conjugate. It will be appreciated that linking groups may alternatively be coupled first to the chelator and then to the HSA linker or HSA linker conjugate of the invention.

In accordance with one aspect of the invention, HSA linker-chelator conjugates of the invention incorporate a diagnostically useful metal capable of forming a complex. Suitable metals include, e.g., radionuclides, such as technetium and rhenium in their various forms (e.g., $^{99m}TcO^{3+}$, $^{99m}TcO_2^+$, ReO^{3+}, and ReO_2^+). Incorporation of the metal within the HSA linker or HSA linker conjugate can be achieved by various methods common in the art of coordination chemistry. When the metal is technetium-99 m, the following general procedure may be used to form a technetium complex. An HSA linker-chelator conjugate solution is formed initially by dissolving the HSA linker or HSA linker conjugate in aqueous alcohol such as ethanol. The solution is then degassed to remove oxygen then thiol protecting groups are removed with a suitable reagent, for example, with sodium hydroxide, and then neutralized with an organic acid, such as acetic acid (pH 6.0-6.5). In the labeling step, a stoichiometric excess of sodium pertechnetate, obtained from a molybdenum generator, is added to a solution of the conjugate with an amount of a reducing agent such as stannous chloride sufficient to reduce technetium and heated. The labeled HSA linker or HSA linker conjugate may be separated from contaminants $^{99m}TcO_4^-$ and colloidal $^{99m}TcO_2$ chromatographically, for example, with a C-18 Sep Pak cartridge.

In an alternative method, labeling of an HSA linker of the invention can be accomplished by a transchelation reaction. The technetium source is a solution of technetium complexed with labile ligands facilitating ligand exchange with the selected chelator. Suitable ligands for transchelation include tartarate, citrate, and heptagluconate. In this instance the preferred reducing reagent is sodium dithionite. It will be appreciated that the HSA linker or HSA linker conjugate may be labeled using the techniques described above, or alternatively the chelator itself may be labeled and subsequently coupled to an HSA linker protein of the invention to form an HSA linker-chelator conjugate; a process referred to as the "prelabeled ligand" method.

Another approach for labeling an HSA linker of the invention, or any agent conjugated thereto, involves immobilizing the HSA linker-chelator conjugate on a solid-phase support through a

linkage that is cleaved upon metal chelation. This is achieved when the chelator is coupled to a functional group of the support by one of the complexing atoms. Preferably, a complexing sulfur atom is coupled to the support which is functionalized with a sulfur protecting group such as maleimide.

When labeled with a diagnostically useful metal, an agent of the invention that includes an HSA linker-chelator conjugate can be used to detect tissue at risk of developing cancer (e.g., lung cancer, breast cancer, colon cancer, and prostate cancer), age-related diseases (e.g., cardiovascular disease, cerebrovascular disease, or Alzheimer's disease), tobacco-related diseases (e.g., emphysema, aortic aneurysms, esophageal cancer, or squamous cell cancer of the head and neck) by procedures established in the art of diagnostic imaging. An agent of the invention that incorporates an HSA linker labeled with a radionuclide metal, such as technetium-99 m, may be administered to a mammal (e.g., a human) by intravenous injection in a pharmaceutically acceptable solution, such as isotonic saline, or by other methods described herein. The amount of a labeled agent of the invention appropriate for administration is dependent upon the distribution profile of the chosen HSA linker or HSA linker conjugate in the sense that an agent of the invention that incorporates a rapidly cleared HSA linker or HSA linker conjugate may be administered at higher doses than an agent that incorporates an HSA linker or HSA linker conjugate that clears less rapidly. Unit doses acceptable for imaging tissues are in the range of about 5-40 mCi for a 70 kg individual. The *in vivo* distribution and localization of an agent of the invention that incorporates a labeled HSA linker or HSA linker conjugate can be tracked by standard techniques described herein at an appropriate time subsequent to administration, typically between 30 minutes and 180 minutes and up to about 5 days depending upon the rate of accumulation at the target site with respect to the rate of clearance at non-target tissue.

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be modified or labeled to facilitate diagnostic or therapeutic uses. Detectable labels such as a radioactive, fluorescent, heavy metal, or other molecules may be bound to any of the agents of the invention. Single, dual, or multiple labeling of an agent may be advantageous. For example, dual labeling with radioactive iodination of one or more residues combined with the additional coupling of, for example, ^{90}Y via a chelating group to amine-containing side or reactive groups, would allow combination labeling. This may be useful for specialized diagnostic needs such as identification of widely dispersed small neoplastic cell masses.

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be modified, for example, by halogenation of the tyrosine residues of the peptide component. Halogens include fluorine, chlorine, bromine, iodine, and astatine. Such halogenated agents may be detectably labeled, e.g., if the halogen is a radioisotope, such as, for example, ^{18}F, ^{75}Br, ^{77}Br, ^{122}I, ^{123}I, ^{124}I, ^{125}I, ^{129}I, ^{131}I, or ^{211}At. Halogenated agents of the invention contain a halogen covalently bound to at least one amino acid, and preferably to D-Tyr residues in each agent molecule. Other suitable detectable modifications include binding of other compounds (e.g., a fluorochrome such as fluorescein) to a lysine residue of the agent of the invention, or analog, particularly an agent or analog having a linker including lysines.

Radioisotopes for radiolabeling an HSA linker of the invention, or any molecule or moiety conjugated thereto, include any radioisotope that can be covalently bound to a residue of the peptide component of the agent of the invention or analog thereof. The radioisotopes can also be selected from radioisotopes that emit either beta or gamma radiation, or alternatively, any of the agents of the invention can be modified to contain chelating groups that, for example, can be covalently bonded to lysine residue(s) of the HSA linker or any peptidic agent conjugated thereto. The chelating groups can then be modified to contain any of a variety of radioisotopes, such as gallium, indium, technetium, ytterbium, rhenium, or thallium (e.g., ^{125}I, ^{67}Ga, ^{111}In, ^{99}mTc, ^{169}Yb, ^{186}Re).

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can be modified by attachment of a radioisotope. Preferable radioisotopes are those having a radioactive half-life corresponding to, or longer than, the biological half-life of the HSA conjugate used. More preferably, the radioisotope is a radioisotope of a halogen atom (e.g. a radioisotope of fluorine, chlorine, bromine, iodine, and astatine), even more preferably ^{75}Br, ^{77}Br, ^{76}Br, ^{122}I, ^{123}I, ^{124}I, ^{125}I, ^{129}I, ^{131}I, or ^{211}At.

An agent of the invention that incorporates an HSA linker, or any molecule or moiety conjugated thereto, can be coupled to radioactive metals and used in radiographic imaging or radiotherapy. Preferred radioisotopes also include ^{99m}Tc, ^{51}Cr, ^{67}Ga, ^{68}Ga, ^{111}In, ^{168}Yb, ^{140}La, ^{90}Y, ^{88}Y, ^{153}Sm, ^{156}Ho, ^{165}Dy, ^{64}Cu, ^{97}Ru, ^{103}Ru, ^{186}Re, ^{188}Re, ^{203}Pb, ^{211}Bi, ^{212}Bi, ^{213}Bi, and ^{214}Bi. The choice of metal is determined based on the desired therapeutic or diagnostic application.

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can be coupled to a metal component, to produce agent of the invention that can be used as a diagnostic or therapeutic agent. A detectable label may be a metal ion from heavy elements or rare earth ions,

such as Gd^{3+}, Fe^{3+}, Mn^{3+}, or Cr^{2+}. An agent of the invention that incorporates an HSA linker having paramagnetic or superparamagnetic metals conjoined thereto are useful as diagnostic agents in MRI imaging applications. Paramagnetic metals that can be coupled to the agents of the invention include, but are not limited to, chromium (III), manganese (II), iron (II), iron (III), cobalt (II), nickel (II), copper (II), praseodymium (III), neodymium (III), samarium (III), gadolinium (III), terbium (III), dysprosium (III), holmium (III), erbium (III), and ytterbium (III).

Chelating groups may be used to indirectly couple detectable labels or other molecules to an HSA linker of the invention or to an agent conjugated thereto. Chelating groups can link agents of the invention with radiolabels, such as a bifunctional stable chelator, or can be linked to one or more terminal or internal amino acid reactive groups. An HSA linker of the invention, or any molecule or moeity conjugated thereto, can be linked via an isothiocyanate β-Ala or appropriate non-α-amino acid linker which prevents Edman degradation. Examples of chelators known in the art include, for example, the ininocarboxylic and polyaminopolycarboxylic reactive groups, ininocarboxylic and polyaminopolycarboxylic reactive groups, diethylenetriaminepentaacetic acid (DTPA), and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA).

An HSA linker of the invention, when expressed recombinantly, can be joined to a peptidic detectable label or diagnostic agent. Peptides and proteins that can be used as a detectable label with an IISA linker include, but are not limited to, fluorescent proteins, bioluminescent proteins, and epitope tags, each of which is discussed in detail below. One or more of these detectable labels can also be incorporated into an HSA linker of the invention that also includes a therapeutic, cytotoxic, or cytostatic agent.

Fluorescent proteins or fluorochromes, such as green fluorescent protein (GFP; SEQ ID NO:47), enhanced GFP (eGFP), yellow fluorescent protein (SEQ ID NO: 48; YFP), cyan fluorescent protein (SEQ ID NO: 49; CFP), and red fluorescent protein (SEQ ID NO: 50; RFP or DsRed), can be used as detectable label joined to an HSA linker of the invention. Fluorescent proteins can be recombinantly expressed in a cell (e.g., a blood cell, such as a lymphocyte) following transfection or transduction of the cell with an expression vector that encodes the nucleotide sequence of the fluorescent protein. Upon exposure of the fluorescent protein to a stimulating frequency of light, the fluorescent protein will emit light at a low, medium, or high intensity that can be observed by eye under a microscope or by an optical imaging device. Exemplary fluorescent proteins suitable for use as the diagnostic sequence in agents of the

invention are described in, e.g., U.S. Patent Nos. 7,417,131 and 7,413,874, each of which is herein incorporated by reference.

Bioluminescent proteins can also be used as a detectable label incorporated into an HSA linker of the invention. Bioluminescent proteins, such as luciferase (e.g., firefly (SEQ ID NO:51), *Renilla* (SEQ ID NO:52), and *Omphalotus* luciferase) and aequorin, emit light as part of a chemical reaction with a substrate (e.g., luciferin and coelenterazine). In one embodiment of the invention, a vector encoding a luciferase gene provides for the *in vivo, in vitro*, or *ex vivo* detection of cells (e.g., blood cells, such as lymphocytes) that have been transduced or transfected according to the methods of the invention. Exemplary bioluminescent proteins suitable for use as diagnostic sequence of the invention and methods for their use are described in, e.g., U.S. Patent Nos. 5,292,658, 5,670,356, 6,171,809, and 7,183,092, each of which is herein incorporated by reference.

Epitope tags are short amino acid sequences, e.g., 5-20 amino acid residues in length, that can be incorporated into an HSA linker of the invention as a detectable label to facilitate detection once expressed in a cell, secreted from the cell, or bound to a target cell. An agent of the invention that incorporates an epitope tag as a diagnostic sequence can be detected by virtue of its interaction with an antibody, antibody fragment, or other binding molecule specific for the epitope tag. Nucleotide sequences encoding the epitope tag are produced either by cloning appropriate portions of natural genes or by synthesizing a polynucleotide that encodes the epitope tag. An antibody, antibody fragment, or other binding molecule that binds an epitope tag can directly incorporate a detectable label (e.g., a fluorochrome, radiolabel, heavy metal, or enzyme such as horseradish peroxidase) or serve itself as a target for a secondary antibody, antibody fragment, or other binding molecule that incorporates such a label. Exemplary epitope tags that can be used as a diagnostic sequence include c-myc (SEQ ID NO:33), hemagglutinin (HA; SEQ ID NO:34), and histidine tag (His_6; SEQ ID NO:35). Furthermore, fluorescent (e.g., GFP) and bioluminescent proteins can also serve as epitope tags, as antibodies, antibody fragments, and other binding molecules are commercially available for the detection of these proteins.

The *in vivo*, *in vitro*, or *ex vivo* detection, imaging, or tracking of a an HSA linker of the invention that incorporates a diagnostic sequence (e.g., a fluorescent protein, bioluminescent protein, or epitope tag) or any cell expressing or bound thereto can be accomplished using a

microscope, flow cytometer, luminometer, or other state of the art optical imaging device, such as an IVIS® Imaging System (Caliper LifeSciences, Hopkinton, MA).

Therapeutic or Cytotoxic Agents Coupled to the HSA Linker of the Invention

An HSA linker protein of the invention, or any molecule or moiety conjugated thereto, can be coupled to any known cytotoxic or therapeutic moiety to form an agent of the invention that can be administered to treat, inhibit, reduce, or ameliorate disease (e.g., a cancer, autoimmune disease, or cardiovascular disease) or one or more symptoms of disease. Examples include but are not limited to antineoplastic agents such as: Acivicin; Aclarubicin; Acodazole Hydrochloride; Acronine; Adozelesin; Adriamycin; Aldesleukin; Altretamine; Ambomycin; A. metantrone Acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperlin; Azacitidine; Azetepa; Azotomycin; Batimastat; Benzodepa; Bicalutamide; Bisantrene Hydrochloride; Bisnafide Dimesylate; Bizelesin; Bleomycin Sulfate; Brequinar Sodium; Bropirimine; Busulfan; Cactinomycin; Calusterone; Camptothecin; Caracemide; Carbetimer; Carboplatin; Carmustine; Carubicin Hydrochloride; Carzelesin; Cedefingol; Chlorambucil; Cirolemycin; Cisplatin; Cladribine; Combretestatin A-4; Crisnatol Mesylate; Cyclophosphamide; Cytarabine; Dacarbazinc; DACA (N-[2- (Dimethyl-amino) ethyl] acridine-4-carboxamide); Dactinomycin; Daunorubicin Hydrochloride; Daunomycin; Decitabine; Dexormaplatin; Dezaguanine; Dezaguaninc Mcsylate; Diaziquone; Docetaxel; Dolasatins; Doxorubicin; Doxorubicin Hydrochloride; Droloxifene; Droloxifene Citrate; Dromostanolone Propionate; Duazomycin; Edatrexate; Eflornithine Hydrochloride; Ellipticine; Elsamitrucin; Enloplatin; Enpromate; Epipropidine; Epirubicin Hydrochloride; Erbulozole; Esorubicin Hydrochloride; Estramustine; Estramustine Phosphate Sodium; Etanidazole; Ethiodized Oil I 131; Etoposide; Etoposide Phosphate; Etoprine; Fadrozole Hydrochloride; Fazarabine; Fenretinide; Floxuridine; Fludarabine Phosphate; Fluorouracil; 5-FdUMP; Flurocitabine; Fosquidone; Fostriecin Sodium; Gemcitabine; Gemcitabine Hydrochloride; Gold Au 198; Homocamptothecin; Hydroxyurea; Idarubicin Hydrochloride; Ifosfamide; Ilmofosine; Interferon Alfa-2a; Interferon Alfa-2b; Interferon Alfa-nl; Interferon Alfa-n3; Interferon Beta-I a; Interferon Gamma-I b; Iproplatin; Irinotecan Hydrochloride; Lanreotide Acetate; Letrozole; Leuprolide Acetate; Liarozole Hydrochloride; Lometrexol Sodium; Lomustine; Losoxantrone Hydrochloride; Masoprocol; Maytansine; Mechlorethamine Hydrochloride; Megestrol Acetate; Melengestrol Acetate; Melphalan; Menogaril; Mercaptopurine; Methotrexate; Methotrexate Sodium; Metoprine;

Meturedepa; Mitindomide; Mitocarcin; Mitocromin; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone Hydrochloride; Mycophenolic Acid; Nocodazole; Nogalamycin;Ormaplatin; Oxisuran; Paclitaxel; Pegaspargase; Peliomycin; Pentamustine; PeploycinSulfate; Perfosfamide; Pipobroman; Piposulfan; Piroxantrone Hydrochloride; Plicamycin; Plomestane; Porfimer Sodium; Porfiromycin; Prednimustine; Procarbazine Hydrochloride; Puromycin; Puromycin Hydrochloride; Pyrazofurin; Rhizoxin; Rhizoxin D; Riboprine; Rogletimide; Safingol; Safingol Hydrochloride; Semustine; Simtrazene; Sparfosate Sodium; Sparsomycin; Spirogermanium Hydrochloride; Spiromustine; Spiroplatin; Streptonigrin; Streptozocin; Strontium Chloride Sr 89; Sulofenur; Talisomycin; Taxane; Taxoid; Tecogalan Sodium; Tegafur; Teloxantrone Hydrochloride; Temoporfin; Teniposide; Teroxirone; Testolactone; Thiamiprine; Thioguanine; Thiotepa; Thymitaq; Tiazofurin; Tirapazamine; Tomudex; TOP53; Topotecan Hydrochloride; Toremifene Citrate; Trestolone Acetate; Triciribine Phosphate; Trimetrexate; Trimetrexate Glucuronate; Triptorelin; Tubulozole Hydrochloride; Uracil Mustard; Uredepa; Vapreotide; Verteporfin; Vinblastine; Vinblastine Sulfate; Vincristine; Vincristine Sulfate; Vindesine; Vindesine Sulfate; Vinepidine Sulfate; Vinglycinate Sulfate; Vinleurosine Sulfate; Vinorelbine Tartrate; Vinrosidine Sulfate; Vinzolidine Sulfate; Vorozole; Zeniplatin; Zinostatin; Zorubicin Hydrochloride; 2-Chlorodeoxyadenosine; 2' Deoxyformycin; 9-aminocamptothecin; raltitrexed; N-propargyl-5,8-dideazafolic acid; 2chloro-2'-arabino-fluoro-2'-deoxyadenosine; 2-chloro-2'-deoxyadenosine; anisomycin; trichostatin A; hPRL-G129R; CEP-751; linomide; sulfur mustard; nitrogen mustard (mechlor ethamine); cyclophosphamide; melphalan; chlorambucil; ifosfamide; busulfan; N-methyl-Nnitrosourea (MNU); N, N'-Bis (2-chloroethyl)-N-nitrosourea (BCNU); N- (2-chloroethyl)-N' cyclohexyl-N-nitrosourea (CCNU); N- (2-chloroethyl)-N'- (trans-4-methylcyclohexyl-N-nitrosourea (MeCCNU); N- (2-chloroethyl)-N'- (diethyl) ethylphosphonate-N-nitrosourea (fotemustine); streptozotocin; diacarbazine (DTIC); mitozolomide; temozolomide; thiotepa; mitomycin C; AZQ; adozelesin; Cisplatin; Carboplatin; Ormaplatin; Oxaliplatin;C1-973; DWA 2114R; JM216; JM335; Bis (platinum); tomudex; azacitidine; cytarabine; gemcitabine; 6-Mercaptopurine; 6-Thioguanine; Hypoxanthine; teniposide 9-amino camptothecin; Topotecan; CPT-11; Doxorubicin; Daunomycin; Epirubicin; darubicin; mitoxantrone; losoxantrone; Dactinomycin (Actinomycin D); amsacrine; pyrazoloacridine; all-trans retinol; 14-hydroxy-retro-retinol; all-trans retinoic acid; N- (4-Hydroxyphenyl) retinamide; 13-cis retinoic acid; 3-Methyl TTNEB; 9-cis retinoic acid; fludarabine (2-F-ara-AMP); or 2-chlorodeoxyadenosine (2-Cda).

Other therapeutic compounds include, but are not limited to, 20-pi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene; adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic acid; ara-CDP-DL-PTBA; argininedeaminase; asulacrine; atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzochlorins; benzoylstaurosporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; bFGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; bleomycin A2; bleomycin B2; bropirimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives (e.g., 10-hydroxy-camptothecin); canarypox IL-2; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetrorelix; chlorins; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A ; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816 ; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentanthraquinones; cycloplatam; cypemycin; cytarabine ocfosfate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrodidemnin B; 2'deoxycoformycin (DCF); deslorelin; dexifosfamide; dexrazoxane; dexverapamil; diaziquone; didemnin B; didox; diethylnorspermine; dihydro-5-azacytidine; dihydrotaxol, 9- ; dioxamycin; diphenyl spiromustine; discodermolide; docosanol; dolasetron; doxifluridine; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin; epothilones (A, R = H; B, R = Me); epithilones; epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole; etoposide; etoposide 4'-phosphate (etopofos); exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride; flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorunicin hydrochloride; forfenimex; formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; homoharringtonine (HHT); hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofosine; ilomastat;

imidazoacridones; imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4- ; irinotecan; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon; leuprolide + estrogen + progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; lovastatin; loxoribine; lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maytansine; mannostatin A; marimastat; masoprocol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone; meterelin; methioninase; metoclopramide; MIF inhibitor; ifepristone; miltefosine; mirimostim; mismatched double stranded RNA; mithracin; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim; monoclonal antibody, human chorionic gonadotrophin; monophosphoryl lipid A + myobacterium cell wall sk; mopidamol; multiple drug resistance gene inhibitor; multiple tumor suppressor 1-based therapy; mustard anticancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyldinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone + pentazocine; napavin; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; neutral endopeptidase; nilutamide; nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; 06-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate sodium; pentostatin; pentrozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride; pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum compounds; platinum-triamine complex; podophyllotoxin; porfimer sodium; porfiromycin; propyl bis-acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase inhibitors; purine nucleoside phosphorylase inhibitors; purpurins; pyrazoloacridine; pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron; ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine

demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rogletimide; rohitukine; romurtide; roquinimex; rubiginone B 1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A; sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor 1; sense oligonucleotides; signal transduction inhibitors; signal transduction modulators; single chain antigen binding protein; sizofiran; sobuzoxane; sodium borocaptate; sodium phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D; spiromustine; splenopentin; spongistatin 1; squalamine; stem cell inhibitor; stem-cell division inhibitors; stipiamide; stromelysin inhibitors; sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; synthetic glycosaminoglycans; tallimustine; tamoxifen methiodide; tauromustine; tazarotene; tecogalan sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; temozolomide; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thalidomide; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene dichloride; topotecan; topsentin; toremifene; totipotent stem cell factor; translation inhibitors; tretinoin; triacetyluridine; triciribine; trimetrexate; triptorelin; tropisetron; turosteride; tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vapreotide; variolin B; vector system, erythrocyte gene therapy; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; and zinostatin stimalamer.

The HSA linker agents of the invention can also include site-specifically conjugated molecules and moieties. Site-specific conjugation allows for the controlled stoichiometric attachment to specific residues in the HSA linker of cytotoxic, immunomodulatory, or cytostatic agents including, e.g., anti tubulin agents, DNA minor groove binders, DNA replication inhibitors, alkylating agents, anthracyclines, antibiotics, antifolates, antimetabolites, chemotherapy or radiation sensitizer, duocarmycins, etoposides, fluorinated pyrimidines, ionophores, lexitropsins, nitrosoureas, platinols, purine antimetabolites, puromycins, steroids, taxanes, topoisomerase inhibitors, and vinca alkaloids or any other molecules or moieties described herein.

Techniques for conjugating therapeutic agents to proteins, and in particular to antibodies, are well-known (e.g., Arnon *et al.,* "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy," in Monoclonal Antibodies And Cancer Therapy (Reisfeld *et al.,* eds., Alan R. Liss, Inc., 1985); Hellstrom *et al.,* "Antibodies For Drug Delivery," in Controlled Drug Delivery (Robinson *et*

al., eds., Marcel Dekker, Inc., 2nd ed. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological And Clinical Applications (Pinchera *et al.,* eds., 1985); "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody In Cancer Therapy," in Monoclonal Antibodies For Cancer Detection And Therapy (Baldwin *et al.,* eds., Academic Press, 1985); Thorpe *et al., Immunol. Rev.* 62:119-58 (1982); and Doronina *et al.,* "Development of potent monoclonal antibody auristatin conjugates for cancer therapy," *Nature Biotech.* 21:(7)778-784 (2003)). See also, e.g., PCT publication WO 89/12624.

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be coupled to a lytic peptide to form an agent of the invention. Such lytic peptides induce cell death and include, but are not limited to, streptolysin O; stoichactis toxin; phallolysin; staphylococcus alpha toxin; holothurin A; digitonin; melittin; lysolecithin; cardiotoxin; and cerebratulus A toxin (Kem *et al., J. Biol. Chem.* 253(16):5752-5757, 1978). An agent of the invention can also be formed by conjugating an HSA linker of the invention, or any molecule or moiety conjugated thereto (e.g., antibody or antibody fragment conjugates), to a synthetic peptide that shares some sequence homology or chemical characteristics with any of the naturally occurring peptide lysins; such characteristics include, but are not limited to, linearity, positive charge, amphipathicity, and formation of alpha-helical structures in a hydrophobic environment (Leuschner *et al., Biology of Reproduction* 73:860-865, 2005). An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be coupled to an agent that induces complement-mediated cell lysis such as, for example, the immunoglobulin F_c subunit. An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also coupled to any member of the phospholipase family of enzymes (including phospholipase A, phospholipase B, phospholipase C, or phospholipase D) or to a catalytically-active subunit thereof.

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be coupled to a radioactive agent to form an agent that can be used for detection or therapeutic applications. Radioactive agents that can be used include but are not limited to Fibrinogen ^{125}I; Fludeoxyglucose ^{18}F; Fluorodopa ^{18}F; Insulin ^{125}I; Insulin ^{131}I; Iobenguane ^{123}I; Iodipamide Sodium ^{131}I; Iodoantipyrine ^{131}I; Iodocholesterol ^{131}I ; Iodohippurate Sodium ^{123}I; Iodohippurate Sodium ^{125}I; Iodohippurate Sodium ^{131}I; Iodopyracet ^{125}I; Iodopyracet ^{131}I; Iofetamine Hydrochloride ^{123}I; Iomethin ^{125}I; Iomethin ^{131}I; Iothalamate Sodium ^{125}I; Iothalamate Sodium ^{131}I; tyrosine ^{131}I;

Liothyronine ^{125}I; Liothyronine ^{131}I; Merisoprol Acetate ^{197}Hg; Merisoprol Acetate ^{203}Hg; Merisoprol ^{197}Hg; Selenomethionine ^{75}Se; Technetium ^{99m}Tc Antimony Trisulfide Colloid; Technetium ^{99m}Tc Bicisate; Technetium ^{99m}Tc Disofenin; Technetium ^{99m}Tc Etidronate; Technetium ^{99m}Tc Exametazime; Technetium ^{99m}Tc Furifosmin; Technetium ^{99m}Tc Gluceptate; Technetium ^{99m}Tc Lidofenin; Technetium ^{99m}Tc Mebrofenin; Technetium ^{99m}Tc Medronate; Technetium ^{99m}Tc Medronate Disodium; Technetium ^{99m}Tc Mertiatide; Technetium ^{99m}Tc Oxidronate; Technetium ^{99m}Tc Pentetate; Technetium ^{99m}Tc Pentetate Calcium Trisodium; Technetium ^{99m}Tc Sestamibi; Technetium ^{99m}Tc Siboroxime; Technetium ^{99m}Tc; Succimer; Technetium ^{99m}Tc Sulfur Colloid; Technetium ^{99m}Tc Teboroxime; Technetium ^{99m}Tc Tetrofosmin; Technetium ^{99m}Tc Tiatide; Thyroxine ^{125}I; Thyroxine ^{131}I; Tolpovidone ^{131}I; Triolein ^{125}I; or Triolein ^{131}I.

Additionally, a radioisotope could be site-specifically conjoined to an HSA linker or HSA linker conjugate. The available reactive groups could be used to conjugate site-specific bifunctional chelating agents for labeling of radioisotopes, including ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{99m}Tc, ^{111}In, ^{64}Cu, ^{67}Cu, ^{186}Re, ^{188}Re, ^{177}Lu, ^{90}Y, ^{77}As, ^{72}As, ^{86}Y, ^{89}Zr, ^{211}At, ^{212}Bi, ^{213}Bi, or ^{225}Ac.

Therapeutic or cytotoxic agents incorporated into or coupled with an HSA linker or an HSA linker conjugate may further include, for example, anti-cancer Supplementary Potentiating Agents, including, but not limited to: tricyclic anti-depressant drugs (e.g., imipramine, desipramine, amitryptyline, clomipramine, trimipramine, doxepin, nortriptyline, protriptyline, amoxapine, and maprotiline); non-tricyclic anti-depressant drugs (e.g., sertraline, trazodone, and citalopram); Ca^{2+} antagonists (e.g., verapamil, nifedipine, nitrendipine, and caroverine); Calmodulin inhibitors (e.g., prenylamine, trifluoroperazine, and clomipramine); Amphotericin B; Triparanol analogs (e.g., tamoxifen); antiarrhythmic drugs (e.g., quinidine); antihypertensive drugs (e.g., reserpine); Thiol depleters (e.g., buthionine and sulfoximine) and Multiple Drug Resistance reducing agents such as Cremaphor EL.

An agent of the invention that includes an HSA linker, or any molecule or moiety conjugated thereto, can also be coupled to or administered with cytokines (e.g., granulocyte colony stimulating factor, interferon-alpha, and tumor necrosis factor-alpha). An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be coupled to an antimetabolic agent. Antimetabolic agents include, but are not limited to, the following compounds and their derivatives: azathioprine, cladribine, cytarabine, dacarbazine, fludarabine phosphate, fluorouracil, gencitabine chlorhydrate, mercaptopurine, methotrexate, mitobronitol, mitotane, proguanil chlorohydrate, pyrimethamine,

raltitrexed, trimetrexate glucuronate, urethane, vinblastine sulfate, vincristine sulfate, etc. More preferably, an HSA linker or conjugate of the invention can be coupled to a folic acid-type antimetabolite, a class of agents that includes, for example, methotrexate, proguanil chlorhydrate, pyrimethanime, trimethoprime, or trimetrexate glucuronate, or derivatives of these compounds.

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be coupled to a member of the anthracycline family of neoplastic agents, including but not limited to aclarubicine chlorhydrate, daunorubicine chlorhydrate, doxorubicine chlorhydrate, epirubicine chlorhydrate, idarubicine chlorhydrate, pirarubicine, or zorubicine chlorhydrate; a camptothecin, or its derivatives or related compounds, such as 10, 11 methylenedioxycamptothecin; or a member of the maytansinoid family of compounds, which includes a variety of structurally-related compounds, e.g., ansamitocin P3, maytansine, 2'-N-demethylmaytanbutine, and maytanbicyclinol.

An HSA linker of the invention, or any molecule or moiety conjugated thereto, can also be coupled directly to a cytotoxic or therapeutic agent using known chemical methods, or coupled indirectly to a cytotoxic or therapeutic agent via an indirect linkage. For example, an HSA linker can be attached to a chelating group that is attached to the cytotoxic or therapeutic agent. Chelating groups include, but are not limited to, ininocarboxylic and polyaminopolycarboxylic reactive groups, diethylenetriaminepentaacetic acid (DTPA), and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). For general methods, see, e.g., Liu *et al., Bioconjugate Chem.* 12(4):653, 2001; Cheng *et al.,* WO 89/12631; Kieffer *et al.,* WO 93/12112; Albert *et al.,* U.S.P.N. 5,753,627; and WO 91/01144 (each of which are hereby incorporated by reference).

An agent of the invention that includes, e.g., an HSA linker, one or more binding moieties (with or without intervening polypeptide connectors, as defined herein), and a therapeutic or cytotoxic agent, can be specifically targeted by the binding moiety (e.g., antibody, antibody fragment, or receptor/ligand) to a cell or tissue, thereby allowing selective destruction of the target cell or tissue to which the binding moiety is directed. For example, an agent of the invention can be used to target and destroy cancer cells of the lung, breast, prostate, and colon in order to prevent, stabilize, inhibit the progression of, or treat cancers originating in these organs when the agent includes a binding moiety that specifically binds to the cancer cells in these organs. Also, for example, the agents of the invention can be used to target and destroy cells of the vasculature, brain, liver, kidney, heart, lung, prostate, colon, nasopharynx, oropharynx, larynx, bronchus, and skin in order to prevent, stabilize, inhibit the progression of, or treat age-related, tobacco-related, or

autoimmune diseases or conditions relating to these organs by targeting, in the case of autoimmune disease for example, autoreactive T cells (e.g., by binding to and agonizing tumor necrosis factor receptor 2 (TNFR2) present on the autoreactive T cells).

An HSA linker of the invention, when expressed recombinantly, can be joined to a cytotoxic polypeptide. Cytotoxic polypeptides, when brought into contact with a target cell (e.g., a cancer cell), exerts cytotoxic or cytostatic effects on the cell. For example, a cytotoxic polypeptide, when joined with an HSA linker of the invention, can induce events in a target cell upon binding of the target cell that leads to cell death through, for example, apoptosis, necrosis, or senescence. Alternatively, a cytotoxic polypeptide joined with an HSA linker of the invention can interfere or inhibit normal cellular biological activities, such as division, metabolism, and growth, or abnormal cellular biological activities, such as metastasis.

For example, an HSA linker of the invention joined to caspase 3 will bind a target cell (e.g., a cancer cell) and undergoe endocytosis. Once internalized by the target cell, the caspase portion of the HSA linker conjugate can initiate the pro-apoptotic caspase cascade, ultimately resulting in the apoptosis of the target cell.

In a preferred embodiment, an HSA linker of the invention includes a cytotoxic polypeptide capable of killing a cancer cell. In another embodiment of the invention, the cytotoxic polypeptide inhibits the growth or metastasis of a cancer cell. The cytotoxic polypeptide joined with an HSA linker of the invention can also be used to kill or inhibit the growth of cells associated with, necessary for, or beneficial to cancer growth, such as endothelial cells that form blood vessels that perfuse solid tumors.

In an embodiment, an HSA linker of the invention can include two or more cytotoxic polypeptides so as to modulate (e.g., increase) the specificity, intensity, or duration of the cytotoxic or cytostatic effect on a target cell (e.g., a cancer cell).

In another embodiment, the HSA linker of the invention is joined to an activatable form of cytotoxic polypeptide (e.g., a biologically-inactive pro-agent that is capable of activation upon cleavage by an enzyme or drug). In this embodiment, texposure (e.g., *in vivo*) of the cytotoxic polypeptide pro-agent to an enzyme or drug capable of cleaving the cytotoxic polypeptide, renders the cytotoxic polypeptide biologically-active (e.g., cytotoxic or cytostatic). An example of a biologically-inactive cytotoxic polypeptide that can be converted to a biologically-active form for use with an HSA linker of the invention is a procaspase (e.g., procaspase 8 or 3). For

example, the procaspase 8 domain of an HSA linker of the invention can be cleaved by TRAIL or FasL upon internalization by a target cell (e.g., a cancer cell). Once cleaved, the biologically active caspase 8 can promote apoptosis of the target cell.

In one embodiment of the invention, the cytotoxic polypeptide joined to an HSA linker of the invention can include a full-length peptide, polypeptide, or protein, or biologically-active fragment thereof (e.g., a "death domain"), known to have cytotoxic or cytostatic properties. Peptides, polypeptides, or proteins with cytotoxic or cytostatic properties can be altered (e.g., by making amino acid substitutions, mutations, truncations, or additions) to facilitate incorporation of the cytotoxic sequence into an agent of the invention. Desirable alterations include, for example, changes to the amino acid sequence that facilitate protein expression, longevity, cell secretion, and target cell toxicity.

The invention also features a nucleic acid molecule encoding a cytotoxic polypeptide as a fusion protein with an HSA linker of the invention, optionally including binding moieities and polypeptide connectors. The nucleic acid molecule can be incorporated into a vector (e.g., an expression vector), such that, upon expression of the HSA linker of the invention in a cell transfected or transduced with the vector, the cytotoxic polypeptide, HSA linker, and binding moieties, if present, are operably linked (e.g., fused, contiguously-joined, or tethered together). Examples of peptides, polypeptides, and proteins that can be used as a cytotoxic polypeptide of the present invention include, but are not limited to, apoptosis-inducing proteins such as cytochrome c (SEQ ID NO:39); caspases (e.g., caspase 3 (SEQ ID NO:36) and caspase 8 (SEQ ID NO:37)); procaspases, granzymes (e.g., granzymes A and B (SEQ ID NO: 38)); tumor necrosis factor (TNF) and TNF receptor family members, including TNF-alpha (TNFα; SEQ ID NO:40)), TNF-beta, Fas (SEQ ID NO:41) and Fas ligand; Fas-associated death domain-like IL-1β converting enzyme (FLICE); TRAIL/APO2L (SEQ ID NO:45) and TWEAK/APO3L (see, e.g., U.S. Patent Application Publication No. 2005/0187177, herein incorporated by reference); pro-apoptotic members of the Bcl-2 family, including Bax (SEQ ID NO:46), Bid, Bik, Bad (SEQ ID NO:42), Bak, and RICK (see, e.g., U.S. Patent Application Publication No. 2004/0224389, herein incorporate by reference); vascular apoptosis inducing proteins 1 and 2 (VAP1 and VAP2; Masuda et al., *Biochem. Biophys. Res. Commun.* 278:197-204 (2000)); pierisin (SEQ ID NO:44; Watanabe et al., *Biochemistry* 96:10608-10613 (1999)); apoptosis-inducing protein (SEQ ID NO:43; AIP; Murawaka et al., *Nature* 8:298-307 (2001)); IL-1α propiece polypeptide (see, e.g., U.S. Patent 6,191,269, herein incorporated by

reference); apoptin and apoptin-associated proteins such as AAP-1 (see, e.g., European Patent Application Publication No. EP 1083224, herein incorporated by reference); anti-angiogenic factors such as endostatin and angiostatin; and other apoptosis-inducing proteins, including those described in the following International and U.S. Patent Application Publications, each herein incorporated by reference: U.S. 2003/0054994, U.S. 2003/0086919, U.S. 2007/0031423, WO 2004/078112, WO 2007/012430, and WO 2006/0125001 (intracellular domain of delta 1 and jagged 1).

Wild-type HSA Linker Conjugates

The present invention also encompasses a naturally-occurring wild-type HSA linker, the amino acid and nucleotide sequences of which are provided in SEQ ID NOS:3 and 4, respectively, in the formation of binding, diagnostic, or therapeutic agents of the invention. In all embodiments of the invention utilizing an HSA linker with the amino acid sequence listed in SEQ ID NO:3, one or more polypeptide connectors, as described above, are covalently attached to the amino and/or carboxy termini of the HSA linker, or to an amino acid residue within the HSA linker sequence, to facilitate conjugation of one or more binding moieties.

Truncations

The invention further features a conjugate that is formed using a truncated wild-type HSA polypeptide, which can be combined with one or more polypeptide connectors or binding moieties. A wild-type HSA polypeptide lacking 1, 2, 3, 4, 5, 10, 15, 20, 50, 100, 200 or more amino acids of the full-length wild-type HSA amino acid sequence (i.e., SEQ ID NO:3) can be conjoined to any of the binding moieties or diagnostic or therapeutic agents described herein. Truncations can occur at one or both ends of the HSA linker, or can include a deletion of internal residues. Truncation of more than one amino acid residue need not be linear. Examples of wild-type HSA linkers of the invention include those having, in combination with one or more polypeptide connectors or binding moieties, one or more of domain I (SEQ ID NO:56; residues 1-197 of SEQ ID NO:3), domain II (SEQ ID NO:54; residues 189-385 of SEQ ID NO:3), or domain III (SEQ ID NO:57; residues 381-585 of SEQ ID NO:3), or combinations thereof, e.g., domains I and II, I and III, and II and III.

Serum clearance rates of a conjugate of the invention (e.g., a bispecific HSA-drug or radioisotope-containing agent), can be optimized by testing conjugates containing a truncated wild-type HSA linker, as describe above.

Additional HSA Linker Modifications

The invention further provides site-specific chemical modification of amino acid residues in the HSA linker polypeptide. The correctly-folded tertiary structure of HSA displays certain amino acid residues on the external face of the protein. Chemically-reactive amino acid residues (e.g., cysteine) can be substituted for these surface-exposed residues to allow site-specific conjugation of a diagnostic or therapeutic agent.

The invention also provides for the addition or removal of asparagine, serine, or threonine residues from an HSA linker polypeptide sequence to alter glycosylation of these amino acid residues. Glycosylation sites added to an HSA linker are preferably surface-exposed, as discussed herein. Glycosyl or other carbohydrate moieties introduced to an HSA linker can be directly conjugated to diagnostic, therapeutic, or cytotoic agents.

Cysteine (Thiol) Conjugation

The invention features the substitution of surface-exposed amino acid residues of the HSA linker polypeptide with cysteine residues to allow for chemical conjugation of diagnostic, therapeutic, or cytotoxic agents. Cysteine residues exposed on the surface of the HSA linker polypeptide (when folded into its native tertiary structure) allow the specific conjugation of a diagnostic, therapeutic, or cytotoxic agent to a thiol reactive group such as maleimide or haloacetyl. The nucleophilic reactivity of the thiol functionality of a cysteine residue to a maleimide group is about 1000 times higher compared to any other amino acid functionality in a protein, such as the amino group of a lysine residue or the N-terminal amino group. Thiol specific functionality in iodoacetyl and maleimide reagents may react with amine groups, but higher pH (>9.0) and longer reaction times are required (Garman, 1997, Non-Radioactive Labelling: A Practical Approach, Academic Press, London). The amount of free thiol in a protein may be estimated using the standard Ellman's assay. In some instances, reduction of the disulfide bonds with a reagent such as dithiothreitol (DTT) or selenol (Singh *et al., Anal. Biochem.* 304:147-156 (2002)) is required to generate the reactive free thiol.

Sites for cysteine substitution can be identified by analysis of surface accessibility of the HSA linker (e.g., the identification of serine and threonine residues as suitable for substitution are described in Example 1 below). The surface accessibility can be expressed as the surface area (e.g., square angstroms) that can be contacted by a solvent molecule, e.g., water. The occupied space of

water is approximated as a sphere with a 1.4 angstrom radius. Software for calculating the surface accessibility of each amino acid of a protein is freely available or licensable. For example, the CCP4 Suite of crystallography programs which employ algorithms to calculate the surface accessibility of each amino acid of a protein with known x-ray crystallography derived coordinates ("The CCP4 Suite: Programs for Protein Crystallography" *Acta. Cryst.* D50:760-763 (1994); www.ccp4.ac.uk/dist/html/INDEX.html). Solvent accessibility may also be assessed using the free software DeepView Swiss PDB Viewer downloaded from the Swiss Institute of Bioinformatics. The substitution of cysteines at surface-exposed sites allows for conjugation of the reactive cysteine to a thiol reactive group linked to the diagnostic or therapeutic agent.

Glycosylation

In addition, altered serum clearance rates can be achieved by engineering glycosylation sites into the HSA linker. In certain embodiments, an HSA linker of the invention is glycosylated. Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of a carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X represents any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition or deletion of glycosylation sites to the HSA linker is conveniently accomplished by altering the amino acid sequence such that one or more of the above-described tripeptide sequences (for N-linked glycosylation sites) is created. The alteration may also be made by the addition, deletion, or substitution of one or more serine or threonine residues to the sequence of the original HSA linker (for O-linked glycosylation sites). The resulting carbohydrate structures on HSA can also be used for site-specific conjugation of cytotoxic, immunomodulatory or cytostatic agents as described above.

HSA Linker Agents in Combination with Therapeutic Agents

The invention further provides for the administration of the HSA linker agents described herein with one or more of the therapeutic, cytotoxic, or cytostatic agents described herein. For example, a patient suffering from breast cancer can be administered an HSA linker containing ErbB2 and ErbB3 scFvs (e.g., B2B3-1) can be co-administered with, e.g., doxorubicin, cyclophosphamide, and paclitaxel, a common chemotherapeutic regimen for the treatment of breast cancer. Additional biological and chemical agents useful for the treatment of cancer are listed in Appendix C.

HSA Linker Agents in Combination with Radiotherapy or Surgery

The invention provides for the administration of an HSA linker agent of the invention prior to, concurrent with, or following radiotherapy or surgery. For example, a patient suffering from a proliferative disorder (e.g., breast cancer) can receive an HSA linker agent, alone or in combination with other therapeutic, cytotoxic, or cytotoxic agents as described herein concurrent with targeted radiotherapy or surgical intervention (e.g., lumpectomy or mastectomy) at the site of the cancerous tissue. Radiotherapies suitable for use in combination with HSA linker agents of the invention include brachytherapy and targeted intraoperative radiotherapy (TARGIT).

Pharmaceutical Compositions

Pharmaceutical compositions of the invention contain a therapeutically or diagnostically effective amount of an HSA linker conjugate of the invention that includes one or more of a binding moiety (e.g., antibodies or antibody fragments), diagnostic agent (e.g., radionuclide or chelating agents), or a therapeutic agent (e.g., cytotoxic or immunomodulatory agents) agent. The active ingredients, an HSA linker conjugate of the invention (prepared with one or more of a binding moiety, diagnostic agent, or therapeutic agent) can be formulated for use in a variety of drug delivery systems. One or more physiologically acceptable excipients or carriers can also be included in the compositions for proper formulation. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer *Science* 249:1527-1533 (1990).

The pharmaceutical compositions are intended for parenteral, intranasal, topical, oral, or local administration, such as by a transdermal means, for prophylactic and/or therapeutic treatment.

Commonly, the pharmaceutical compositions are administered parenterally (e.g., by intravenous, intramuscular, or subcutaneous injection), or by oral ingestion, or by topical application. Thus, the invention provides compositions for parenteral administration that include an HSA linker of the invention, with or without one or more binding, diagnostic, and/or therapeutic agent conjugated thereto, dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, e.g., water, buffered water, saline, PBS, and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. The invention also provides compositions for oral delivery, which may contain inert ingredients such as binders or fillers for the formulation of a tablet, a capsule, and the like. Furthermore, this invention provides compositions for local administration, which may contain inert ingredients such as solvents or emulsifiers for the formulation of a cream, an ointment, and the like.

These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably between 5 and 9 or between 6 and 8, and most preferably between 7 and 8, such as 7 to 7.5. The resulting compositions in solid form may be packaged in multiple single dose units, each containing a fixed amount of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) in a sealed package of tablets or capsules, for example. The composition in solid form can also be packaged in a container for a flexible quantity, such as in a squeezable tube designed for a topically applicable cream or ointment.

The compositions containing an effective amount of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) can be administered to a mammal (e.g., a human) for prophylactic and/or therapeutic treatments. In prophylactic applications, compositions of the invention containing an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) are administered to a patient susceptible to or otherwise at risk of developing a disease or condition (e.g., a cancer, autoimmune disease, or cardiovascular disease). Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health, but generally range from about 0.5 mg to about 400 mg of an HSA linker conjugate (prepared with one or more of a binding,

diagnostic, and/or therapeutic agent) per dose (e.g., 10 mg, 50 mg, 100 mg, 200 mg, 300 mg, or 400 mg per dose) and from about 0.1 μg to about 300 mg of one or more immunomodulatory agents per dose (e.g., 10 μg, 30 μg, 50 μg, 0.1 mg, 10 mg, 50 mg, 100 mg, or 200 mg per dose). A dose of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) can be administered prophylactically to a patient one or more times per hour, day, week, month, or year (e.g., 2, 4, 5, 6, 7, 8, 9, 10, 11, or 12 times per hour, day, week, month, or year). More commonly, a single dose per week of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) is administered.

In therapeutic applications, a dose of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) is administered to a mammal (e.g., a human) already suffering from a disease or condition (e.g., a cancer, autoimmune disease, or cardiovascular disease) in an amount sufficient to cure or at least partially arrest or alleviate one or more of the symptoms of the disease or condition and its complications. An amount adequate to accomplish this purpose is defined as a "therapeutically effective dose." Amounts effective for this use may depend on the severity of the disease or condition and general state of the patient, but generally range from about 0.5 mg to about 400 mg of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) per dose (e.g., 10 mg, 50 mg, 100 mg, 200 mg, 300 mg, or 400 mg per dose). A dose of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) can be administered therapeutically to a patient one or more times per hour, day, week, month, or year (e.g., 2, 4, 5, 6, 7, 8, 9, 10, 11, or 12 times per hour, day, week, month, or year). More commonly, a single dose per week of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) is administered.

In several embodiments, the patient may receive an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) in the range of about 0.5 to about 400 mg per dose one or more times per week (e.g., 2, 3, 4, 5, 6, or 7 or more per week), preferably about 5 mg to about 300 mg per dose one or more times per week, and even more preferably about 5 mg to about 200 mg per dose one or more times per week. The patient may also receive a biweekly dose of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) in the range of about 50 mg to about 800 mg or a monthly dose of an HSA linker of the invention, or any binding, diagnostic, and/or therapeutic agent conjugated thereto, in the range of about 50 mg to about 1,200 mg.

In other embodiments, an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) may be administered to a patient in a typical dosage range of about 0.5 mg per week to about 400 mg per week, about 1.0 mg per week to about 300 mg per week, about 5 mg per week to about 200 mg per week, about 10 mg per week to about 100 mg per week, about 20 mg per week to about 80 mg per week, about 100 mg per week to about 300 mg per week, or about 100 mg per week to about 200 mg per week. An HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) may be administered in the range of about 0.5 mg every other day to about 100 mg every other day, preferably about 5 mg every other day to about 75 mg every other day, more preferably about 10 mg every other day to about 50 mg every other day, and even more preferably 20 mg every other day to about 40 mg every other day. An HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) may also be administered in the range of about 0.5 mg three times per week to about 100 mg three times per week, preferably about 5 mg three times per week to about 75 mg three times per week, more preferably about 10 mg three times per week to about 50 mg three times per week, and even more preferably about 20 mg three times per week to about 40 mg three times per week.

In non-limiting embodiments of the methods of the present invention, an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) is administered to a mammal (e.g., a human) continuously for 1, 2, 3, or 4 hours; 1, 2, 3, or 4 times a day; every other day or every third, fourth, fifth, or sixth day; 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 times a week; biweekly; 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 times a month; bimonthly; 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 times every six months; 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 times a year; or biannually. An HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) may be administered at different frequencies during a therapeutic regime. In additional embodiments, an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) may be administered to a patient at the same frequency or at a different frequency.

The amount of one or more diagnostic or therapeutic agents and an HSA linker, or any agent conjugated thereto, required to achieve the desired therapeutic effect depends on a number of factors, such as the specific diagnostic or therapeutic agent(s) chosen, the mode of administration, and clinical condition of the recipient. A skilled artisan will be able to determine the appropriate dosages

of one or more diagnostic or therapeutic agents and an HSA linker, or any agent conjugated thereto, to achieve the desired results.

Single or multiple administrations of the compositions comprising an effective amount of an HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) can be carried out with dose levels and pattern being selected by the treating physician. The dose and administration schedule can be determined and adjusted based on the severity of the disease or condition in a mammal (e.g., a human), which may be monitored throughout the course of treatment according to the methods commonly practiced by clinicians or those described herein.

An HSA linker conjugate (prepared with one or more of a binding, diagnostic, and/or therapeutic agent) can be administered to a mammalian subject, such as a human, directly or in combination with any pharmaceutically acceptable carrier or salt known in the art. Pharmaceutically acceptable salts may include non-toxic acid addition salts or metal complexes that are commonly used in the pharmaceutical industry. Examples of acid addition salts include organic acids such as acetic, lactic, pamoic, maleic, citric, malic, ascorbic, succinic, benzoic, palmitic, suberic, salicylic, tartaric, methanesulfonic, toluenesulfonic, or trifluoroacetic acids or the like; polymeric acids such as tannic acid, carboxymethyl cellulose, or the like; and inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid phosphoric acid, or the like. Metal complexes include zinc, iron, and the like. One exemplary pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (18^{th} edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA.

Diagnostic and Therapeutic Applications

The HSA linker conjugates of the invention, as described herein, can be used for diagnostic and therapeutic applications in a human, including, for example, the diagnosis or treatment of proliferative diseases (e.g., cancers, such as melanoma, clear cell sarcoma, and renal cancer) and autoimmune diseases (e.g., multiple sclerosis, rheumatoid arthritis, and uveitis). The following discussion of human proliferative and autoimmune diseases is meant to provide the skilled practitioner with a general understanding of how HSA linker conjugates of the invention can be applied in diagnostic and therapeutic applications and is not meant to limit the scope of the present invention.

Proliferative Diseases (Cancer)

An HSA linker conjugate of the invention can be used to diagnose, treat, prevent, or eliminate proliferative diseases such as, but not limited to, breast cancer, melanoma, clear cell sarcoma, renal cancer (e.g., renal cell carcinoma), prostate cancer, lung cancer, gastric cancer, and ovarian cancer. Binding moieties to be conjoined with an HSA linker of the invention for diagnostic or therapeutic application in a patient suspected of having or suffering from a proliferative disease may be chosen based on its ability to specifically bind, agonize, activate, antagonize, or inhibit target molecules (e.g., cell surface receptors such as tyrosine kinase receptors) associated with a proliferative disease. Binding moieties that target, for example, insulin-like growth factor receptor (IGFR, e.g., IGF1R and IGF2R), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), tumor necrosis factor receptor (TNFR), epidermal growth factor receptor (EGFR, e.g., ErbB2 (HER2/neu)), Fc receptor, c-kit receptor, or mesenchymal epithelial transition factor receptor (c-met; also known as hepatocyte growth factor receptor (HGFR)) can be conjoined to an HSA linker of the invention to diagnose or treat a proliferative disease. Specific binding of a cancer cell by an HSA linker conjugate can allow for detection (e.g., an HSA linker conjoined to a detectable label, as defined herein) or destruction (e.g., an HSA linker conjoined to a cytotoxic agent) of the bound cancer cell. Specific application of HSA linker conjugates of the invention for the treatment of breast and renal cancer is described below.

Breast Cancer

Common forms of breast cancer include invasive ductal carcinoma, a malignant cancer in the breast's ducts, and invasive lobular carcinoma, a malignant cancer in the breast's lobules. Some types of breast cancer cells are known to highly express high levels of epidermal growth factor receptors, especially ErbB2 (i.e., HER2/neu). Aberrant signaling or unregulated activation of EGFRs has been linked to the development and progression of many cancers, including breast cancer. Uncontrolled cellular proliferation mediated via dysfunctional EGFR pathways can be found in a wide variety of solid tumors of epithelial origin and data have linked tumor EGFR expression, overexpression, and dysregulation to advanced disease, metastatic phenotype, resistance to chemotherapy, and an overall poorer prognosis.

An HSA linker of the invention conjoined to one or more binding moieties specific for an EGFR (e.g., anti-ErbB2; trastuzumab) can be used with a diagnostic (e.g., a detectable label) or

cytotoxic, cytostatic, or therapeutic agent, as described herein, to diagnose or treat breast cancer. Alternatively, a bispecific HSA linker conjugate that consists of binding moieties specific for ErbB2 and ErbB3, such as "B2B3-1," described further herein, can be employed to diagnose or treat cancers, e.g., breast, kidney, ovarian, and lung cancers.

As described above, an HSA linker conjugate of the invention used to treat breast cancer can be administered prior to (e.g., neoadjuvant chemotherapy), concurrent with, or following (e.g., adjuvant chemotherapy) radiotherapy or surgical intervention. An HSA linker conjugate can also be co-administered with other compounds (e.g., antineoplastic agents, such as biological or chemical therapeutics) useful for the treatment of breast cancer. For example, the antineoplastic agents listed in Table 1, including mitotic inhibitors (e.g., taxanes), topoisomerase inhibitors, alkylating agents (including, e.g., platinum-based agents), selective estrogen modulators (SERM), aromatase inhibitors, antimetabolites, antitumor antibiotics (e.g., anthracycline antibiotics), anti-VEGF agents, anti-ErbB2 (HER2/neu) agents, and anti-ErbB3 agents, are known to be particularly useful for the treatment of breast cancer. An HSA linker conjugate of the invention can be administered by a clinician in combination with any compound, including those listed in Appendix C, known or thought to be beneficial for the treatment of breast cancer.

Table 1: Exemplary antineoplastic agents for treatment of breast cancer in combination with HSA linker conjugates of the invention.

Therapeutic Class	**Exemplary Agent (Generic/Tradename)**	**Exemplary Dose**
Mitotic Inhibitors	paclitaxel (TAXOL®; ABRAXANE®)	175 mg/m^2
	docetaxel (TAXOTERE®)	60-100 mg/m^2
Topoisomerase Inhibitors	camptothecin	
	topotecan hydrochloride (HYCAMTIN®)	
	etoposide (EPOSIN®)	
Alkylating Agents	cyclophosphamide (CYTOXAN®)	600 mg/m^2
Platinum-Based Agents	cisplatin	20-100 mg/m^2
	carboplatin (PARAPLATIN®)	300 mg/m^2
	nedaplatin (AQUPLA®)	
	oxaliplatin (ELOXATIN®)	65-85 mg/m^2
	satraplatin (SPERA®)	
	triplatin tetranitrate	
Selective Estrogen Modulators (SERM)	tamoxifen (NOLVADEX®)	20-40 mg/day
	raloxifene (EVISTA®)	60 mg/day

	toremifene (FARESTON®)	
Antimetabolites	methotrexate	40 mg/m^2
	fluorouracil (5-FU)	500 mg/m^2
	raltitrexed	
Antitumor Antibiotics	doxorubicin (ADRIAMYCIN®)	40-75 mg/m^2
	epirubicin (ELLENCE®)	60-120 mg/m^2
Aromatase Inhibitors	aminoglutethimide (CYTADREN®)	250-2000 mg/day
	anastrozole (ARIMIDEX®)	1 mg/day
	letrozole (FEMARA®)	2.5 mg/day
	vorozole	
	exemestane (AROMASIN®)	25-50 mg/day
	testolactone	
	fadrozole (AFEMA®)	
Anti-VEGF Agents	bevacizumab (AVASTIN®)	10 mg/kg
Anti-ErbB2 (HER2/neu) Agents	trastuzumab (HERCEPTIN®)	2-8 mg/kg
	pertuzumab (OMNITARG®)	
Anti-ErbB3 (HER3) Agents	U3-1287 (AMG 888)	

Renal Cancer

Kidney cancers, such as renal cell carcinoma, are particularly resistant to traditional radiological and chemical therapies. As such, the application of biological therapeutics, conjoined with an HSA linker of the invention represents an attractive option for patients suffering from these cancers. For example, an HSA linker conjoined with binding moieties that agonize type I interferon or interleukin 2 receptors can be used to treat a renal cancer. As a solid tumor, binding moieties that target and inhibit tumor vascularization (e.g., anti-vascular endothelial growth factor (VEGF) antibodies such as bevacizumab) can also be used for therapeutic effect.

Autoimmune Diseases

An HSA linker conjugate of the invention can be used to diagnose, treat, prevent, or stabilize autoimmune diseases and disorders in e.g., a human patient, such as, e.g., multiple sclerosis (MS), insulin-dependent diabetes mellitus (IDDM), rheumatoid arthritis (RA), uveitis, Sjögren's syndrome, Grave's disease, psoriasis, and myasthenia gravis. Autoimmune diseases and disorders are caused by self-reactive elements of the immune system (e.g., T cells, B cells, and self-reactive antibodies). As such, binding moieties that inhibit, block, antagonize, or deplete (e.g., anti-lymphocyte or anti-thymocyte globulins; basiliximab, daclizumab, or muromonab-CD3 monoclonal antibodies) self-

reactive immune cells and antibodies can be conjoined with an HSA linker of the invention for therapeutic use. Binding moieties that function as inflammatory signaling inhibitors (ISI), as defined herein, can be conjoined to an HSA linker of the invention for the treatment of autoimmunity. In addition, binding moieties that inhibit or antagonize integrin function (e.g., an integrin antagonist, as defined herein) can ameliorate or halt disease progression.

In other embodiments of the invention, the binding moiety is a soluble TNF receptor, such as etanercept or lenercept; an antibody directed against a pro-inflammatory cytokine or a pro-inflammatory cell surface signaling molecule, such as adalimumab, certolizumab, inflixamab, golimumab, and rituxan; a dominant-negative pro-inflammatory cytokine variant, such as XENP345, XPROTM1595, anakinra, and variants disclosed in U.S. Patent Application Publication Nos. 20030166559 and 20050265962; an inhibitor of the signaling pathways downstream of pro-inflammatory cytokine or pro-inflammatory cell surface signaling molecules, such as DE 096, 5-amino-2-carbonylthiopene derivatives (as described in WO2004089929), ARRY-797, BIRB 796 BS, (1-5-tert-butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-2(morpholin-4-yl-ethoxy)-naphtalen-1-yl]-urea, CHR-3620, CNI-1493, FR-167653 (Fujisawa Pharmaceutical, Osaka, Japan), ISIS 101757 (Isis Pharmaceuticals), ML3404, NPC31145, PD169316, PHZ1112, RJW67657, 4-(4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl)-3-butyn-1-ol, SCIO-469, SB202190, SB203580, (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), SB239063, trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl-methoxypyridimidin-4-yl)imidazole, SB242235, SD-282, SKF-86002, TAK 715, VX702, and VX745; or an inhibitor of TNF-alpha converting enzyme (TACE), such as BB-1101, BB-3103, BMS-561392, butynyloxyphenyl β-sulfone piperidine hydroxomates, CH4474, DPC333, DPH-067517, GM6001, GW3333, Ro 32-7315, TAPI-1, TAPI-2, and TMI 005); or an anti-idiotypic agent, such as monoclonal antibodies, LJP 394 (abetimus, RIQUENT®, La Jolla Pharmaceuticals).

In other embodiments, the binding moiety is an interferon, as described herein. Binding moieties that can be conjoined to an HSA linker of the invention include, e.g., interferon-beta (REBIF® (IFN-β-1a), AVONEX® (IFN- β-1a), and BETASERON® (IFN- β-1b)), interferon-t (TAUFERONTM), interferon-alpha (e.g., ROFERON-A® (IFN-α-2a), INTRON-A® (IFN-α-2b), REBETRON® (IFN- α-2b), ALFERON-N® (IFN-α-n3), PEG-INTRON® (IFN-α-2b covalently conjugated with monomethoxy polyethylene glycol), INFERGEN® (a non-naturally occurring type 1

interferon with 88% homology to IFN-α-2b), or PEGASYS® (pegylated IFN-α-1a)), and ACTIMMUNE® (IFN-g-1b).

The present invention further provides HSA linker conjugates with binding moieties that antagonize these pro-inflammatory molecules or their specific receptors to treat autoimmunity. Specific application of HSA linker conjugates of the invention for the diagnosis and treatment of MS and RA are described below.

Multiple Sclerosis

Multiple sclerosis (MS) is a neurological disease characterized by irreversible degeneration of the nerves of the central nervous system (CNS). Although the underlying cause is unclear, the neurodegeneration in MS is the direct result of demyelination, or the stripping of myelin, a protein that normally lines the outer layer and insulates the nerves. T cells play a key role in the development of MS. Inflamed MS lesions, but not normal white matter, can have infiltrating $CD4^+$ T cells that respond to self antigens presented by MHC class II-linked molecules such as human HLA-DR2. The infiltrating CD4 T cells (T_H1 cells) produce the pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α that activate antigen-presenting cells (APCs) such as macrophages to produce additional pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, and IL-12. IL-12 induces further IFN-γ synthesis. The result is progressive demyelination of neuronal sheaths, leading to human disease.

HSA linker conjugates can be used to aid in the diagnosis of MS. Diagnostic HSA linker conjugates that include binding moieties, as defined herein, that specifically target one or more (e.g., a bispecific HSA linker conjugate) immune cell activation markers (e.g., CD69, CD28, HLA-DR, and CD45). An imbalance of one or more of these pro-inflammatory or immune cell activation mediators relative to other factors or cells may be using measured by an HSA linker conjugate conjoined with a diagnostic agent (e.g., a radioisotope or fluorochrome).

An HSA linker conjugate of the invention can be used to treat a person at risk of developing or suffering from MS or to prevent, ameliorate, or cure the symptoms of the disease. For example, binding moieties, as defined herein, that specifically target and antagonize α4 integrin (e.g., natalizumab), CD52 (e.g., alemtuzumab), CD80, P-selectin, sphingosine-1-phosphate receptor-1 (S1PR1), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD11 (e.g., efalizumab), CD18, CD20 (e.g., rituximab), CD85, ICOS ligand, CCR2, CXCR3, or CCR5 can be useful when conjoined to an HSA linker of the invention for therapeutic use in a patient suffering from MS.

Similary, binding moieties that neutralize type I interferons (e.g., interferons -alpha and –beta) or that antagonize type I interferon receptors (e.g., IFNαR1) can also be conjoined to an HSA linker of the invention for therapeutic application.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disorder that causes the immune system to attack the joints. It is a disabling and painful inflammatory condition, which can lead to substantial loss of mobility due to pain and joint destruction. RA is a systemic disease, often affecting extra-articular tissues throughout the body including the skin, blood vessels, heart, lungs, and muscles.

Patients suffering from RA frequently have an increase in cellular expression of the HLA-DR4/DR1 cluster. HSA linker conjugates specific for one or both of these cell surface molecules are useful for the diagnosis of RA.

An HSA linker conjugate of the invention can be used to treat a person at risk of developing of suffering from RA to prevent, ameliorate, or cure the symptoms of the disease. For example, binding moieties, as defined herein, that specifically target and antagonize TNF-α (e.g., etanercept, infliximab, and adalimumab), IL-1 (e.g., anakinra), or CTLA-4 (e.g., abatacept). Binding moieties that specifically target and deplete B cells (e.g., an anti-CD20 antibody, such as rituximab) can also be conjoined to the HSA linker described herein to treat or prevent RA.

Uveitis

Uveitis specifically refers to inflammation of the middle layer of the eye, but may refer to any inflammatory process involving the interior of the eye. Uveitis may be autoimmune or idiopathic in origin

An HSA linker conjugate of the invention can be used to treat a person at risk of developing of suffering from autoimmune uveitis to prevent, ameliorate, or cure the symptoms of the disease. For example, alpha-fetoprotein (e.g., human AFP; NCBI Accession No. NM_001134), or biologically-active fragments thereof, can be conjoined to an HSA linker of the invention to reduce or eliminate inflammation associated with autoimmune or idiopathic uveitis.

Kits

The invention provides kits that include a pharmaceutical composition containing an HSA linker of the invention, and one or more of a binding moiety (e.g., antibodies or antibody fragments), a diagnostic agent (e.g., radionuclide or chelating agents), and a therapeutic agent (e.g., cytotoxic or immunomodulatory agents) with reagents that can be used to conjugate them to the HSA linker, if necessary, and including a pharmaceutically-acceptable carrier, in a therapeutically effective amount for treating a disease or condition (e.g., a cancer, autoimmune disease, or cardiovascular disease).. The kits include instructions to allow a practitioner (e.g., a physician, nurse, or patient) to administer the composition contained therein.

Preferably, the kits include multiple packages of the single-dose pharmaceutical composition(s) containing an effective amount of an HSA linker of the invention, or any binding (e.g., antibodies or antibody fragments (e.g., scFv)), diagnostic (e.g., radionuclide or chelating agents), and/or therapeutic (e.g., cytotoxic or immunomodulatory agents) conjugate thereof. Optionally, instruments or devices necessary for administering the pharmaceutical composition(s) may be included in the kits. For instance, a kit of this invention may provide one or more pre-filled syringes containing an effective amount of an HSA linker of the invention, or any binding, diagnostic, and/or therapeutic agent conjugated thereto. Furthermore, the kits may also include additional components such as instructions or administration schedules for a patient suffering from a disease or condition (e.g., a cancer, autoimmune disease, or cardiovascular disease) to use the pharmaceutical composition(s) containing an HSA linker of the invention, or any binding, diagnostic, and/or therapeutic agent conjugated thereto.

It will be apparent to those skilled in the art that various modifications and variations can be made in the compositions, methods, and kits of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially the same or similar results.

Example 1: *Methods to identify residues in the HSA linker for site-specific conjugation of cytotoxic or cytostatic drugs.*

To identify sites for specific conjugation of drug to HSA the crystal structure was studied and surface exposed serine and threonine residues were identified. These particular surface-exposed amino acids can then be mutated to cysteine allowing drug conjugation to the substituted cysteine using a thiol-specific conjugating agent such as maleimide. Mild reduction may be required prior to drug conjugation. The number of drugs conjugated is controlled by the number of surface exposed cysteine residues introduced into HSA. Serine and threonine are selected as the most suitable residues for mutation as they share the most structural identity with cysteine, however, other surface exposed residues may also be mutated to cysteine and successfully conjugated to cytostatic or cytotoxic drugs.

The crystal structure of HSA is deposited in the RSCB Protein Data Bank (1bm0 – Sugio *et al.*, "Crystal structure of human serum albumin at 2.5 A resolution," *Protein Eng.* 12:439-446 (1999)). This structure was analyzed using the DeepView Swiss PDB Viewer downloaded from the Swiss Institute of Bioinformatics. Serine and threonine residues with 50%, 40%, and 30% surface exposure were identified as the most suitable for mutation to cysteine (Table 2). Mutations can be introduced using standard molecular biology procedures. Conjugation of a thiol reactive drug or chelating agent to introduced cysteines can be tested using standard protein chemistry techniques.

Table 2

% Surface Exposure	Residue
50	T496
40	S58
30	T76, T79, T83, T125, T236, S270, S273, S304, S435, T478, T506, T508

Example 2: *Methods to identify residues in the HSA linker for introduction of asparagine-linked glycosylation sites.*

To identify regions for introduction of asparagine-linked glycosylation sites in HSA, the crystal structure was studied to identify surface exposed (>30%) asparagine, serine and threonine residues that would be suitable for mutation. Glycosylation occurs on asparagine residues when the

consensus sequence asparagine – x – serine/threonine is present, where x cannot be a proline. Table 2 lists possible mutation sites in HSA for the introduction of asparagine-linked glycosylation.

Table 3

Residue	Proposed Mutation
Gln32	Asn
Val46	Ser/Thr
Asp56	Asn
Asp63	Ser/Thr*
Glu231	Asn
Asp237	Asn
Gln268	Asn
Asp269	Ser/Thr
Glu285	Asn
Ala320	Ser/Thr*
Asp340	Asn
Glu354	Asn
Gln437	Asn
Glu425	Asn
Glu465	Asn
Asp494	Asn*

*These mutations have also been reported to occur very rarely in HSA (Carlson *et al.*, "Alloalbuminemia in Sweden: Structural study and phenotypic distribution of nine albumin variants," *Proc.Nat.Acad.Sci.USA* 89:8225- 8229 (1992); Madison *et al.*, "Genetic variants of human serum albumin in Italy: point mutants and a carboxyl-terminal variant," *Proc.Nat.Acad.Sci.USA* 91:6476-6480 (1994); Hutchinson *et al.*, "The N-terminal sequence of albumin Redhill, a variant of human serum albumin," *FEBS Lett.* 193:211-212 (1985); Brennan *et al.*, "Albumin Redhill (-1 Arg, 320 Ala-Thr): a glycoprotein variant of human serum albumin whose precursor has an aberrant signal peptidase cleavage site," *Proc.Nat.Acad.Sci.USA* 87:26-30 (1990); Minchiotti *et al.*, "Structural characterization of four genetic variants of human serum albumin associated with alloalbuminemia in Italy," *Eur.J.Biochem.* 247:476-482 (1997); Peach *et al.*, "Structural characterization of a glycoprotein variant of human serum albumin: albumin Casebrook (494 Asp-Asn)," *Biochim.Biophys.Acta* 1097:49-54 (1991)).

Example 3

B2B3-1 is a bispecific scFv antibody fusion molecule consisting of B1D2, a human anti-ErbB2 scFv antibody (SEQ ID NO:27) and H3, a human anti-ErbB3 scFv (SEQ ID NO:26). The two scFvs are joined by a modified human serum albumin (HSA) linker. The anti-ErbB3 scFv, H3, is recombinantly fused to the amino terminus of the HSA linker incorporating a short connector polypeptide and the anti-ErbB2 scFv, B1D2, is recombinantly fused to the carboxy terminus of the

modified HSA linker incorporating an additional short connector polypeptide. Each connector polypeptide was selected based on protease resistance properties. The modified HSA linker contains two amino acid substitutions. A cysteine residue at position 34 of native HSA was mutated to serine in order to reduce potential protein heterogeneity due to oxidation at this site. An asparagine residue at amino acid 503 of native HSA, which in native HSA is sensitive to deamidation and can result in decreased pharmacologic half-life, was mutated to glutamine. B2B3-1 selectively binds ErbB2 over-expressing tumors by virtue of its high affinity anti-ErbB2 scFv binding moiety, which has a kD in the range of 10.0 nM to 0.01 nM and more preferably a kD of about 0.3 nM. Subsequent binding of ErbB3 by the anti-ErbB3 scFv, which has a kD in the range of 50 to 1 nM and more preferably about 16 nM, prevents HRG induced phosphorylation of ErbB3. The modified HSA linker confers an extended circulating half-life on the bispecific molecule. B2B3-1 has a molecular weight of 119.6 kDa and is not glycosylated.

B2B3-1 inhibits ligand-induced phosphorylation of ErbB3 with sub-nanomolar potency by exploiting the abundant expression of its dimerization partner, ErbB2, to specifically target cancer cells that express both receptors.

Example 4

As shown in Figure 2, B2B3-1 variants inhibit HRG-induced pErbB3 in ZR75-1 breast cancer cells. ZR75-1 breast cancer cells were treated with a dose range of B2B3-1 variants for 24 hours followed by HRG stimulation. pErbB3 levels were measured in cell lysates and IC_{50} values were calculated together with the percent of inhibition. Shown are the mean IC_{50} values (Y axis) with error bars representing replicate experiments. Percent inhibition values are shown above the corresponding bar.

Example 5

Inhibition of phosphorylated ErbB3 (Figures 3A-D), AKT (Figures 4A-D), and ERK (Figures 5A-D) following 24 hour pre-treatment with B2B3-1 variants A5-HSA-B1D2 (panel A of Figures 3-5), H3-HSA-B1D2 (panel B of Figures 3-5), H3-HSA-F5B6H2 (panel C of Figures 3-5), and F4-HSA-F5B6H2 (panel D of Figures 3-5). BT474 breast cancer cells were treated with a dose range of B2B3-1 variants for 24 hours followed by HRG stimulation. pErbB3, pAKT, and pERK levels were measured in cell lysates and IC_{50} values were calculated together with the percent of inhibition.

Example 6

As shown in Figure 6, treatment of BT474 breast tumor cells with B2B3-1 variants causes G1 cell cycle arrest and a decrease in the population of cells in S phase. BT474 cells were treated with 1 μM of B2B3-1 variants and controls for 72 hours. After the end of treatment, cells were trypsinized, gently resuspended in hypotonic solution containing propidium iodide and single cells were analyzed by flow cytometry. Cell cycle distribution in G1 and S phases was measured using curve-fitting algorithms designed for cell cycle analysis (FlowJo software cell cycle platform).

Example 7

B2B3-1 (SEQ ID NO:16) inhibits ErbB3 activation, utilizing the abundant expression of its dimerization partner, ErbB2, to target tumor cells. A high affinity anti-ErbB2 scFv antibody, B1D2, facilitates targeting of B2B3-1 to tumor cells over-expressing ErbB2. B1D2 is connected by a modified HSA linker to a lower affinity anti-ErbB3 scFv antibody, H3, which blocks binding of ErbB3's ligand, HRG, thereby inhibiting ErbB3 phosphorylation and downstream AKT signaling. The ErbB2 binding scFv, B1D2, is derived from parent scFv C6.5, which possesses neither agonistic nor antagonistic activity. B1D2, therefore, likely functions solely as a targeting agent. The lower affinity binding of the ErbB3 binding scFv prevents strong binding of B2B3-1 to normal, non-cancerous tissues which express ErbB3 but little or no ErbB2, thereby reducing the potential for non-specific toxicity. In tumor cells expressing both ErbB2 and ErbB3, the avidity effect of bispecific B2B3-1 binding to both receptors overcomes the low affinity of the ErbB3 scFv allowing strong inhibition of HRG interaction.

The ability of B2B3-1 to inhibit HRG binding to ErbB3 was investigated using flow cytometry (FACS). Cells of the breast cancer cell line BT-474-M3 (a variant of BT-474 that over-express ErbB2), were pretreated with 1 μM B2B3-1 then incubated with 10 nM biotinylated HRG 1β EGF domain. After extensive washing, binding was assessed using streptavidin-AlexaFluor 647 conjugate. All incubations were performed at 4°C. Figure 7 shows that B2B3-1 is capable of blocking the binding of HRG to ErbB3.

Example 8

After demonstrating B2B3-1's ability to block HRG binding to ErbB3, we investigated the effect of B2B3-1 on ErbB3 signaling *in vitro* on two cell lines that express ErbB3 and over-express ErbB2. Breast cancer cell lines BT-474-M3 and ZR75-3 0 were serum starved overnight, pre-treated with a dose titration of B2B3-1 for 24 hours and then stimulated for 10 minutes with 5 nM of HRG 1β EGF domain. The phosphorylation status of ErbB3 and AKT was then examined using an ELISA assay. The results show that B2B3-1 inhibits HRG induced activation of both ErbB3 and AKT phosphorylation in a dose-dependent manner and with potent IC_{50}s in both cell lines (Figures 8A-D).

Example 9

Figure 9 shows the effect of B2B3-1 treatment on signaling proteins in BT474 breast cancer cells. Cells were treated with a dose range of B2B3-1 for 24 hours, followed by heregulin stimulation. Levels of pErbB2, pErbB3, pErk and pAKT and their corresponding total protein levels were determined on cell lysates by Western blot analysis.

Example 10

Figure 10 shows the immunoprecipitation-Western blot analysis of B2B3-1 treated BT474 breast cancer cells. Cells were treated with a dose range of B2B3-1 for 24 hours, followed by heregulin stimulation. ErbB2-associated complexes were isolated from cell lysates using an anti-ErbB2 antibody followed by Western blot analysis to detect pErbB2 and pErbB3 and their corresponding total protein levels.

Example 11

The anti-tumor activity of B2B3-1 was investigated *in vitro* using a number of assays. In the first assay, the effect of B2B3-1 on the accumulation of BT-474 or SKBR3 cells in G1 phase and the concomitant decrease in S phase of the cell cycle was examined. Briefly, cells were treated with 1 μM B2B3-1 or PBS vehicle for 72 hours. After the end of treatment, cells were trypsinized, gently resuspended in hypotonic solution containing propidium iodide and single cells were analyzed by flow cytometry. Cell cycle distribution in G1 and S phases was measured using curve –fitting algorithms designed for cell cycle analysis (FlowJo software cell cycle platform). B2B3-1 was found to modestly decrease the percentage of cells in S phase and increase the population of cells in

G1 phase (Figure 11A). In a second experiment, the number of cell colonies formed following treatment with B2B3-1 was studied. BT-474 and SKBR3 breast cancer cells were plated in the presence of 1 μM B2B3-1 and compared to cells plated in media only. Media only or media including treatment was replenished every 3 days. After 14 days the number of colonies were counted and compared to untreated cells. Figure 11B illustrates the 40-45% decrease in the number of colonies formed when cells are treated with B2B3-1 compared to control cells. Finally, the ability of B2B3-1 to inhibit cell proliferation was assessed in a cell impedance assay using a Real-Time Cell Electronic Sensing System (RT-CES: ACEA Biosciences). BT- 474 cells were seeded on plates integrated with microelectronic sensor arrays and treated with a dose titration of B2B3-1 or media only for 72 hours. Data reflecting the generation of cell-electrode impedance response was collected every hour for 72 hours and IC_{50} values were calculated 68 hours after treatment. Figure 11C illustrates that B2B3-1 was able to inhibit impedance of BT-474 cells with an IC_{50} of 33 nM.

Example 12

We also investigated whether B2B3-1 exhibits agonistic activity based on its ability to simultaneously bind and cross-link ErbB2 and ErbB3 receptors. Serum starved ZR75-1 breast cancer cells were incubated with 1 μM B2B3-1 or PBS vehicle for 24 hours. Cells were also treated with B2B3-1 or PBS vehicle for 24 hours followed by a 10-minute stimulation with 5 nM HRG 1β EGF domain. Cells were lysed and the pErbB3 content of the lysates was assessed by ELISA. Figure 12 shows that cells treated with B2B3-1 alone contained comparable levels of phosphorylated ErbB3 as untreated cells, indicating that B2B3-1 is non-agonistic.

Example 13

The ability of B2B3-1 to bind with specificity to ErbB2 and ErbB3 and not to related ErbB family members, EGFR and ErbB4, was investigated by ELISA. Plates were coated with the recombinant extracellular domain of either ErbB2 or ErbB3. Plates were blocked and incubated with a half-maximal binding concentration of B2B3-1 in the presence of a dilution series of competing recombinant extracellular domains of EGFR, ErbB2, ErbB3 or ErbB4. The results show only soluble ErbB2 extracellular domain blocked B2B3-1 binding to ErbB2-coated plates (Figure 13A). Likewise, only soluble ErbB3 extracellular domain blocked B2B3-1 binding to ErbB3-coated plates (Figure 13B). These results demonstrate the specificity of the anti-ErbB2 arm B1D2 for ErbB2, and

of the anti-ErbB3 arm H3 for ErbB3. The increased signal observed when soluble ErbB2 extracellular domain was incubated with B2B3-1 on the ErbB3 coated plate is assumed to be due to formation of an ErbB2, ErbB3, B2B3-1 complex on the plate.

Example 14

The ability of B2B3-1 to bind avidly to tumor cells expressing both ErbB2 and ErbB3 was studied. SKO2 and SKO3 are variants of B2B3-1 that lack the ability to interact with ErbB2 or ErbB3, respectively. MALME-3M melanoma cells, which express approximately equal numbers of ErbB2 and ErbB3 receptors, were incubated with a dilution series of B2B3-1, SKO2, or SKO3 in the presence of saturating concentrations of a goat anti-HSA Alexafluor-647 conjugated antibody. Cell binding was assessed by flow cytometry and apparent binding affinities were determined for each molecule. Control cells were incubated with secondary antibody alone. No measurable cell binding was observed for SKO2, which retains only low affinity binding to ErbB3 and lacks ErbB2 binding activity. SKO3, which retains a functional, high affinity ErbB2 binding scFv but lacks the ability to bind ErbB3 had a K_D of 6.7 nM. B2B3-1 bound cells with a K_D of 0.2 nM, demonstrating avidity binding of this bispecific molecule (Figure 14).

Example 15

The stability of B2B3-1 under physiological conditions was assessed by incubating 100 nM B2B3-1 in human, Cynomolgus monkey, or mouse serum at 37°C for a period of 120 hours. Samples were removed at 0, 24, 48, 72, 96 and 120 hours and the ability of B2B3-1 to bind both ErbB2 and ErbB3 was measured by ELISA. This ELISA involves coating a 96-well plate with recombinant ErbB2 extracellular domain overnight followed by a blocking step and then incubation with a dilution series of B2B3-1. Plates are then incubated with an Fc-ErbB3 extracellular domain fusion protein followed by a goat antihuman-Fc-HRP conjugate. Plates are developed by addition of supersignal chemiluminescense substrate. Figures 15A-C show that B2B3-1 remains stable in serum from all three species under physiological conditions, retaining comparable ability to bind both ErbB2 and ErbB3 at all time points measured.

Example 16: *B2B3-1 dose response in BT-474-M3 breast cancer xenograft model.*

The efficacy of B2B3-1 *in vivo* was assessed using nude mice bearing BT-474-M3 xenografts. Ten mice per group were treated with 12 doses of 0.3, 1, 3, 10, 30 or 90 mg/kg of B2B3-1 every 3 days. Control groups were administered PBS vehicle or HSA at an equimolar dose to the 90 mg/kg B2B3-1 dose. All doses were administered interperitoneally (i.p.). Tumor size was measured twice a week and the corresponding tumor volume was calculated. The results show that B2B3-1 treatment leads to significant reduction in BT-474-M3 tumor size as compared to the control group (Figure 16). Complete regressions were observed in each of the B2B3-1 treatment groups except mice treated with the lowest dose of B2B3-1 (0.1 mg/kg).

Example 17

As shown in Figures 17A-E, B2B3-1 is effective in multiple xenograft models in an ErbB2 dependent manner. B2B3-1 was efficacious in the Calu-3 (Figure 17A), SKOV-3 (Figure 17B), NCI-N87 (Figure 17C), and MDA-MB-361 (Figure 17E) xenograft models expressing ErbB2 at > 1 x 10^5 receptors/cell but worked less well in the ACHN (Figure 17D) xenograft model which expresses 4.5 x 10^4 ErbB2 receptors/cell. Mice were treated with 30 mg/kg of B2B3-1 every 3 days.

Example 18

Over-expression of ErbB2 converts B2B3-1 non-responder ADRr breast cancer xenograft model into a responder to B2B3-1 (Figures 18A B). ErbB2 was over-expressed in ADRr breast cancer cells using a retroviral expression system. Transfected cells expressing high levels of ErbB2 (ADRr –E2) were selected using FACS and subsequently injected subcutaneously into nude mice to establish xenograft tumors. Mice were treated with 30 mg/kg of B2B3-1 every 3 days. While no response to B2B3-1 was observed in wild type ADRr xenografts (Figure 18A), ADRr-E2 xenografts (Figure 18B) responded to B2B3-1.

Example 19

As shown in Figures 19A-B, B2B3-1 activity correlates positively with ErbB2 expression levels *in vitro* (Figure 19A) and *in vivo* (Figure 19B). B2B3-1 inhibition of ErbB3 phosphorylation was determined in 9 tumor cell lines with expression levels of ErbB2 ranging from 5 x 10^4 receptors/cell to 2.4 x 10^6 receptors/cell using an ELISA assay. The extent of B2B3-1's ability to

inhibit ErbB3 phosphorylation to basal levels (% pErbB3 inhibition) was found to correlate positively with ErbB2 expression levels. Similarly, B2B3-1 activity was assessed in 10 tumor xenograft models expressing low to high levels of ErbB2. Xenograft response also correlated positively with ErbB2 expression levels.

Example 20

B2B3-1 treatment of BT474-M3 breast tumor cells results in translocation of $p27^{kip1}$ to the nucleus (Figure 20A). BT474-M3 cells were treated with 1 μM of B2B3-1 for 6 hours. The sub-cellular location of $p27^{kip1}$ was assessed using immunofluorescence techniques. In cells treated with B2B3-1, $p27^{kip1}$ translocated to the nucleus, which has been shown to result in inhibition of cell proliferation. $p27^{kip1}$ remained in the cytoplasm of untreated cells.

To further study the effect of B2B3-1 on the cell cycle, BT-474-M3 cells treated with B2B3-1 for 72 hours were probed for the cell cycle regulator Cyclin D1 using Western blot analysis (Figure 20B). The cytoskeleton protein vinculin was used as a protein loading control in this experiment. B2B3-1 treatment resulted in a decrease in the levels of Cyclin D1 compared to untreated cells.

Example 21

As shown in Figures 21A-B, B2B3-1 treatment of BT474-M3 breast tumor xenografts results in translocation of $p27^{kip1}$ to the nucleus. BT474 breast tumor xenografts were treated with B2B3-1 (Figures 21A) at a dose of 30 mg/kg or an equimolar dose of HSA (Figures 21B) every 3 days for a total of 4 doses. Increased nuclear staining for $p27^{kip1}$ was observed in B2B3-1 treated tumors compared to HSA control tumors indicating an anti-proliferative effect of B2B3-1 *in vivo*.

Example 22

B2B3-1 treatment results in a reduction of the proliferation marker Ki67 in BT474 breast cancer xenograft tumors. BT474-M3 breast tumor xenografts were treated with B2B3-1 (Figure 22A) at a dose of 30 mg/kg or an equimolar dose of HSA (Figure 22B) every 3 days for a total of 4 doses. Subsequent staining of tumor sections for Ki67 demonstrated a reduced expression pattern for B2B3-1 treated tumors compared to HSA treated tumors.

Example 23

B2B3-1 treatment results in a reduction of vessel density in BT474-M3 breast cancer xenograft tumors, as determined by assaying for CD31 expression (Figures 23A-B). BT474 breast tumor xenografts were treated with B2B3-1 (Figure 23A) at a dose of 30 mg/kg or an equimolar dose of HSA (Figure 23B) every 3 days for a total of 4 doses. Tumors were stained for the presence of vascular marker CD31. Tumors treated with B2B3-1 show a marked decrease in vessel density compared to control tumors treated with HSA.

Example 24: *B2B3-1 inhibits phosphorylation of ErbB3 in vivo.*

BT-474-M3 xenograft tumors were treated with 30 mg/kg B2B3-1 or 17.5 mg/kg HSA every 3 days for a total of 4 doses and tumors were harvested 24 hours after the final dose. Tumors were lysed and subjected to SDS-PAGE followed by Western analysis to assess relative levels of phosphorylation of B2B3-1's target ErbB3. Equal quantities of protein were loaded in each lane and total protein levels were controlled by probing for beta tubulin. Western blot analysis using antibodies specific for phosphorylated ErbB3 show that B2B3-1 treated tumors contain less pErbB3 than HSA treated tumors (Figure 24A). Densitometry of the western blot analysis followed by normalization of the mean pErbB3 integral band intensity to the mean beta tubulin integral band intensity demonstrated that B2B3-1 treated tumors contained significantly less pErbB3 than control HSA treated tumors (Figure 24B). These data confirmed that B2B3-1 inhibits phosphorylation of its target ErbB3 *in vivo.*

Example 25: *In vivo activity of B2B3-1 in BT-474-M3 xenografts which have reduced PTEN activity.*

ShRNA technology was applied to knock out the activity of the tumor suppressor gene phosphatase and tensin homolog (PTEN) in BT-474-M3 breast cancer cells. Briefly, cultured BT-474-M3 cells were transfected with shPTEN or shControlRNA by retroviral transfection. Transfected cells with reduced PTEN were selected using FACS and subsequently injected subcutaneously into the right flank of nude mice to establish xenograft tumors. Cells transfected with a control vector were injected into the left flank of the same mouse. Mice were treated with 30mg/kg B2B3-1 every 3 days or 10mg/kg Herceptin every week. HSA was injected as a control at an equimolar dose to B2B3-1. All injections were done i.p.

B2B3-1 and Herceptin promoted a reduction in the size of tumors formed by control BT-474-M3 breast cancer cells (Figure 25A), whereas only B2B3-1 (and not Herceptin) promoted a reduction in the size of tumors formed by BT-474-M3 breast cancer cells lacking expression of PTEN (Figure 25B).

Example 26: *B2B3-1 inhibits ErbB3 signaling in BT-474-M3 breast cancer cells having reduced PTEN activity.*

The ability of B2B3-1 to inhibit phosphorylation of ErbB3 signaling in tumor xenografts was studied using the PTEN deficient BT-474-M3 model. Xenograft tumors of the engineered cell line or control cell line were treated with 30 mg/kg B2B3-1, 17.5 mg/kg HSA every 3 days or 10 mg/kg Herceptin weekly and tumors were harvested 24 hours after the final dose. Tumors were lysed and subjected to SDS-PAGE followed by Western analysis to assess relative levels of phosphorylation of B2B3-1's target ErbB3, AKT and total PTEN levels. Equal quantities of protein were loaded in each lane and total protein levels were controlled by probing for PCNA. Western blot analysis using antibodies specific for phosphorylated ErbB3 show that B2B3-1 treated tumors contain less pAKT than HSA treated or Herceptin treated tumors (Figure 26A). Densitometry of the western blot analysis followed by normalization of the mean pAKT integral band intensity to the mean PCNA integral band intensity demonstrated that B2B3-1 treated tumors contained significantly less pAKT than control HSA treated and Herceptin-treated tumors (Figure 26B).

Example 27

The pharmacokinetic parameters for B2B3-1 were investigated in nu/nu mice. Animals were randomized into groups and administered intravenous (IV) with a single dose of 5, 15, 30, or 45 mg/kg B2B3-1 (Figures 27A-D, respectively). Blood was collected pre-dose and at 0.5, 4, 8, 24, 48, 72, and 120 hours post dose. Three mice were used for each time point. Serum levels of B2B3-1 were measured using two ELISA methods. The first method requires functional binding of B2B3-1 to both ErbB2 and ErbB3 while the second method measures only the HSA component of B2B3-1 in the serum. The HSA ELISA utilizes a polyclonal-anti HSA capture antibody and a HRP-conjugated polyclonal anti-HSA detection antibody. A reduction in B2B3-1 serum concentration measured using the ErbB2/ErbB3 binding method versus the HSA method would indicate a loss in functional

B2B3-1. Figures 27A-D show that the pharmacokinetic properties of B2B3-1 are comparable when assessed using either ELISA method, indicating that B2B3-1 is stable in circulation in mice.

Example 28

B2B3-1 serum concentrations were fit using a two-compartment, biexponential model and showed biphasic disposition. Terminal half-lives were calculated to be 17, 16, 23, and 18 hrs for the 5, 15, 30, or 45 mg/kg doses, respectively, and are shown in Table 4. Increases in B2B3-1 dose resulted in a linear increase in exposure (Figure 28).

Table 4: Pharmacokinetic properties of B2B3-1 in mice and Cynomolgus monkeys.

Dose (mg/kg)	Species	N	T½β (hrs)	AUC (hr-µg/ml)	Clearance (ml/hr/kg)
5	Mouse (single dose)	3	16.9	1.58E+03	3.19
15	Mouse (single dose)	3	16.2	6.10E+03	2.47
30	Mouse (single dose)	3	22.6	1.18E+04	2.54
45	Mouse (single dose)	3	17.5	1.84E+04	2.46
4	Cynomolgus monkey (1st dose)	2	39.1	3.44E+03	1.17
4	Cynomolgus monkey (4th dose)	2	44.9	7.20E+03	0.60
20	Cynomolgus monkey (1st dose)	2	33.1	2.29E+04	0.88
20	Cynomolgus monkey (4th dose)	2	122.5	8.20E+04	0.25
200	Cynomolgus monkey (1st dose)	4	68.8	3.18E+05	0.64
200	Cynomolgus monkey (4th dose)	2	69.7	5.72E+05	0.35
200	Cynomolgus monkey (4th dose)*	2	66.6	5.99E+05	0.34

*recovery animals

Example 29

Blood samples for pharmacokinetic analysis were also obtained from a dose range-finding toxicology study in female Cynomolgus monkeys. In this study, animals were infused with 4, 20 or 200 mg/kg of B2B3-1 administered every 3 days for 4 doses. Sampling occurred prior to and 5 minutes after dosing on each dosing day (study days 1, 4, 7 and 10) to provide pre-dose and

peak/trough concentrations, and at 1, 2, 4, 8, 24 and 48 hours after the end of the first infusion on day 1 and at 1, 2, 4, 8, 24, 48, 72 and 120 hours after the last infusion on day 10. For recovery animals dosed at 200 mg/kg serum samples were also collected at 168, 336 and 456 hours after the last infusion.

Cynomolgus monkey serum samples were assayed using the ErbB2/ErbB3 ELISA method described previously. Serum concentrations for each dose over the time course are shown in Figure 29. The analysis showed that mean concentration-time profiles for serum B2B3-1 after dosing on days 1 and 10 were qualitatively similar with concentrations generally declining with time from Cmax. Mean half-life estimates ranged from 38.3-67.2 hours on day 1 and 45.0 to 121.0 hours on day 10 (Table 4).

Example 30

The plasmid encoding the B2B3-1 bispecific scFv antibody fusion protein was created combining gene sequences of a unique human anti-ErbB3 scFv (designated "H3"), a human anti-ErbB2 scFv (designated "B1D2"), and a modified human serum albumin (HSA) linker. The anti-ErbB3 scFv, H3, is recombinantly linked to the amino terminus of the HSA linker via a connecting peptide (Ala-Ala-Ser) and the anti-ErbB2 scFv, B1D2, is genetically linked the carboxy terminus of the HSA linker via a connecting peptide (Ala-Ala- Ala-Leu). The peptide connectors were formed through the introduction of restriction sites during construction of the mammalian expression vector and were synthesized with optimized codon usage for mammalian expression together with the single chain antibody fragments and HSA linker.

The B1D2 scFv was selected from a combinatorial phage display library created by mutagenesis of the ErbB2-binding scFv C6.5, which was selected from a non-immune phage display library. The H3 scFv was selected from a non-immune phage display library originally made by Sheets et al. The gene sequences encoding the B1D2 and H3 single chain antibody fragments were optimized for CHO cell codon preferences and synthesized for subsequent construction of the B2B3-1 encoding plasmid.

The modified HSA linker contains two amino acid substitutions. A cysteine residue at position 34 was mutated to serine in order to reduce potential protein heterogeneity due to oxidation at this site. An asparagine residue at amino acid 503 was mutated to glutamine, which in wild type HSA is sensitive to deamination and can result in decreased pharmacologic half-life.

The gene sequence encoding the modified HSA linker was synthesized with optimized codon usage for mammalian expression for subsequent construction of the B2B3-1 encoding plasmid.

Example 31

The B2B3-1 coding sequence was cloned into pMP10k base vector using standard molecular biology techniques to create plasmid pMP10k4H3-mHSA-B1D2, shown in Figure 30. For the most part this construct employs commonly used genetic elements. B2B3-1 expression is driven by the human GAPD promoter. This vector utilizes genetic elements referred to as Matrix Attachment Regions or MAR elements. The MAR genetic elements control the dynamic organization of chromatin, and insulate nearby genes from the effect of surrounding chromatin thereby increasing copy number dependent, position-independent, expression of genes. MAR elements have been shown to improve the probability of isolating a clone exhibiting the desired level of expression for the production of a recombinant protein and to increase the stability of production. The MAR elements used in the B2B3-1 constructs are non-coding human MAR elements. In addition to the B2B3-1 plasmid, a neomycin antibiotic resistance plasmid (Figure 31) and a hygromycin resistance plasmid (Figure 32) were also used to select for stable transformants.

Example 32: *First round of Gene Transfection*

Chinese Hamster Ovary CHO-K1 cells were purchased from ATCC (ATCC # CCL-61). The CHO-K1 cell line is a serum and proline dependent adherent sub-clone of the parental CHO cell line created by T.T. Puck. The CHO-K1 cells used for B2B3-1 transfection were pre-adapted for suspension growth in serum free media prior to transfection. An iterative transfection procedure was used to develop the B2B3-1 cell line. Twenty-four hours before transfection, CHO-K1 cells were sub passaged to $1.0x10^6$ cells/mL in SFM4CHO (Serum Free) medium (HyClone, Logan, UT) supplemented with 8mM L-glutamine, 0.1 mM sodium hypoxanthine, and 0.016 mM thymidine. On the day of transfection, cells were resuspended in OptiMEM medium (Invitrogen Corp, Carlsbad, CA) and 40,000 cells were placed in each well of a twenty-four well plate. In the first transfection, the B2B3-1 expression plasmid (pMP10k4H3-mHSA-B1D2) and the neomycin resistance plasmid (Figure 30; pSV2-neo (Selexis, Inc., Marlborough, MA) were mixed together using a molar plasmid ratio of 75:1 (B2B3-1:neomycin resistance). The plasmid mixture was subsequently mixed with a cationic lipid transfection reagent (Lipofectamine LTX, Invitrogen Corp, Carlsbad, CA) and

lipid/DNA complexes were allowed to form for thirty minutes. The DNA/Lipid complex was than added to the CHO-K1 cells and the 24-well plates were placed in a 37°C, 5% CO_2 incubator.

Example 33: *Selection and Screening for High Producers*

The contents of each transfection well were washed with PBS, trypsinized and distributed across two, ninety-six well plates. The growth media used consisted of DMEM/F12 (Invitrogen Corp, Carlsbad, CA) with 10% FBS (Invitrogen Corp, Carlsbad, CA) and 500 mg/L of geneticin (G418; Invitrogen Corp, Carlsbad, CA). Media in the 96-well plates was changed on day 4 to SFM4CHO medium supplemented with 8mM L-glutamine, 0.1 mM sodium hypoxanthine, 0.016 mM thymidine, and 500mg/L geneticin. Following an additional two weeks of culture in selection medium, surviving cells had formed well-defined colonies. The clones were evaluated using quantitative spot blot techniques. The top producing colonies were trypsinized, and expanded to a single well of a 24-well plate.

A seven day productivity assay was used to screen for high B2B3-1 producing colonies. Upon expansion the cells in 24-well plates were allowed to proliferate for seven days in SFM4CHO medium supplemented with 8 mM L-glutamine, 0.1 mM sodium hypoxanthine, and 0.016 mM thymidine. The B2B3-1 concentration in the spent media was determined. Top clones from the 24-well scale were expanded into 125 mL baffled shake flasks. A seven day study in the shake flask in SFM4CHO medium supplemented with 8mM L-glutamine, 0.1 mM sodium hypoxanthine, and 0.016 mM thymidine was used to screen the cell pools for growth and B2B3-1 production.

Example 34: *Second Round of Gene Transfection*

The highest producing cell pool determined from the first round of transfection (*supra*) was transfected a second time to increase production. Twenty-four hours before transfection, the cell pool was sub passaged to $1.0x10^6$ cells/mL in SFM4CHO (Serum Free) medium supplemented with 8mM L-glutamine, 0.1 mM sodium hypoxanthine, and 0.016 mM thymidine. On the day of transfection, cells were resuspended in OptiMEM medium (Invitrogen Corp, Carlsbad, CA) and 40,000 cells were placed in each well of a twenty-four well plate. In the first transfection, the B2B3-1 and hygromycin resistance plasmid (Figure 32; pTK-Hyg (Clontech, Mountain View, CA)) were mixed together using a molar plasmid ratio of 50:1 (B2B3-1:hygromycin resistance). The plasmid mixture was subsequently mixed with a cationic lipid transfection reagent (Lipofectamine LTX,

Invitrogen Corp) and lipid/DNA complexes were allowed to form for thirty minutes. The DNA/Lipid complex was than added to the cell pool and the 24-well plates were placed in a 37°C, 5% CO_2 incubator.

Example 35: *Selection and Screening for High Producers From Second Transfection*

The contents of each transfection well were washed with PBS, trypsinized and distributed across two, 96-well plates. The growth media used consisted of DMEM/F12 supplemented with 10% FBS and 400 mg/L of hygromycin B (Invitrogen Corp). Media in the 96-well plates was changed on day 4 to Hyclone SFM4CHO medium supplemented with 8mM L-glutamine, 0.1 mM sodium hypoxanthine, 0.016 mM thymidine , and 400 mg/L of hygromycin B. After an additional two weeks of selection, surviving cells had formed well-defined colonies. The clones were evaluated using quantitative spot blot techniques. The top producing colonies were trypsinized, and expanded to a single well of a 24-well plate.

A seven day productivity assay was used to screen for high B2B3-1 producing colonies. Upon expansion the cells were allowed to proliferate for seven days, and the B2B3-1 concentration in the spent media was determined.

Top clones from the 24-well plates were expanded into 125 mL baffled shaker flasks in the Hyclone SFM4CHO medium supplemented with 8mM L-glutamine, 0.1 mM sodium hypoxanthine, and 0.016 mM thymidine. A seven day study in shake flask was used to screen the cell pools for growth and B2B3-1 production. The spent media was quantitated using Protein Aresin and an HPLC instrument.

Example 36: *Limiting Dilution Cloning*

The best growing and highest B2B3-1-producing colony identified by the productivity assay was transferred from the 125 mL shaker flask and plated in five 96-well plates at a cell concentration calculated to give one cell/well. The 96-well plates were placed in an incubator at 37°C and 5% CO_2. The wells were examined bi-weekly to track formation of colonies. Colonies arising from a single cell were identified based on the symmetrical shape of the colony. Wells containing such colonies were marked for further screening by 24-well 7-day assessment, and 125mL shaker flask 7-day assessment.

The second round of limiting dilution cloning was performed in a similar manner to the first round. An additional 100 mL fed batch evaluation was performed to confirm clone choice. A pre-seed bank was cryopreserved.

Example 37: *Formulation of B2B3-1*

B2B3-1 can be administered once a week via intravenous infusion over a period of 60 or 90 minutes, depending on patient tolerability. B2B3-1 can be formulated in a sterile 20 mM L-histidine hydrochloride, 150 mM sodium chloride, pH 6.5 solution at a concentration of 25mg/mL for administration to a patient (e.g., a human).

Example 38: *Treatment of Breast Cancer*

An HSA linker of the invention (e.g., B2B3-1; SEQ ID NO:16) can be administered to a patient diagnosed with breast cancer. For example, a patient whose cancer is diagnosed as expressing high levels of epidermal growth factor receptors, such as ErbB2 (HER2/neu) can be treated with an HSA linker conjoined to an ErbB2 biding moiety. A physician providing care to a patient diagnosed with breast cancer can administer an HSA linker of the invention suitable and capable of providing the best therapeutic benefit to the patient. Selection of, for example, the binding moieties, conjoined to an HSA linker of the invention can be based on clinical determinants of the patient and specific cancer variant. For example, genotypic or histologic screens of cancer biopsies can reveal increased expression of ErbB2, indicating that an HSA linker of the invention that incorporates and anti-ErbB2 binding moiety (e.g., B2B3-1) can be used therapeutically.

In one embodiment of the invention, B2B3-1 can be administered to a patient diagnosed with breast cancer once a week via intravenous infusion over a period of, e.g., 60 or 90 minutes, depending on patient tolerability. B2B3-1 can be formulated in a sterile 20 mM L-histidine hydrochloride, 150 mM sodium chloride, pH 6.5 solution at a concentration of 25mg/mL for administration to a patient (e.g., a human). A clinician supervising the administration of B2B3-1 or any other HSA linker of the invention can follow common formulation and dosing practices to determine the proper course of therapy for any given patient.

The invention further provides for the co-administration of one or more therapeutic drugs or compounds in combination with the HSA linker of the invention (e.g., B2B3-1; SEQ ID

NO:16), such as the common chemotherapeutic regimen for the treatment of breast cancer includes doxorubicin, cyclophosphamide, and paclitaxel. Alternatively, a clinician can administer the HSA linker of invention (e.g., B2B3-1) in combination with surgical or radiation therapy to treat breast cancer in a patient in need thereof.

Example 39: *Treatment of Ovarian Cancer*

An HSA linker of the invention can be administered to a patient diagnosed with ovarian cancer (ovarian carcinoma), for example, a patient whose cancer is diagnosed as expressing high levels of epidermal growth factor receptors, such as ErbB2 (HER2/neu) can be treated with an HSA linker conjoined to an ErbB2 biding moiety. A physician providing care to a patient diagnosed with ovarian cancer can administer an HSA linker of the invention suitable and capable of providing the best therapeutic benefit to the patient. Selection of, for example, the binding moieties, conjoined to an HSA linker of the invention can be based on clinical determinants of the patient and specific cancer variant. For example, genotypic or histologic screens of cancer biopsies can reveal increased expression of ErbB2 or other targeting molecules (e.g., overexpressed receptors or ligands specific to the cancer), which can be targeted by an HSA linker of the invention that incorporates an anti-ErbB2 binding moiety (e.g., B2B3-1) or other target specific binding moiety.

In one embodiment of the invention, B2B3-1 can be administered to a patient diagnosed with ovarian cancer once a week via intravenous infusion over a period of, e.g., 60 or 90 minutes, depending on patient tolerability. B2B3-1 can be formulated in a sterile 20 mM L-histidine hydrochloride, 150 mM sodium chloride, pH 6.5 solution at a concentration of 25mg/mL for administration to a patient (e.g., a human). A clinician supervising the administration of B2B3-1 or any other HSA linker of the invention for the treatment of ovarian cancer can follow common formulation and dosing practices (e.g., intraperitoneal injection) to determine the proper course of therapy for any given patient.

The invention further provides for the co-administration of one or more therapeutic drugs or compounds in combination with the HSA linker of the invention (e.g., B2B3-1; SEQ ID NO:16). Alternatively, a clinician can administer an HSA linker of invention in combination with surgical or radiation therapy to treat ovarian cancer in a patient in need thereof.

Example 40

HSA linker conjugates of the invention can be constructed using one or more of the elements (groups A-E) listed in Table 5 below. In particular, an HSA linker of the invention, which is shown as Group C in Table 5 below, can incorporate one or more binding moieties selected from groups A and E shown in Table 5. In addition, the HSA linker conjugates can also include one or more peptide connectors, which are selected from groups B and D in Table 5, at each of the amino and carboxy terminal ends of the HSA linker. Peptide connectors can be repeated or truncated to increase or decrease the length of the connector sequence.

The invention provides specific embodiments of the HSA linkers, polypeptide connectors, and binding moieties discussed above. Table 6 lists ten HSA linker conjugates with varying ErbB2-specific or ErbB3-specific binding moieties, as well as polypeptide connectors, at the amino and carboxy termini of an HSA linker of the invention.

Table 6

Agent of the Invention	Amino Terminal Binding Moiety	N-Terminal Connector	HSA	C-Terminal Connector	Carboxy Terminal Binding Moiety
B2B3-1 (SEQ ID NO:16)	H3 (SEQ ID NO:26)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	B1D2 (SEQ ID NO:27)
B2B3-2 (SEQ ID NO:17)	A5 (SEQ ID NO:28)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	B1D2 (SEQ ID NO:27)
B2B3-3 (SEQ ID NO:18)	A5 (SEQ ID NO:28)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	F5B6H2 (SEQ ID NO:32)
B2B3-4 (SEQ ID NO:19)	A5 (SEQ ID NO:28)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	ML3.9 (SEQ ID NO:29)
B2B3-5 (SEQ ID NO:20)	B12 (SEQ ID NO:30)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	B1D2 (SEQ ID NO:27)
B2B3-6 (SEQ ID NO:21)	B12 (SEQ ID NO:30)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	F5B6H2 (SEQ ID NO:32)
B2B3-7 (SEQ ID NO:22)	F4 (SEQ ID NO:31)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	B1D2 (SEQ ID NO:27)
B2B3-8 (SEQ ID NO:23)	F4 (SEQ ID NO:31)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	F5B6H2 (SEQ ID NO:32)
B2B3-9 (SEQ ID NO:24)	H3 (SEQ ID NO:26)	AAS	HSA (SEQ ID NO:3)	AAAL (SEQ ID NO:5)	B1D2 (SEQ ID NO:27)
B2B3-10 (SEQ ID NO:25)	H3 (SEQ ID NO:26)	AAS	mHSA (SEQ ID NO:1)	AAAL (SEQ ID NO:5)	F5B6H2 (SEQ ID NO:32)

APPENDIX A

SEQUENCE LISTINGS

SEQ ID NO:1
Human Serum Albumin (HSA) Linker with C34S and N503Q substitutions ("mHSA")
Amino Acid Sequence

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAE
NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV
DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP
KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTK
VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPAD
LPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCC
AAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPT
LVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLV
NRRPCFSALEVDETYVPKEFQAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQL
KAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

SEQ ID NO:2
Human Serum Albumin (HSA) Linker w/C34S and N503Q substitutions ("mHSA")
Nucleotide Sequence

GACGCTCACAAGAGCGAAGTGGCACATAGGTTCAAAGATCTGGGCGAAGAGAACTTTA
AGGCCCTCGTCCTGATCGCTTTCGCACAGTACCTCCAGCAGTCTCCCTTTGAAGATCAC
GTGAAACTGGTCAATGAGGTGACCGAATTTGCCAAGACATGCGTGGCTGATGAGAGTG
CAGAAAACTGTGACAAATCACTGCATACTCTCTTTGGAGATAAGCTGTGCACCGTCGCC
ACACTCAGAGAGACTTATGGGGAAATGGCTGACTGTTGCGCAAAACAGGAGCCTGAAC
GGAATGAGTGTTTCCTCCAGCACAAGGATGACAACCCAAATCTGCCCCGCCTCGTGCGA
CCTGAGGTCGATGTGATGTGCACCGCCTTTCATGACAACGAAGAGACATTCCTGAAGA
AATACCTGTATGAAATTGCTCGTAGGCACCCATACTTTTATGCCCCCGAGCTCCTGTTCT
TTGCAAAGAGATACAAAGCTGCCTTCACTGAATGTTGCCAGGCAGCTGATAAGGCCGC
ATGTCTCCTGCCTAAACTGGACGAGCTCCGGGATGAAGGTAAGGCTTCCAGCGCCAAA
CAGCGCCTGAAGTGCGCTTCTCTCCAGAAGTTTGGCGAGCGAGCATTCAAAGCCTGGGC
TGTGGCCCGTCTCAGTCAGAGGTTTCCAAAGGCAGAATTTGCTGAGGTCTCAAAACTGG
TGACCGACCTCACAAAGGTCCATACTGAGTGTTGCCACGGAGATCTGCTGGAATGTGCC
GACGATAGAGCAGACCTCGCTAAATATATCTGCGAGAATCAGGATTCCATTAGCTCTAA
GCTGAAAGAATGTTGCGAGAAGCCCCTCCTGGAAAAGAGTCATTGTATCGCCGAGGTG
GAAAACGACGAGATGCCAGCAGATCTGCCATCACTCGCTGCCGACTTTGTGGAATCCA
AAGATGTCTGCAAGAATTACGCAGAGGCTAAAGACGTGTTCCTGGGGATGTTTCTGTAT
GAGTACGCCCGGCGTCACCCCGATTATAGCGTCGTGCTCCTGCTCCGACTGGCAAAGAC
CTACGAAACAACTCTGGAGAAATGTTGCGCTGCCGCAGACCCTCATGAATGTTATGCTA
AGGTGTTCGATGAGTTTAAGCCACTCGTCGAAGAGCCCCAGAACCTGATTAAACAGAA
TTGCGAACTGTTCGAGCAGCTCGGTGAATACAAGTTTCAGAACGCCCTGCTCGTGCGTT
ATACCAAAAAGGTCCCTCAGGTGTCTACACCAACTCTGGTGGAGGTCAGTAGGAATCT
GGGCAAAGTGGGATCAAAGTGTTGCAAACACCCCGAGGCAAAGAGAATGCCTTGTGCT

GAAGATTACCTCTCCGTCGTGCTGAACCAGCTCTGCGTGCTGCATGAAAAGACCCCAGT
CAGCGATCGGGTGACAAAATGTTGCACCGAATCTCTGGTCAATCGCCGACCCTGTTTCA
GTGCCCTCGAAGTGGACGAAACTTATGTGCCTAAGGAGTTTCAGGCTGAAACATTCACC
TTTCACGCCGATATCTGCACTCTGTCCGAGAAAGAAAGGCAGATTAAGAAACAGACAG
CACTGGTCGAGCTCGTGAAGCATAAACCAAAGGCTACCAAGGAGCAGCTGAAAGCCGT
CATGGACGATTTCGCAGCTTTTGTGGAAAAGTGTTGCAAAGCCGACGATAAGGAGACT
TGTTTCGCAGAAGAGGGGAAAAAGCTCGTGGCTGCCAGCCAGGCAGCTCTGGGTCTG

SEQ ID NO:3
Human Serum Albumin (HSA)
Amino Acid Sequence

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAE
NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV
DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP
KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTK
VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPAD
LPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCC
AAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPT
LVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLV
NRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQL
KAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

SEQ ID NO:4
Human Serum Albumin (HSA)
Nucleotide Sequence

GATGCACACAAGAGTGAGGTTGCTCATCGGTTTAAAGATTTGGGAGAAGAAAATTTCA
AAGCCTTGGTGTTGATTGCCTTTGCTCAGTATCTTCAGCAGTGTCCATTTGAAGATCATG
TAAAATTAGTGAATGAAGTAACTGAATTTGCAAAAACATGTGTAGCTGATGAGTCAGC
TGAAAATTGTGACAAATCACTTCATACCCTTTTTGGAGACAAATTATGCACAGTTGCAA
CTCTTCGTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAACCTGAGAG
AAATGAATGCTTCTTGCAACACAAAGATGACAACCCAAACCTCCCCCGATTGGTGAGA
CCAGAGGTTGATGTGATGTGCACTGCTTTTCATGACAATGAAGAGACATTTTTGAAAAA
ATACTTATATGAAATTGCCAGAAGACATCCTTACTTTTATGCCCCGGAACTCCTTTTCTT
TGCTAAAAGGTATAAAGCTGCTTTTACAGAATGTTGCCAAGCTGCTGATAAAGCTGCCT
GCCTGTTGCCAAAGCTCGATGAACTTCGGGATGAAGGGAAGGCTTCGTCTGCCAAACA
GAGACTCAAATGTGCCAGTCTCCAAAAATTTGGAGAAAGAGCTTTCAAAGCATGGGCA
GTGGCTCGCCTGAGCCAGAGATTTCCCAAAGCTGAGTTTGCAGAAGTTTCCAAGTTAGT
GACAGATCTTACCAAAGTCCACACGGAATGCTGCCATGGAGATCTGCTTGAATGTGCTG
ATGACAGGGCGGACCTTGCCAAGTATATCTGTGAAAATCAGGATTCGATCTCCAGTAA
ACTGAAGGAATGCTGTGAAAAACCTCTGTTGGAAAAATCCCACTGCATTGCCGAAGTG
GAAAATGATGAGATGCCTGCTGACTTGCCTTCATTAGCTGCTGATTTTGTTGAAAGTAA

GGATGTTTGCAAAAACTATGCTGAGGCAAAGGATGTCTTCCTGGGCATGTTTTTGTATG
AATATGCAAGAAGGCATCCTGATTACTCTGTCGTGCTGCTGCTGAGACTTGCCAAGACA
TATGAAACCACTCTAGAGAAGTGCTGTGCCGCTGCAGATCCTCATGAATGCTATGCCAA
AGTGTTCGATGAATTTAAACCTCTTGTGGAAGAGCCTCAGAATTTAATCAAACAAAACT
GTGAGCTTTTTAAGCAGCTTGGAGAGTACAAATTCCAGAATGCGCTATTAGTTCGTTAC
ACCAAGAAAGTACCCCAAGTGTCAACTCCAACTCTTGTAGAGGTCTCAAGAAACCTAG
GAAAAGTGGGCAGCAAATGTTGTAAACATCCTGAAGCAAAAAGAATGCCCTGTGCAGA
AGACTATCTATCCGTGGTCCTGAACCAGTTATGTGTGTTGCATGAGAAAACGCCAGTAA
GTGACAGAGTCACAAAATGCTGCACAGAGTCCTTGGTGAACAGGCGACCATGCTTTTC
AGCTCTGGAAGTCGATGAAACATACGTTCCCAAAGAGTTTAATGCTGAAACATTCACCT
TCCATGCAGATATATGCACACTTTCTGAGAAGGAGAGACAAATCAAGAAACAAACTGC
ACTTGTTGAGCTTGTGAAACACAAGCCCAAGGCAACAAAAGAGCAACTGAAAGCTGTT
ATGGATGATTTCGCAGCTTTTGTAGAGAAGTGCTGCAAGGCTGACGATAAGGAGACCT
GCTTTGCCGAGGAGGGTAAAAAACTTGTTGCTGCAAGTCAAGCTGCCTTAGGCTTA

SEQ ID NO:5
Polypeptide Connector
Amino Acid Sequence

AAAL

SEQ ID NO:6
Human Serum Albumin (HSA) Linker with C34S and N503Q substitutions ("mHSA") and Polypeptide Connector
Amino Acid Sequence

AASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP
RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGL

SEQ ID NO:7
Human Serum Albumin (HSA) Linker with C34S and N503Q substitutions ("mHSA") and Polypeptide Connector
Amino Acid Sequence

AAQDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP

RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGL

SEQ ID NO:8
Human Serum Albumin (HSA) Linker with C34S and N503Qsubstitutions ("mHSA") and Polypeptide Connector
Amino Acid Sequence

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLV
RPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADK
AACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVS
KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAE
VENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRL
AKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALL
VRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEK
TPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADICTLSEKERQIKKQ
TALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAAL
GLAAAL

SEQ ID NO:9
Human Serum Albumin (HSA) Linker with C34S and N503Q substitutions ("mHSA") and Polypeptide Connector
Amino Acid Sequence

AASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP
RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGLAAAL

SEQ ID NO:10
Human Serum Albumin (HSA) Linker with C34S and N503Q substitutions ("mHSA") and Polypeptide Connector
Amino Acid Sequence

AAQDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP
RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGLAAAL

SEQ ID NO:11
Human Serum Albumin (HSA) Linker and Polypeptide Connector
Amino Acid Sequence

AASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP
RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGL

SEQ ID NO:12
Human Serum Albumin (HSA) Linker and Polypeptide Connector
Amino Acid Sequence

AAQDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP
RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH

EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGL

SEQ ID NO:13
Human Serum Albumin (HSA) Linker and Polypeptide Connector
Amino Acid Sequence

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLV
RPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADK
AACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVS
KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAE
VENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRL
AKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALL
VRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEK
TPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQ
TALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAAL
GLAAAL

SEQ ID NO:14
Human Serum Albumin (HSA) Linker and Polypeptide Connector
Amino Acid Sequence

AASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP
RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL
RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGLAAAL

SEQ ID NO:15
Human Serum Albumin (HSA) Linker and Polypeptide Connector
Amino Acid Sequence

AAQDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVA
DESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLP
RLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA
DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI
AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL

RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNA
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIK
KQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQA
ALGLAAAL

SEQ ID NO:16

mHSA Linker, Polypeptide Connectors, H3 (anti-ErbB3) scFv, and B1D2 (anti-ErbB2) scFv ("B2B3-1")
Amino Acid Sequence

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANINRDGSA
SYYVDSVKGRFTISRDDAKNSLYLQMNSLRAEDTAVYYCARDRGVGYFDLWGRGTLV
TVSSASTGGGGSGGGGSGGGGSQSALTQPASVSGSPGQSITISCTGTSSDVGGYNFVSWY
QQHPGKAPKLMIYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADDEADYYCSSYGSSST
HVIFGGGTKVTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKL
VNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAK
RYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAV
ARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKL
KECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYE
YARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC
ELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAE
DYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTF
HADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETC
FAEEGKKLVAASQAALGLAAALQVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAW
VRQMPGKGLEYMGLIYPGDSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYF
CARHDVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGSQSVLTQPP
SVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFSGSKS
GTSASLAISGFRSEDEADYYCASWDYTLSGWVFGGGTKLTVLG

SEQ ID NO:17
B2B3-2 (A5-mHSA-B1D2)

QVQLVQSGGGLVKPGGSLRLSCAASGFSFNTYDMNWVRQAPGKGLEWVSSISSSSSYIY
YADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDGVATTPFDYWGQGTLVT
VSSGGGGSGGGGSGGGGSQSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQL
PGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDSSLSALF
GGGTKLTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNE
VTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYK
AAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARL
SQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKEC
CEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYAR
RHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELF
EQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYL

SVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADI
CTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEE
GKKLVAASQAALGLAAALQVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAWVRQ
MPGKGLEYMGLIYPGDSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR
HDVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGSQSVLTQPPSVS
AAPGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFSGSKSGTS
ASLAISGFRSEDEADYYCASWDYTLSGWVFGGGTKLTVLG

SEQ ID NO:18
B2B3-3 (A5-mHSA-F5B6H2)

QVQLVQSGGGLVKPGGSLRLSCAASGFSFNTYDMNWVRQAPGKGLEWVSSISSSSSYIY
YADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDGVATTPFDYWGQGTLVT
VSSGGGGSGGGGSGGGGSQSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQL
PGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDSSLSALF
GGGTKLTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNE
VTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYK
AAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARL
SQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKEC
CEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYAR
RHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELF
EQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYL
SVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADI
CTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEE
GKKLVAASQAALGLAAALQVQLVESGGGLVQPGGSLRLSCAASGFTFRSYAMSWVRQ
APGKGLEWVSAISGRGDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA
KMTSNAVGFDYWGQGTLVTVSSGGGGSGGGSGGGGSGQSVLTQPPSVSGAPGQRVTIS
CTGRHSNIGLGYGVHWYQQLPGTAPKLLIYGNTNRPSGVPDRFSGFKSGTSASLAITGLQ
AEDEADYYCQSYDRRTPGWVFGGGTKLTVLG

SEQ ID NO:19
B2B3-4 (A5-mHSA-ML3.9)

QVQLVQSGGGLVKPGGSLRLSCAASGFSFNTYDMNWVRQAPGKGLEWVSSISSSSSYIY
YADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDGVATTPFDYWGQGTLVT
VSSGGGGSGGGGSGGGGSQSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQL
PGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDSSLSALF
GGGTKLTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNE
VTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYK
AAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARL
SQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKEC
CEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYAR
RHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELF
EQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYL

SVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTFHADI
CTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEE
GKKLVAASQAALGLAAALQVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAWVRQ
MPGKGLEYMGLIYPGDSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR
HDVGYCSSSNCAKWPEYFQHWGQGTLVTVSSGGGGSSGGGSGGGGSQSVLTQPPSVSA
APGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFSGSKSGTSA
SLAISGFRSEDEADYYCASWDYTLSGWVFGGGTKLTVLG

SEQ ID NO:20
B2B3-5 (B12-mHSA-B1D2)

QVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSGISWNSGSI
GYADSVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYCARDLGAKQWLEGFDYWGQG
TLVTVSSASTGGGGSGGGGSGGGGSSYELTQDPAVSVALGQTVRITCQGDSLRSYYAS
WYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSTSGNSASLTITGAQAEDEADYYCNSRDS
SGNHWVFGGGTKVTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFED
HVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP
ERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELL
FFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKA
WAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSIS
SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMF
LYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIK
QNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMP
CAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAET
FTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDK
ETCFAEEGKKLVAASQAALGLAAALQVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWI
AWVRQMPGKGLEYMGLIYPGDSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSA
VYFCARHDVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGSQSVL
TQPPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFS
GSKSGTSASLAISGFRSEDEADYYCASWDYTLSGWVFGGGTKLTVLG

SEQ ID NO:21
B2B3-6 (B12-mHSA-F5B6H2)

QVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSGISWNSGSI
GYADSVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYCARDLGAKQWLEGFDYWGQG
TLVTVSSASTGGGGSGGGGSGGGGSSYELTQDPAVSVALGQTVRITCQGDSLRSYYAS
WYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSTSGNSASLTITGAQAEDEADYYCNSRDS
SGNHWVFGGGTKVTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFED
HVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP
ERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFKKYLYEIARRHPYFYAPELLF
FAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKA
WAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSIS
SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMF
LYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIK
QNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMP

CAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAET
FTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDK
ETCFAEEGKKLVAASQAALGLAAALQVQLVESGGGLVQPGGSLRLSCAASGFTFRSYA
MSWVRQAPGKGLEWVSAISGRGDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAED
TAVYYCAKMTSNAVGFDYWGQGTLVTVSSGGGGSGGGSGGGGSGQSVLTQPPSVSGA
PGQRVTISCTGRHSNIGLGYGVHWYQQLPGTAPKLLIYGNTNRPSGVPDRFSGFKSGTSA
SLAITGLQAEDEADYYCQSYDRRTPGWVFGGGTKLTVLG

SEQ ID NO:22
B2B3-7 (F4-mHSA-B1D2)

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTISGSGGST
YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGYSSSWSEVASGYWGQG
TLVTVSSASTGGGGSGGGGSGGGGSAIVMTQSPSSLSASVGDRVTITCRASQGIRNDLG
WYQQKAGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPDDFATYFCQQAHSF
PPTFGGGTKVEIKRGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVK
LVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERN
ECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFA
KRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWA
VARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSK
LKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY
EYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQN
CELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCA
EDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKET
CFAEEGKKLVAASQAALGLAAALQVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIA
WVRQMPGKGLEYMGLIYPGDSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAV
YFCARHDVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGSQSVLT
QPPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFSG
SKSGTSASLAISGFRSEDEADYYCASWDYTLSGWVFGGGTKLTVLG

SEQ ID NO:23
B2B3-8 (F4-mHSA-F5B6H2)

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTISGSGGST
YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGYSSSWSEVASGYWGQG
TLVTVSSASTGGGGSGGGGSGGGGSAIVMTQSPSSLSASVGDRVTITCRASQGIRNDLG
WYQQKAGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPDDFATYFCQQAHSF
PPTFGGGTKVEIKRGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVK
LVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERN
ECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFA
KRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWA
VARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSK
LKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY
EYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQN
CELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCA

EDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKET
CFAEEGKKLVAASQAALGLAAALQVQLVESGGGLVQPGGSLRLSCAASGFTFRSYAMS
WVRQAPGKGLEWVSAISGRGDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA
VYYCAKMTSNAVGFDYWGQGTLVTVSSGGGGSGGGSGGGGSGQSVLTQPPSVSGAPG
QRVTISCTGRHSNIGLGYGVHWYQQLPGTAPKLLIYGNTNRPSGVPDRFSGFKSGTSASL
AITGLQAEDEADYYCQSYDRRTPGWVFGGGTKLTVLG

SEQ ID NO:24
B2B3-9 (H3-HSA-B1D2)

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANINRDGSA
SYYVDSVKGRFTISRDDAKNSLYLQMNSLRAEDTAVYYCARDRGVGYFDLWGRGTLV
TVSSASTGGGGSGGGGSGGGGSQSALTQPASVSGSPGQSITISCTGTSSDVGGYNFVSWY
QQHPGKAPKLMIYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADDEADYYCSSYGSSST
HVIFGGGTKVTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVK
LVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERN
ECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFA
KRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWA
VARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSK
LKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY
EYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQN
CELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCA
EDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFT
FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKET
CFAEEGKKLVAASQAALGLAAALQVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIA
WVRQMPGKGLEYMGLIYPGDSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAV
YFCARHDVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGSQSVLT
QPPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFSG
SKSGTSASLAISGFRSEDEADYYCASWDYTLSGWVFGGGTKLTVLG

SEQ ID NO:25
B2B3-10 (H3-mHSA-F5B6H2)

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANINRDGSA
SYYVDSVKGRFTISRDDAKNSLYLQMNSLRAEDTAVYYCARDRGVGYFDLWGRGTLV
TVSSASTGGGGSGGGGSGGGGSQSALTQPASVSGSPGQSITISCTGTSSDVGGYNFVSWY
QQHPGKAPKLMIYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADDEADYYCSSYGSSST
HVIFGGGTKVTVLGAASDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKL
VNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAK
RYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAV
ARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKL
KECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYE
YARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC
ELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAE

DYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFTF
HADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETC
FAEEGKKLVAASQAALGLAAALQVQLVESGGGLVQPGGSLRLSCAASGFTFRSYAMSW
VRQAPGKGLEWVSAISGRGDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY
YCAKMTSNAVGFDYWGQGTLVTVSSGGGGSGGGSGGGGSGQSVLTQPPSVSGAPGQR
VTISCTGRHSNIGLGYGVHWYQQLPGTAPKLLIYGNTNRPSGVPDRFSGFKSGTSASLAI
TGLQAEDEADYYCQSYDRRTPGWVFGGGTKLTVLG

SEQ ID NO:26
H3 (anti-ErbB3) scFv

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANINRDGSA
SYYVDSVKGRFTISRDDAKNSLYLQMNSLRAEDTAVYYCARDRGVGYFDLWGRGTLV
TVSSASTGGGGSGGGGSGGGGSQSALTQPASVSGSPGQSITISCTGTSSDVGGYNFVSWY
QQHPGKAPKLMIYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADDEADYYCSSYGSSST
HVIFGGGTKVTVLG

SEQ ID NO:27
B1D2 (anti-ErbB2)

QVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPGDSDT
KYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHDVGYCTDRTCAKWPEWL
GVWGQGTLVTVSSGGGGSSGGGSGGGGSQSVLTQPPSVSAAPGQKVTISCSGSSSNIGN
NYVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYCA
SWDYTLSGWVFGGGTKLTVLG

SEQ ID NO:28
A5 (anti-ErbB3) scFv

QVQLVQSGGGLVKPGGSLRLSCAASGFSFNTYDMNWVRQAPGKGLEWVSSISSSSSYIY
YADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDGVATTPFDYWGQGTLVT
VSSGGGGSGGGGSGGGGSQSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQL
PGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDSSLSALF
GGGTKLTVLG

SEQ ID NO:29
ML3.9 (anti-ErbB2) scFv

QVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPGDSDT
KYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHDVGYCSSSNCAKWPEYFQ
HWGQGTLVTVSSGGGGSSGGGSGGGGSQSVLTQPPSVSAAPGQKVTISCSGSSSNIGNN
YVSWYQQLPGTAPKLLIYDHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYCAS
WDYTLSGWVFGGGTKLTVLG

SEQ ID NO:30
B12 (anti-ErbB3) scFv

QVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSGISWNSGSI
GYADSVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYCARDLGAKQWLEGFDYWGQG
TLVTVSSASTGGGGSGGGGSGGGGSSYELTQDPAVSVALGQTVRITCQGDSLRSYYAS
WYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSTSGNSASLTITGAQAEDEADYYCNSRDS
SGNHWVFGGGTKVTVLG

SEQ ID NO:31
F4 (anti-ErbB3) scFv

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTISGSGGST
YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGYSSSWSEVASGYWGQG
TLVTVSSASTGGGGSGGGGSGGGGSAIVMTQSPSSLSASVGDRVTITCRASQGIRNDLG
WYQQKAGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPDDFATYFCQQAHSF
PPTFGGGTKVEIKRG

SEQ ID NO:32
F5B6H2 (anti-ErbB2) scFv

QVQLVESGGGLVQPGGSLRLSCAASGFTFRSYAMSWVRQAPGKGLEWVSAISGRGDNT
YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKMTSNAVGFDYWGQGTLV
TVSSGGGGSGGGSGGGGSGQSVLTQPPSVSGAPGQRVTISCTGRHSNIGLGYGVHWYQ
QLPGTAPKLLIYGNTNRPSGVPDRFSGFKSGTSASLAITGLQAEDEADYYCQSYDRRTPG
WVFGGGTKLTVLG

SEQ ID NO:33
c-myc
EQKLISEEDL

SEQ ID NO:34
hemagglutinin
YPYDVPDYA

SEQ ID NO:35
Histidine tag (HIS_6)
HHHHHH

SEQ ID NO:36
Caspase 3
Homo sapiens
CAC88866

MENTENSVDSKSIKNLEPKIIHGSESMDSGMSWDTGYKMDYPEMGLCIIINNKNFHKST
GMTSRSGTDVDAANLRETFRNLKYEVRNKNDLTREEIVELMRDVSKEDHSKRSSFVCV

LLSHGEEGIIFGTNGPVDLKKITNFFRGDRCRSLTGKPKLFIIQACRGTELDCGIETDSGVD
DDMACHKIPVDADFLYAYSTAPGYYSWRNSKDGSWFIQSLCAMLKQYADKLEFMHILT
RVNRKVATEFESFSFDATFHAKKQIPCIVSMLTKELYFYH

SEQ ID NO:37
Caspase 8
Homo sapiens
AAD24962

MDFSRNLYDIGEQLDSEDLASLKFLSLDYIPQRKQEPIKDALMLFQRLQEKRMLEESNLS
FLKELLFRINRLDLLITYLNTRKEEMERELQTPGRAQISAYRVMLYQISEEVSRSELRSFK
FLLQEEISKCKLDDDMNLLDIFIEMEKRVILGEGKLDILKRVCAQINKSLLKIINDYEEFSK
ERSSSLEGSPDEFSNGEELCGVMTISDSPREQDSESQTLDKVYQMKSKPRGYCLIINNHN
FAKAREKVPKLHSIRDRNGTHLDAGALTTTFEELHFEIKPHDDCTVEQIYDILKIYQLMD
HSNMDCFICCILSHGDKGIIYGTDGQEPPIYELTSQFTGLKCPSLAGKPKVFFIQACQGDN
YQKGIPVETDSEEQPYLEMDLSSPQTRYIPDEADFLLGMATVNNCVSYRNPAEGTWYIQ
SLCQSLRERCPRGDDILTILTEVNYEVSNKDDKKNMGKQMPQPTFTLRKKLVFPSD

SEQ ID NO:38
Granzyme B
Homo sapiens
AAA75490

MQPILLLLAFLLLPRADAGEIIGGHEAKPHSRPYMAYLMIWDQKSLKRCGGFLIQDDFVL
TAAHCWGSSINVTLGAHNIKEQEPTQQFIPVKRAIPHPAYNPKNFSNDIMLLQLERKAKR
TRAVQPLRLPSNKAQVKPGQTCSVAGWGQTAPLGKHSHTLQEVKMTVQEDRKCESDL
RHYYDSTIELCVGDPEIKKTSFKGDSGGPLVCNKVAQGIVSYGRNNGMPPRACTKVSSF
VHWIKKTMKRY

SEQ ID NO:39
Cytochrome c
Homo sapiens
NP_061820

MGDVEKGKKIFIMKCSQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGYSYTAANKNKGI
IWGEDTLMEYLENPKKYIPGTKMIFVGIKKKEERADLIAYLKKATNE

SEQ ID NO:40
Tumor Necrosis Factor Alpha (TNFα)
Homo sapiens
CAA26669

MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQRE
EFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELR
DNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRET
PEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

SEQ ID NO:41
Fas
Homo sapiens
CAI13871

MLGIWTLLPLVLTSVARLSSKSVNAQVTDINSKGLELRKTVTTVETQNLEGLHHDGQFC
HKPCPPGERKARDCTVNGDEPDCVPCQEGKEYTDKAHFSSKCRRCRLCDEGHGLEVEI
NCTRTQNTKCRCKPNFFCNSTVCEHCDPCTKCEHGIIKECTLTSNTKCKEEVKRKEVQK
TCRKHRKENQGSHESPTLNPETVAINLSDVDLSKYITTIAGVMTLSQVKGFVRKNGVNE
AKIDEIKNDNVQDTAEQKVQLLRNWHQLHGKKEAYDTLIKDLKKANLCTLAEKIQTIIL
KDITSDSENSNFRNEIQSLV

SEQ ID NO:42
Bad (Bcl2 antagonist of cell death)
Homo sapiens
CAG46757

MFQIPEFEPSEQEDSSSAERGLGPSPAGDGPSGSGKHHRQAPGLLWDASHQQEQPTSSSH
HGGAGAVEIRSRHSSYPAGTEDDEGMGEEPSPFRGRSRSAPPNLWAAQRYGRELRRMS
DEFVDSFKKGLPRPKSAGTATQMRQSSSWTRVFQSWWDRNLGRGSSAPSQ

SEQ ID NO:43
Apoptosis Inducing Protein (AIP)
Homo sapiens
AAK67626

MVDHLANTEINSQRIAAVESCFGASGQPLALPGRVLLGEGVLTKECRKKAKPRIFFLFND
ILVYGSIVLNKRKYRSQHIIPLEEVTLELLPETLQAKNRWMIKTAKKSFVVSAASATERQ
EWISHIEECVRRQLKATGRPPSTEHAAPWIPDKATDICMRCTQTRFSALTRRHHCRKCGF
VVCAECSRQRFLLPRLSPKPVRVCSLCYRELAAQQRQEEAEEQGAGSPRQPAHLARPIC
GASSGDDDDSDEDKEGSRDGDWPSSVEFYASGVAWSAFHS

SEQ ID NO:44
Pierisin-1
Pieris rapae
Q9U8Q4

MADRQPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPMALLATSELRWRPTVLTDTDN
VVRLDRRQRLVRWDRRPPNEIFLDGFVPIVTRENPDWEETDLYGFAKNNHPSIFVSTTKT
QRNKKKYVWTPRNANRGIVYQYEIYAPGGVDVNDSFSDASPWPNQMEVAFPGGIQNIY
IRSARELHNGRIQRIWINPNFLDPGDLEPIVSSSRTPQVIWRMNHPDGGHRDQRSERSASS
YDDLMYGGTGNVQEDTFGDEPNNPKPIAAGEFMIESIKDKNSFLDLSKNVNGGVIHSNL
YSGGDNQIWVFSYDDNKKAYRIQSYQNSYLYLSWDSNASSKEMILRGYTNSGSNNQY
WQIEQTGKNYRLRNLLNLDMIITAQDKPSAFGGKEVIVNTEISNSNTKISQEWKMIPFDF
RPIIDGDYNIFNVDLSNQVVDFSNQPDLLVHGHIFCDNENQTWHFTYNSTYHAYKIWSG
RKSNLLLTWDSNAASKEMVVRAYTESRSKNQYWRIEQTGSKSYKVRNLENSSMILGLT

RVSTPYGGLNLMVEDDSDGHSDLHSDWDIKPIFYQDIPDGDYNIFNDNFPNIAIDFTNQE
GSLIHGHNFCSNNNQKWSFVFDGKRKAYRIKSGVRSNLWLSWDSNASSKEMVLRAYTE
SGSSNQYWRLDEANDGSYRIRNLQDYYKLIALTNKNTPYGGKELIVSDNKESGNTWYL
KKLGEVPLPNRKFRIATKLNYKKVIDSSTSYNLIITHDLNFASSIWELVYDSSKKAYNIYS
SDINNLGWIYQNKNFFVKLGNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLHDPANAVGY
TDSESVITDTSTYSDNQLFHFILM

SEQ ID NO:45
TRAIL (TNF-related apoptosis-inducing ligand)
Homo sapiens
P50591

MAMMEVQGGPSLGQTCVLIVIFTVLLQSLCVAVTYVYFTNELKQMQDKYSKSGIACFL
KEDDSYWDPNDEESMNSPCWQVKWQLRQLVRKMILRTSEETISTVQEKQQNISPLVRE
RGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGELVI
HEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSKDA
EYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG

SEQ ID NO:46
Bax
Homo sapiens
Q07812

MDGSGEQPRGGGPTSSEQIMKTGALLLQGFIQDRAGRMGGEAPELALDPVPQDASTKK
LSECLKRIGDELDSNMELQRMIAAVDTDSPREVFFRVAADMFSDGNFNWGRVVALFYF
ASKLVLKALCTKVPELIRTIMGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTI
FVAGVLTASLTIWKKMG

SEQ ID NO:47
Green Fluorescent Protein (GFP)
Aequorea victoria
P42212

MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
LVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQ
LADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDE
LYK

SEQ ID NO:48
Yellow Fluorescent Protein (YFP)
Aliivibrio fischeri
P21578

MFKGIVEGIGIIEKIDIYTDLDKYAIRFPENMLNGIKKESSIMFNGCFLTVTSVNSNIVWFD
IFEKEARKLDTFREYKVGDRVNLGTFPKFGAASGGHILSARISCVASIIEIIENEDYQQMW
IQIPENFTEFLIDKDYIAVDGISLTIDTIKNNQFFISLPLKIAQNTNMKWRKKGDKVNVELS
NKINANQCW

SEQ ID NO:49
Cyan Fluorescent Protein (CFP)
Montastraea cavernosa
AAL17905

MSVIKSVMKIKLRMDGIVNGHKFMITGEGEGKPFEGTHTIILKVKEGGPLPFAYDILTTAF
QYGNRVFTKYPKDIPDYFKQSFPEGYSWERSMTFEDQGVCTVTSDIKLEGDCFFYEIRFY
GVNFPSSGPVMQKKTLKWEPSTENMYVRDGVLLGDVSRTLLLEGDKHHRCNFRSTYG
AKKGVVLPEYHFVDHRIEILSHDKDYNTVEVYENAVARPSMLPVKAK

SEQ ID NO:50
Red Fluorescent Protein (RFP)
Discosoma sp. SSAL-2000
AAG16224

MSCSKNVIKEFMRFKVRMEGTVNGHEFEIKGEGEGRPYEGHCSVKLMVTKGGPLPFAF
DILSPQFQYGSKVYVKHPADIPDYKKLSFPEGFKWERVMNFEDGGVVTVSQDSSLKDG
CFIYEVKFIGVNFPSDGPVMQRRTRGWEASSERLYPRDGVLKGDIHMALRLEGGGHYL
VEFKSIYMVKKPSVQLPGYYYVDSKLDMTSHNEDYTVVEQYEKTQGRHHPFIKPLQ

SEQ ID NO:51
Luciferase
Photinus pyralis
CAA59282

MEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAHIEVNITYAEYFEM
SVRLAEAMKRYGLNTNHRIVVCSENSLQFFMPVLGALFIGVAVAPANDIYNERELLNSM
NISQPTVVFVSKKGLQKILNVQKKLPIIQKIIIMDSKTDYQGFQSMYTFVTSHLPPGFNEY
DFVPESFDRDKTIALIMNSSGSTGSPKGVALPHRTACVRFSHARDPIFGNQIIPDTAILSVV
PFHHGFGMFTTLGYLICGFRVVLMYRFEEELFLRSLQDYKIQSALLVPTLFSFFAKSTLID
KYDLSNLHEIASGGAPLSKEVGEAVAKRFHLPGIRQGYGLTETTSAILITPEGDDKPGAV
GKVVPFFEAKVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNDPEATNALIDKDGWLH
SGDIAYWDEDEHFFIVDRLKSLIKYKGCQVAPAELESILLQHPNIFDAGVAGLPGDDAGE
LPAAVVVLEHGKTMTEKEIVDYVASQVTTAKKLRGGVVFVDEVPKGLTGKLDARKIRE
ILIKAKKGGKSKL

SEQ ID NO:52
Luciferase
Renilla reniformis
AAA29804

MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNAASS
YLWRHVVPHIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELLNLPKKIIFV
GHDWGACLAFHYSYEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGEKMVL
ENNFFVETMLPSKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPDVVQI
VRNYNAYLRASDDLPKMFIESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQEDAPDEM
GKYIKSFVERVLKNEQ

SEQ ID NO:53
Human Serum Albumin (HSA) Linker with C34S substitution, Domain I
Amino Acid Sequence

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADESAE
NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV
DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP
KLDELRDEGKASSAKQR

SEQ ID NO:54
Human Serum Albumin (HSA) Linker, Domain II
Amino Acid Sequence

GKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECC
HGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLA
ADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAA
ADPHECYAKVFDEFKPLVEEPQ

SEQ ID NO:55
Human Serum Albumin (HSA) Linker with N503Q substitution, Domain III
Amino Acid Sequence

VEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCK
HPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETY
VPKEFQAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVE
KCCKADDKETCFAEEGKKLVAASQAALGL

SEQ ID NO:56
Human Serum Albumin (HSA) Linker, Domain I
Amino Acid Sequence

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLV

RPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADK
AACLLPKLDELRDEGKASSAKQR

SEQ ID NO:57
Human Serum Albumin (HSA) Linker, Domain III
Amino Acid Sequence

VEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCK
HPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETY
VPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVE
KCCKADDKETCFAEEGKKLVAASQAALGL

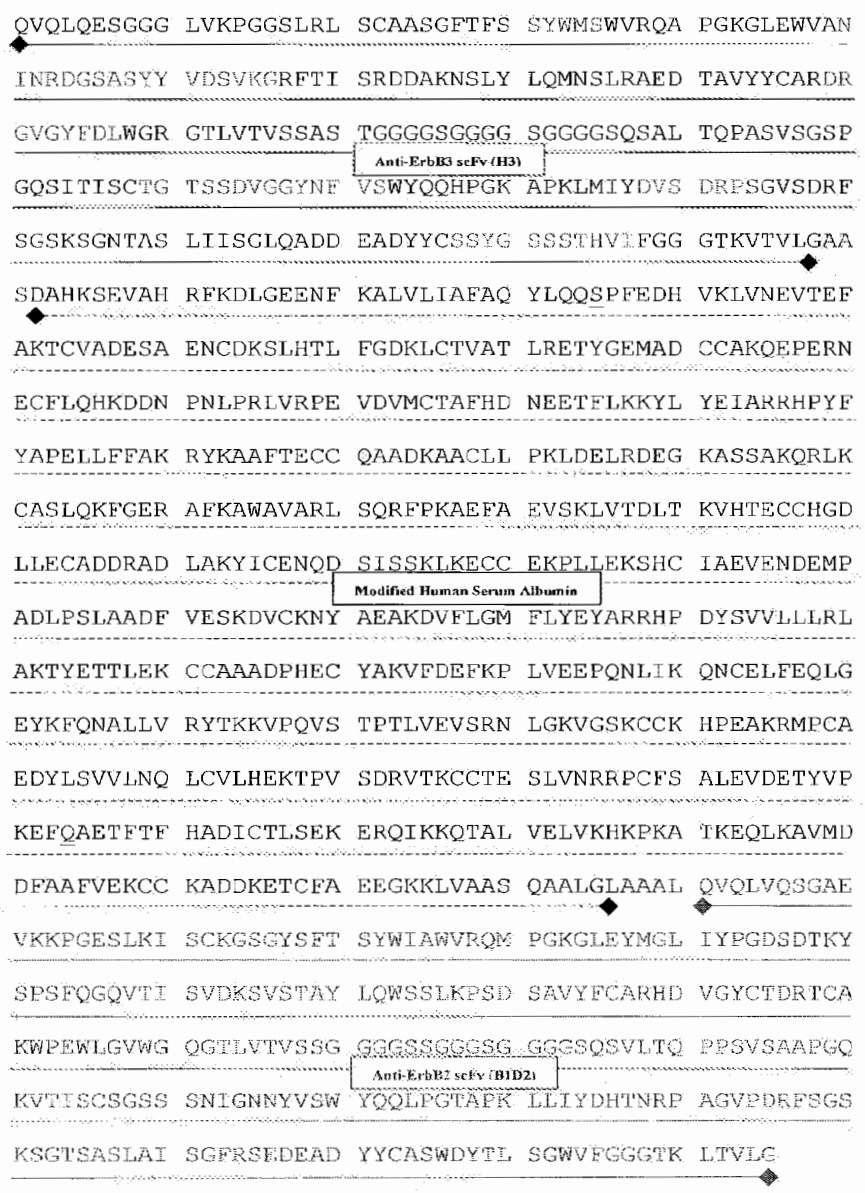
SEQ ID NO:58
Human Alpha-Fetoprotein
Amino Acid Sequence

MKWVESIFLIFLLNFTESRTLHRNEYGIASILDSYQCTAEISLADLATIFFAQFVQEATYKE
VSKMVKDALTAIEKPTGDEQSSGCLENQLPAFLEELCHEKEILEKYGHSDCCSQSEEGRH
NCFLAHKKPTPASIPLFQVPEPVTSCEAYEEDRETFMNKFIYEIARRHPFLYAPTILLWAA
RYDKIIPSCCKAENAVECFQTKAATVTKELRESSLLNQHACAVMKNFGTRTFQAITVTK
LSQKFTKVNFTEIQKLVLDVAHVHEHCCRGDVLDCLQDGEKIMSYICSQQDTLSNKITE
CCKLTTLERGQCIIHAENDEKPEGLSPNLNRFLGDRDFNQFSSGEKNIFLASFVHEYSRRH
PQLAVSVILRVAKGYQELLEKCFQTENPLECQDKGEEELQKYIQESQALAKRSCGLFQK
LGEYYLQNAFLVAYTKKAPQLTSSELMAITRKMAATAATCCQLSEDKLLACGEGAADII
IGHLCIRHEMTPVNPGVGQCCTSSYANRRPCFSSLVVDETYVPPAFSDDKFIFHKDLCQA
QGVALQTMKQEFLINLVKQKPQITEEQLEAVIADFSGLLEKCCQGQEQEVCFAEEGQKLI
SKTRAALGV

APPENDIX B

SEQUENCE ALIGNMENTS

B2B3-1 (H3-mHSA-B1D2)



CDR loops are highlighted within H3 (blue with pink CDRs) and B1D2 (pink with cyan CDRs). Connectors to modified HSA are shown in red.

Sequence alignments for B2B3/mHSA variants

```
                          1                                           45
   A5-mHSA-ML3.9    (1)   QVQLVQSGGGLVKPGGSLRLSCAASGFSFNTYDMNWVRQAPGKGL
   A5-mHSA-B1D2     (1)   QVQLVQSGGGLVKPGGSLRLSCAASGFSFNTYDMNWVRQAPGKGL
   A5-mHSA-F5B6H2   (1)   QVQLVQSGGGLVKPGGSLRLSCAASGFSFNTYDMNWVRQAPGKGL
   B12-mHSA-B1D2    (1)   QVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGL
   B12-mHSA-F5B6H2  (1)   QVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGL
   F4-mHSA-B1D2     (1)   QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGL
   F4-mHSA-F5B6H2   (1)   QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGL
   H3-mHSA-B1D2     (1)   QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGL
   H3-mHSA-F5B6H2   (1)   QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGL

                          46                                          90
   A5-mHSA-ML3.9    (46)  EWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAED
   A5-mHSA-B1D2     (46)  EWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAED
   A5-mHSA-F5B6H2   (46)  EWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAED
   B12-mHSA-B1D2    (46)  EWVSGISWNSGSIGYADSVKGRFTISRDNAKNSLYLQMNSLRPED
   B12-mHSA-F5B6H2  (46)  EWVSGISWNSGSIGYADSVKGRFTISRDNAKNSLYLQMNSLRPED
   F4-mHSA-B1D2     (46)  EWVSTISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAED
   F4-mHSA-F5B6H2   (46)  EWVSTISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAED
   H3-mHSA-B1D2     (46)  EWVANINRDGSASYYVDSVKGRFTISRDDAKNSLYLQMNSLRAED
   H3-mHSA-F5B6H2   (46)  EWVANINRDGSASYYVDSVKGRFTISRDDAKNSLYLQMNSLRAED

                          91                                          135
   A5-mHSA-ML3.9    (91)  TAVYYCARDG---VATTPFDYWGQGTLVTVS---SGGGGSGGGGS
   A5-mHSA-B1D2     (91)  TAVYYCARDG---VATTPFDYWGQGTLVTVS---SGGGGSGGGGS
   A5-mHSA-F5B6H2   (91)  TAVYYCARDG---VATTPFDYWGQGTLVTVS---SGGGGSGGGGS
   B12-mHSA-B1D2    (91)  TAVYYCARDLGAKQWLEGFDYWGQGTLVTVSSASTGGGGSGGGGS
   B12-mHSA-F5B6H2  (91)  TAVYYCARDLGAKQWLEGFDYWGQGTLVTVSSASTGGGGSGGGGS
   F4-mHSA-B1D2     (91)  TAVYYCAKGYSSSWSEVASGYWGQGTLVTVSSASTGGGGSGGGGS
   F4-mHSA-F5B6H2   (91)  TAVYYCAKGYSSSWSEVASGYWGQGTLVTVSSASTGGGGSGGGGS
   H3-mHSA-B1D2     (91)  TAVYYCARDR----GVGYFDLWGRGTLVTVSSASTGGGGSGGGGS
   H3-mHSA-F5B6H2   (91)  TAVYYCARDR----GVGYFDLWGRGTLVTVSSASTGGGGSGGGGS

                          136                                         180
   A5-mHSA-ML3.9    (130) GGGGSQSVLTQPPS-VSGAPGQRVTISCTGSSSNIGAGYDVHWYQ
   A5-mHSA-B1D2     (130) GGGGSQSVLTQPPS-VSGAPGQRVTISCTGSSSNIGAGYDVHWYQ
   A5-mHSA-F5B6H2   (130) GGGGSQSVLTQPPS-VSGAPGQRVTISCTGSSSNIGAGYDVHWYQ
   B12-mHSA-B1D2    (136) GGGGSSYELTQDPA-VSVALGQTVRITCQGDSLRS---YYASWYQ
   B12-mHSA-F5B6H2  (136) GGGGSSYELTQDPA-VSVALGQTVRITCQGDSLRS---YYASWYQ
   F4-mHSA-B1D2     (136) GGGGSAIVMTQSPSSLSASVGDRVTITCRASQGIR---NDLGWYQ
   F4-mHSA-F5B6H2   (136) GGGGSAIVMTQSPSSLSASVGDRVTITCRASQGIR---NDLGWYQ
   H3-mHSA-B1D2     (132) GGGGSQSALTQPAS-VSGSPGQSITISCTGTSSDVGGYNFVSWYQ
   H3-mHSA-F5B6H2   (132) GGGGSQSALTQPAS-VSGSPGQSITISCTGTSSDVGGYNFVSWYQ

                          181                                         225
   A5-mHSA-ML3.9    (174) QLPGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAITGLQAED
   A5-mHSA-B1D2     (174) QLPGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAITGLQAED
   A5-mHSA-F5B6H2   (174) QLPGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAITGLQAED
   B12-mHSA-B1D2    (177) QKPGQAPVLVIYGKNNRPSGIPDRFSGSTSGNSASLTITGAQAED
   B12-mHSA-F5B6H2  (177) QKPGQAPVLVIYGKNNRPSGIPDRFSGSTSGNSASLTITGAQAED
   F4-mHSA-B1D2     (178) QKAGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPDD
   F4-mHSA-F5B6H2   (178) QKAGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPDD
   H3-mHSA-B1D2     (176) QHPGKAPKLMIYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADD
   H3-mHSA-F5B6H2   (176) QHPGKAPKLMIYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADD
```

		226 270
A5-mHSA-ML3.9	(219)	EADYYCQSYDSS-LSALFGGGTKLTVLG-AASDAHKSEVAHRFKD
A5-mHSA-B1D2	(219)	EADYYCQSYDSS-LSALFGGGTKLTVLG-AASDAHKSEVAHRFKD
A5-mHSA-F5B6H2	(219)	EADYYCQSYDSS-LSALFGGGTKLTVLG-AASDAHKSEVAHRFKD
B12-mHSA-B1D2	(222)	EADYYCNSRDSSGNHWVFGGGTKVTVLG-AASDAHKSEVAHRFKD
B12-mHSA-F5B6H2	(222)	EADYYCNSRDSSGNHWVFGGGTKVTVLG-AASDAHKSEVAHRFKD
F4-mHSA-B1D2	(223)	FATYFCQQAHSF--PPTFGGGTKVEIKRGAASDAHKSEVAHRFKD
F4-mHSA-F5B6H2	(223)	FATYFCQQAHSF--PPTFGGGTKVEIKRGAASDAHKSEVAHRFKD
H3-mHSA-B1D2	(221)	EADYYCSSYGSSSTHVIFGGGTKVTVLG-AASDAHKSEVAHRFKD
H3-mHSA-F5B6H2	(221)	EADYYCSSYGSSSTHVIFGGGTKVTVLG-AASDAHKSEVAHRFKD
		271 315
A5-mHSA-ML3.9	(262)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
A5-mHSA-B1D2	(262)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
A5-mHSA-F5B6H2	(262)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
B12-mHSA-B1D2	(266)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
B12-mHSA-F5B6H2	(266)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
F4-mHSA-B1D2	(266)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
F4-mHSA-F5B6H2	(266)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
H3-mHSA-B1D2	(265)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
H3-mHSA-F5B6H2	(265)	LGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVADES
		316 360
A5-mHSA-ML3.9	(307)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
A5-mHSA-B1D2	(307)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
A5-mHSA-F5B6H2	(307)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
B12-mHSA-B1D2	(311)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
B12-mHSA-F5B6H2	(311)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
F4-mHSA-B1D2	(311)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
F4-mHSA-F5B6H2	(311)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
H3-mHSA-B1D2	(310)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
H3-mHSA-F5B6H2	(310)	AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFL
		361 405
A5-mHSA-ML3.9	(352)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
A5-mHSA-B1D2	(352)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
A5-mHSA-F5B6H2	(352)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
B12-mHSA-B1D2	(356)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
B12-mHSA-F5B6H2	(356)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
F4-mHSA-B1D2	(356)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
F4-mHSA-F5B6H2	(356)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
H3-mHSA-B1D2	(355)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
H3-mHSA-F5B6H2	(355)	QHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPY
		406 450
A5-mHSA-ML3.9	(397)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
A5-mHSA-B1D2	(397)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
A5-mHSA-F5B6H2	(397)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
B12-mHSA-B1D2	(401)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
B12-mHSA-F5B6H2	(401)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
F4-mHSA-B1D2	(401)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
F4-mHSA-F5B6H2	(401)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
H3-mHSA-B1D2	(400)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
H3-mHSA-F5B6H2	(400)	FYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASS
		451 495
A5-mHSA-ML3.9	(442)	AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL

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A5-mHSA-B1D2    (442) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL
A5-mHSA-F5B6H2  (442) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL
B12-mHSA-B1D2   (446) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL
B12-mHSA-F5B6H2 (446) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL
F4-mHSA-B1D2    (446) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL
F4-mHSA-F5B6H2  (446) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL
H3-mHSA-B1D2    (445) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL
H3-mHSA-F5B6H2  (445) AKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDL

                      496                                         540
A5-mHSA-ML3.9   (487) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
A5-mHSA-B1D2    (487) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
A5-mHSA-F5B6H2  (487) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
B12-mHSA-B1D2   (491) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
B12-mHSA-F5B6H2 (491) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
F4-mHSA-B1D2    (491) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
F4-mHSA-F5B6H2  (491) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
H3-mHSA-B1D2    (490) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL
H3-mHSA-F5B6H2  (490) TKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPL

                      541                                         585
A5-mHSA-ML3.9   (532) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
A5-mHSA-B1D2    (532) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
A5-mHSA-F5B6H2  (532) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
B12-mHSA-B1D2   (536) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
B12-mHSA-F5B6H2 (536) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
F4-mHSA-B1D2    (536) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
F4-mHSA-F5B6H2  (536) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
H3-mHSA-B1D2    (535) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
H3-mHSA-F5B6H2  (535) LEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG

                      586                                         630
A5-mHSA-ML3.9   (577) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
A5-mHSA-B1D2    (577) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
A5-mHSA-F5B6H2  (577) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
B12-mHSA-B1D2   (581) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
B12-mHSA-F5B6H2 (581) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
F4-mHSA-B1D2    (581) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
F4-mHSA-F5B6H2  (581) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
H3-mHSA-B1D2    (580) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV
H3-mHSA-F5B6H2  (580) MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKV

                      631                                         675
A5-mHSA-ML3.9   (622) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
A5-mHSA-B1D2    (622) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
A5-mHSA-F5B6H2  (622) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
B12-mHSA-B1D2   (626) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
B12-mHSA-F5B6H2 (626) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
F4-mHSA-B1D2    (626) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
F4-mHSA-F5B6H2  (626) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
H3-mHSA-B1D2    (625) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV
H3-mHSA-F5B6H2  (625) FDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQV

                      676                                         720
A5-mHSA-ML3.9   (667) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
A5-mHSA-B1D2    (667) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
A5-mHSA-F5B6H2  (667) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
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B12-mHSA-B1D2    (671) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
B12-mHSA-F5B6H2  (671) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
F4-mHSA-B1D2     (671) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
F4-mHSA-F5B6H2   (671) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
H3-mHSA-B1D2     (670) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL
H3-mHSA-F5B6H2   (670) STPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVL

                       721                                        765
A5-mHSA-ML3.9    (712) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
A5-mHSA-B1D2     (712) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
A5-mHSA-F5B6H2   (712) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
B12-mHSA-B1D2    (716) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
B12-mHSA-F5B6H2  (716) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
F4-mHSA-B1D2     (716) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
F4-mHSA-F5B6H2   (716) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
H3-mHSA-B1D2     (715) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT
H3-mHSA-F5B6H2   (715) HEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFQAETFT

                       766                                        810
A5-mHSA-ML3.9    (757) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
A5-mHSA-B1D2     (757) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
A5-mHSA-F5B6H2   (757) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
B12-mHSA-B1D2    (761) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
B12-mHSA-F5B6H2  (761) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
F4-mHSA-B1D2     (761) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
F4-mHSA-F5B6H2   (761) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
H3-mHSA-B1D2     (760) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
H3-mHSA-F5B6H2   (760) FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA

                       811                                        855
A5-mHSA-ML3.9    (802) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVQSGA
A5-mHSA-B1D2     (802) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVQSGA
A5-mHSA-F5B6H2   (802) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVESGG
B12-mHSA-B1D2    (806) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVQSGA
B12-mHSA-F5B6H2  (806) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVESGG
F4-mHSA-B1D2     (806) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVQSGA
F4-mHSA-F5B6H2   (806) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVESGG
H3-mHSA-B1D2     (805) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVQSGA
H3-mHSA-F5B6H2   (805) FVEKCCKADDKETCFAEEGKKLVAASQAALGLAAALQVQLVESGG

                       856                                        900
A5-mHSA-ML3.9    (847) EVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPG
A5-mHSA-B1D2     (847) EVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPG
A5-mHSA-F5B6H2   (847) GLVQPGGSLRLSCAASGFTFRSYAMSWVRQAPGKGLEWVSAISGR
B12-mHSA-B1D2    (851) EVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPG
B12-mHSA-F5B6H2  (851) GLVQPGGSLRLSCAASGFTFRSYAMSWVRQAPGKGLEWVSAISGR
F4-mHSA-B1D2     (851) EVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPG
F4-mHSA-F5B6H2   (851) GLVQPGGSLRLSCAASGFTFRSYAMSWVRQAPGKGLEWVSAISGR
H3-mHSA-B1D2     (850) EVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPG
H3-mHSA-F5B6H2   (850) GLVQPGGSLRLSCAASGFTFRSYAMSWVRQAPGKGLEWVSAISGR

                       901                                        945
A5-mHSA-ML3.9    (892) DSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARH
A5-mHSA-B1D2     (892) DSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARH
A5-mHSA-F5B6H2   (892) GDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKM
B12-mHSA-B1D2    (896) DSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARH
B12-mHSA-F5B6H2  (896) GDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKM
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      F4-mHSA-B1D2    (896) DSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARH
    F4-mHSA-F5B6H2    (896) GDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKM
      H3-mHSA-B1D2    (895) DSDTKYSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARH
    H3-mHSA-F5B6H2    (895) GDNTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKM

                              946                                       990
      A5-mHSA-ML3.9   (937) DVGYCSSSNCAKWPEYFQHWGQGTLVTVSSGGGGSSGGGSGGGGS
        A5-mHSA-B1D2  (937) DVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGS
      A5-mHSA-F5B6H2  (937) TSNAVG----------FDYWGQGTLVTVSSGGGGSGGGSGGGGSG
      B12-mHSA-B1D2   (941) DVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGS
    B12-mHSA-F5B6H2   (941) TSNAVG----------FDYWGQGTLVTVSSGGGGSGGGSGGGGSG
      F4-mHSA-B1D2    (941) DVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGS
    F4-mHSA-F5B6H2    (941) TS----------NAVGFDYWGQGTLVTVSSGGGGSGGGSGGGGSG
      H3-mHSA-B1D2    (940) DVGYCTDRTCAKWPEWLGVWGQGTLVTVSSGGGGSSGGGSGGGGS
    H3-mHSA-F5B6H2    (940) TSNAVG---------FDYWGQGTLVTVSSGGGGSGGGSGGGGSG

                              991                                      1035
      A5-mHSA-ML3.9   (982) QSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNY-VSWYQQLPGTA
        A5-mHSA-B1D2  (982) QSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNY-VSWYQQLPGTA
      A5-mHSA-F5B6H2  (972) QSVLTQPPSVSGAPGQRVTISCTGRHSNIGLGYGVHWYQQLPGTA
      B12-mHSA-B1D2   (986) QSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNY-VSWYQQLPGTA
    B12-mHSA-F5B6H2   (976) QSVLTQPPSVSGAPGQRVTISCTGRHSNIGLGYGVHWYQQLPGTA
      F4-mHSA-B1D2    (986) QSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNY-VSWYQQLPGTA
    F4-mHSA-F5B6H2    (976) QSVLTQPPSVSGAPGQRVTISCTGRHSNIGLGYGVHWYQQLPGTA
      H3-mHSA-B1D2    (985) QSVLTQPPSVSAAPGQKVTISCSGSSSNIGNNY-VSWYQQLPGTA
    H3-mHSA-F5B6H2    (975) QSVLTQPPSVSGAPGQRVTISCTGRHSNIGLGYGVHWYQQLPGTA

                              1036                                     1080
      A5-mHSA-ML3.9  (1026) PKLLIYDHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYC
        A5-mHSA-B1D2 (1026) PKLLIYDHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYC
      A5-mHSA-F5B6H2 (1017) PKLLIYGNTNRPSGVPDRFSGFKSGTSASLAITGLQAEDEADYYC
      B12-mHSA-B1D2  (1030) PKLLIYDHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYC
    B12-mHSA-F5B6H2  (1021) PKLLIYGNTNRPSGVPDRFSGFKSGTSASLAITGLQAEDEADYYC
      F4-mHSA-B1D2   (1030) PKLLIYDHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYC
    F4-mHSA-F5B6H2   (1021) PKLLIYGNTNRPSGVPDRFSGFKSGTSASLAITGLQAEDEADYYC
      H3-mHSA-B1D2   (1029) PKLLIYDHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYC
    H3-mHSA-F5B6H2   (1020) PKLLIYGNTNRPSGVPDRFSGFKSGTSASLAITGLQAEDEADYYC

                              1081                1104
      A5-mHSA-ML3.9  (1071) ASWDYTLSGWVFGGGTKLTVLG--
        A5-mHSA-B1D2 (1071) ASWDYTLSGWVFGGGTKLTVLG--
      A5-mHSA-F5B6H2 (1062) QSYDRRTPGWVFGGGTKLTVLG--
      B12-mHSA-B1D2  (1075) ASWDYTLSGWVFGGGTKLTVLG--
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APPENDIX C

ANTICANCER AGENTS

Anticancer agents for combination with B2B3-1	Brand Name(s)	Manufacturer/Proprietor
Anti-IGF1R Antibodies		
AMG 479 (fully humanized mAb)		Amgen
IMCA12 (fully humanized mAb)		ImClone
NSC-742460		Dyax
19D12 (fully humanized mAb)		
CP751-871 (fully humanized mAb)		Pfizer
H7C10 (humanized mAb)		
alphaIR3 (mouse)		
scFV/FC (mouse/human chimera)		
EM/164 (mouse)		
MK-0646, F50035		Pierre Fabre Medicament, Merck
Small Molecules Targeting IGF1R		
NVP-AEW541		Novartis
BMS-536,924 (1H-benzoimidazol-2-yl)-1H-pyridin-2-one)		Bristol-Myers Squibb
BMS-554,417		Bristol-Myers Squibb
Cycloligan		
TAE226		
PQ401		
Anti-EGFR Monoclonal Antibodies		
INCB7839		Incyte
Bevacizumab	Avastin	Genentech
Cetuximab	Erbitux®	IMCLONE
mAb 806		
Matuzumab (EMD72000)		
Nimotuzumab (TheraCIM)		
Panitumumab	Vectibix®	Amgen
Anti-ErbB3 Therapeutics		
U3-1287 / AMG888		U3 Pharma/Amgen
MM-121		Merrimack Pharmaceuticals
Anti-ErbB2 Therapeutics		
Trastuzumab	Herceptin	Genentech
HKI-272	Neratinib	Wyeth

KOS-953	Tanespimycin	Kosan Biosciences
Her/ErbB Dimerization Inhibitors		
2C4, R1273	Pertuzumab, Omintarg	Genentech, Roche
Small Molecules Targeting EGFR		
CI-1033 (PD 183805)		Pzifer, Inc.
EKB-569		
Gefitinib	IRessa®	AstraZeneca
Lapatinib (GW572016)		GlaxoSmithKline
Lapatinib Ditosylate	Tykerb®	SmithKline Beecham
Erlotinib HCl (OSI-774)	Tarceva®	OSI Pharms
PD158780		
PKI-166		Novartis
Tyrphostin AG 1478 (4-(3-Chloroanillino)-6,7-dimethoxyquinazoline)		
Anti-cmet Antibody Therapies		
AVEO (AV299)		AVEO
AMG102		Amgen
5D5 (OA-5D5)		Genentech
Small Molecules Targeting cmet		
PHA665752		
ARQ-650RP		ArQule
ARQ 197		ArQule
Alkylating Agents		
BCNU→ 1 ,3-bis t2-chloroethyl)-nitrosourea		
Bendamustine		
Busulfan	Myleran	GlaxoSmithKline
Carboplatin	Paraplatin	Bristol-Myers Squibb
Carboquone		
Carmustine		
CCNU→ 1,-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (methyl CCNU)		
Chlorambucil	Leukeran®	Smithkline Beecham
Chlormethine		
Cisplatin (Cisplatinum, CDDP)	Platinol	Bristol-Myers
Cyclophosphamide	Cytoxan	Bristol-Myers Squibb
	Neosar	Teva Parenteral
Dacarbazine (DTIC)		

Fotemustine		
Hexamethylmelamine (Altretamine, HMM)	Hexalen®	MGI Pharma, Inc.
Ifosfamide	Mitoxana®	ASTA Medica
Lomustine		
Mannosulfan		
Melphalan	Alkeran®	GlaxoSmithKline
Nedaplatin		
Nimustine		
Oxaliplatin	Eloxatin®	Sanofi-Aventis US
Prednimustine,		
Procarbazine HCL	Matulane	Sigma-Tau Pharmaceuticals, Inc.
Ribonucleotide Reductase Inhibitor (RNR)		
Ranimustine		
Satraplatin		
Semustine		
Streptozocin		
Temozolomide		
Treosulfan		
Triaziquone		
Triethylene Melamine		
ThioTEPA		Bedford, Abraxis, Teva
Triplatin tetranitrate		
Trofosfamide		
Uramustine		
Antimetabolites		
5-azacytidine		
Flourouracil (5-FU)/Capecitabine		
6-mercaptopurine (Mercaptopurine, 6-MP)		
6-Thioguanine (6-TG)	Purinethol®	Teva
Cytosine Arabinoside (Cytarabine, Ara-C)	Thioguanine®	GlaxoSmithKline
Azathioprine	Azasan®	AAIPHARMA LLC
Capecitabine	XELODA®	HLR (Roche)
Cladribine (2-CdA, 2-chlorodeoxyadenosine)	Leustatin®	Ortho Biotech
5-Trifluoromethyl-2'-deoxyuridine		
Fludarabine phosphate	Fludara®	Bayer Health Care
Floxuridine (5-fluoro-2)	FUDR®	Hospira, Inc.
Methotrexate sodium	Trexall	Barr

Pemetrexed	Alimta®	Lilly
Pentostatin	Nipent®	Hospira, Inc.
Raltitrexed	Tomudex®	AstraZeneca
Tegafur		
Aromatose Inhibitor		
Ketoconazole		
Glucocorticoids		
Dexamethasone	Decadron® Dexasone, Diodex, Hexadrol, Maxidex	Wyeth, Inc.
Prednisolone		
Prednisone	Deltasone, Orasone, Liquid Pred, Sterapred®	
Immunotherapeutics		
Alpha interferon		
Angiogenesis Inhibitor	Avastin®	Genentech
IL-12→ Interleukin 12		
IL-2→ Interleukin 2 (Aldesleukin)	Proleukin ®	Chiron
Kinase Inhibitors		
AMG 386		Amgen
Axitinib ((AG-013736)		Pfizer, Inc
Bosutinib (SKI-606)		Wyeth
Brivanib alalinate (BMS-582664)		BMS
Cediranib (AZD2171)	Recentin	AstraVeneca
Dasatinib (BMS-354825)	Sprycel®	Bristol-Myers Squibb
Imatinib mesylate	Gleevec	Novartis
Lestaurtinib (CEP-701)		Cephalon
Motesanib diphosphate (AMG-706)		Amgen/Takeda
Nilotinib hydrochloride monohydrate	Tasigna®	Novartis
Pazopanib HCL (GW786034)	Armala	GSK
Semaxanib (SU5416)		Pharmacia,
Sorafenib tosylate	Nexavar®	Bayer
Sunitinib malate	Sutent®	Pfizer, Inc.
Vandetanib (AZD647)	Zactima	AstraZeneca
Vatalanib; PTK-787		Novartis; Bayer Schering Pharma
XL184, NSC718781		Exelixis, GSK
Microtubule-Targeting Agents		
Docetaxel	Taxotere®	Sanofi-Aventis US
Ixabepilone	IXEMPRA™	Bristol-Myers Squibb

Larotaxel		
Ortataxel		
Nanoparticle paclitaxel (ABI-007)	Abraxane®	Abraxis BioScience, Inc.
Paclitaxel	Taxol®	Bristol-Myers Squibb
Tesetaxel		
Vinblastine sulfate	Velban®	Lilly
Vincristine	Oncovin®	Lilly
Vindesine sulphate	Eldisine®	Lilly
Vinflunine		
Vinorelbine tartrate	Navelbine®	Pierre Fabre
mTOR Inhibitors		
Deforolimus (AP23573, MK 8669)		ARIAD Pharmaceuticals, Inc
Everolimus (RAD001, RAD001C)	Certican®, Afinitor	Novartis
Sirolimus (Rapamycin)	Rapamune®	Wyeth Pharama
Temsirolimus (CCI-779)	Torisel®	Wyeth Pharama
Protein Synthesis Inhibitor		
L-asparaginase	Elspar®	Merck & Co.
Somatostatin Analogue		
Octreotide acetate	Sandostatin®	Novartis
Topoisomerase Inhibitors		
Actinomycin D		
Camptothecin (CPT)		
Belotecan		
Daunorubicin citrate	Daunoxome®	Gilead
Doxorubicin hydrochloride	Doxil®	Alza
Etoposide	Vepesid®	Bristol-Myers Squibb
	Etopophos	Hospira, Bedford, Teva Parenteral, Etc.
Irinotecan HCL (CPT-11)	Camptosar®	Pharmacia & Upjohn
Mitoxantrone HCL	Novantrone	EMD Serono
Rubitecan		
Teniposide (VM-26)	Vumon®	Bristol-Myers Squibb
Topotecan HCL	Hycamtin®	GlaxoSmithKline

Chemotherapeutic Agents Adriamycin, 5-Fluorouracil, Cytoxin, Bleomycin, Mitomycin C, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins Clofarabine, Mercaptopurine, Pentostatin, Thioguanine, Cytarabine, Decitabine, Floxuridine, Gemcitabine (Gemzar), Enocitabine, Sapacitabine		
Hormonal Therapy		
Abarelix	Plenaxis™	Amgen
Abiraterone acetate	CB7630	BTG plc
Afimoxifene	TamoGel	Ascend Therapeutics, Inc.
Anastrazole	Arimidex®	AstraZeneca
Aromatase inhibitor	Atamestane plus toremifene	Intarcia Therapeutics, Inc.
	Arzoxifene	Eli Lilly & Co.
Asentar; DN-101		Novartis; Oregon Health & Science Univ.
Bicalutamide	Casodex®	AstraZeneca
Buserelin	Suprefact®	Sanofi Aventis
Cetrorelix	Cetrotide®	EMD Serono
Exemestane	Aromasin®	Pfizer
Exemestane	Xtane	Natco Pharma, Ltd.
Fadrozole (CGS 16949A)		
Flutamide	Eulexin®	Schering
Flutamide	Prostacur	Laboratorios Almirall, S.A.
Fulvestrant	Faslodex®	AstraZeneca
Goserelin acetate	Zoladex®	AstraZeneca
Letrozole	Femara®	Novartis
Letrozole (CGS20267)	Femara	Chugai Pharmaceutical Co., Ltd.
Letrozole	Estrochek	Jagsonpal Pharmaceuticals, Ltd.
Letrozole	Letrozole	Indchemie Health Specialities
Leuprolide acetate	Eligard®	Sanofi Aventis
Leuprolide acetate	Leopril	VHB Life Sciences, Inc.
Leuprolide acetate	Lupron®/Lupron Depot	TAP Pharma
Leuprolide acetate	Viador	Bayer AG
Megestrol acetate	Megace®	Bristol-Myers Squibb
Magestrol acetate	Estradiol Valerate (Delestrogen)	Jagsonpal Pharmaceuticals, Ltd.
Medroxyprogesterone acetate	Veraplex	Combiphar

MT206		Medisyn Technologies, Inc.
Nafarelin		
Nandrolone decanoate	Zestabolin	Mankind Pharma, Ltd.
Nilutamide	Nilandron®	Aventis Pharmaceuticals
Raloxifene HCL	Evista®	Lilly
Tamoxifen	Taxifen	Yung Shin Pharmaceutical
Tamoxifen	Tomifen	Alkem Laboratories, Ltd.
Tamoxifen citrate	Nolvadex	AstraZeneca
Tamoxifen citrate	Soltamox	EUSA Pharma, Inc.
Tamoxifen citrate	Tamoxifen citrate SOPHARMA	Sopharma JSCo.
Toremifene citrate	Fareston®	GTX, Inc.
Triptorelin pamoate	Trelstar®	Watson Labs
Triptorelin pamoate	Trelstar Depot	Paladin Labs, Inc.
Protein Kinase B (PKB) Inhibitor		
Akt Inhibitor ASTEX		Astex Therapeutics
Akt Inhibitors NERVIANO		Nerviano Medical Sciences
AKT Kinase Inhibitor TELIK		Telik, Inc.
AKT DECIPHERA		Deciphera Pharmaceuticals, LLC
Perifosine (KRX0401, D-21266)		Keryx Biopharmaceuticals, Inc., AEterna Zentaris, Inc.
Perifosine with Docetaxel		Keryx Biopharmaceuticals, Inc., AEterna Zentaris, Inc.
Perifosine with Gemcitabine		AEterna Zentaris, Inc.
Perifosine with Paclitaxel		Keryx Biopharmaceuticals, Inc, AEterna Zentaris, Inc.
Protein Kinase-B inhibitor DEVELOGEN		DeveloGen AG
PX316		Oncothyreon, Inc.
RX0183		Rexahn Pharmaceuticals, Inc.
RX0201		Rexahn Pharmaceuticals, Inc.
VQD002		VioQuest Pharmaceuticals, Inc.
XL418		Exelixis, Inc.
ZEN027		AEterna Zentaris, Inc.
Phosphatidylinositol 3-Kinase (PI3K) Inhibitor		
BEZ235		Novartis AG
BGT226		Novartis AG
CAL101		Calistoga Pharmaceuticals, Inc.
CHR4432		Chroma Therapeutics, Ltd.
Erk/PI3K Inhibitors ETERNA		AEterna Zentaris, Inc.

GDC0941		Genentech Inc./Piramed Limited/Roche Holdings, Ltd.
Enzastaurin HCL (LY317615)	Enzastaurin	Eli Lilly
LY294002/Wortmannin		
PI3K Inhibitors SEMAFORE		Semafore Pharmaceuticals
PX866		Oncothyreon, Inc.
SF1126		Semafore Pharmaceuticals
VMD-8000		VM Discovery, Inc.
XL147		Exelixis, Inc.
XL147 with XL647		Exelixis, Inc.
XL765		Exelixis, Inc.
PI-103		Roche/Piramed
Cyclin-dependent kinase inhibitors		
CYC200, r-roscovitine	Seliciclib	Cyclacel Pharma
NSC-649890, L86-8275, HMR-1275	Alvocidib	NCI
TLr9, CD289		
IMOxine		Merck KGaA
HYB2055		Idera
IMO-2055		Isis Pharma
1018 ISS		Dynavax Technologies/UCSF
PF-3512676		Pfizer
Enzyme Inhibitor		
Lonafarnib (SCH66336)	Sarasar	SuperGen, U Arizona
Anti-TRAIL		
AMG-655		Aeterna Zentaris, Keryx Biopharma
Apo2L/TRAIL, AMG951		Genentech, Amgen
Apomab (fully humanized mAb		Genentech

Anticancer agents for combination with B2B3-1	Target	Manufacturer/Proprietor
Other		
Imprime PGG		Biothera
CHR-2797	AminopeptidaseM1	Chroma Therapeutics
E7820, NSC 719239	Integrin-alpha2	Eisai
INCB007839	ADAM 17, TACE	Incyte
CNF2024, BIIB021	Hsp90	Biogen Idec
MP470, HPK-56	Kit/Met/Ret	Shering-Plough

SNDX-275/MS-275	HDAC	Syndax
Zarnestra, Tipifarnib, R115777	Ras	Janssen Pharma
Volociximab; Eos 200-4, M200	alpha581 integrin	Biogen Idec; Eli Lilly/UCSF/PDL BioPharma
Apricoxib (TP2001)	COX-2 Inhibitor	Daiichi Sankyo; Tragara Pharma

Other Embodiments

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference in their entirety.

What is claimed is:

CLAIMS

1. An agent comprising:
   i) a human serum albumin (HSA) linker comprising an amino acid sequence set forth in any one of SEQ ID NOS:6-10; and
   ii) first and second binding moieties selected from the group consisting of antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, receptors, ligands, aptamers, and biologically-active fragments thereof, wherein said first binding moiety is bonded to the amino terminus of said HSA linker and said second binding moiety is bonded to the carboxy terminus of said HSA linker.

2. An agent comprising:
   i) a human serum albumin (HSA) linker comprising an amino acid sequence set forth in any one SEQ ID NOS:11-15; and
   ii) first and second binding moieties selected from the group consisting of antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, receptors, ligands, aptamers, and biologically-active fragments thereof, wherein said first binding moiety is bonded to the amino terminus and said second binding moiety is bonded to the carboxy terminus of said sequence.

3. The agent of claim 1 or 2, wherein said first binding moiety specifically binds ErbB3 and said second binding moiety specifically binds ErbB2.

4. The agent of claim 1 comprising the amino acid sequence set forth in SEQ ID NO:9.

5. The agent of claim 1 comprising the amino acid sequence set forth in SEQ ID NO:10.

6. The agent of claim 2 comprising the amino acid sequence set forth in SEQ ID NO:14.

7. The agent of claim 2 comprising the amino acid sequence set forth in SEQ ID NO:15.

8. A human serum albumin (HSA) linker comprising an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO:1 and comprising a serine residue at position 34 of SEQ ID NO: 1 and a glutamine residue at position 503 of SEQ ID NO: 1.

9. The HSA linker of claim 8, comprising an amino acid sequence having at least 95% sequence identity to the sequence set forth in SEQ ID NO:1.

10. The HSA linker of claim 8, comprising the amino acid sequence set forth in SEQ ID NO:1.

11. An agent comprising the HSA linker of claim 8 and at least a first binding moiety.

12. The agent of claim 11 further comprising a first polypeptide connector that binds said first binding moiety to said HSA linker.

13. The agent of claim 12, wherein said connector binds said first binding moiety to the amino terminus of said HSA linker.

14. The agent of claim 12, wherein said connector binds said first binding moiety to the carboxy terminus of said HSA linker.

15. The agent of claim 12, wherein said connector covalently binds said first binding moiety to said HSA linker.

16. The agent of claim 11 further comprising a second binding moiety.

17. The agent of claim 16 further comprising a second polypeptide connector that binds said second binding moiety to said HSA linker.

18. The agent of claim 17, wherein said connector binds said second binding moiety to the amino terminus of said HSA linker.

19. The agent of claim 17, wherein said connector binds said second binding moiety to the carboxy terminus of said HSA linker.

20. The agent of claim 17, wherein said connector covalently binds said second binding moiety to said HSA linker.

21. The agent of claim 16 further comprising a first connector that covalently binds said first binding moiety to the amino terminus of said HSA linker and a second connector that covalently binds a second binding moiety to the carboxy terminus of said HSA linker.

22. The agent of claim 21, wherein said first connector comprises the amino acid sequence AAS or AAQ and said second connector comprises the amino acid sequence set forth in SEQ ID NO:5.

23. The agent of claim 16, wherein said first binding moiety or second binding moiety is selected from the group consisting of antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, cell surface receptors or ligands, aptamers, and biologically-active fragments thereof,.

24. The agent of claim 16, wherein said first or second binding moiety specifically binds a protein selected from the group consisting of insulin-like growth factor 1 receptor (IGF1R), IGF2R, insulin-like growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, α4 integrin, P-selectin, sphingosine-1-phosphate

receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-related apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, receptors alpha and beta, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), $Lewis^Y$, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and fragments thereof.

25. The agent of claim 24, wherein said EGFR is erythroblastic leukemia viral oncogene homolog (ErbB) receptor.

26. The agent of claim 16, wherein said first or second binding moiety is alpha-fetoprotein (AFP) or an interferon, or a biologically-active fragment thereof.

27. The agent of claim 16, wherein said first or second binding moiety is selected from the group consisting of natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, panitumumab, and anakinra.

28. The agent of claim 16, wherein said first or second binding moiety is a single-chain Fv molecule.

29. The agent of claim 28, wherein said single-chain Fv molecule is human or humanized.

30. The agent of claim 11 further comprising a diagnostic or therapeutic agent.

31. The agent of claim 30, wherein said diagnostic agent is a detectable label.

32. The agent of claim 31, wherein said detectable label is a radioactive, fluorescent, or heavy metal label.

33. The agent of claim 30, wherein said therapeutic agent is a cytotoxic, cytostatic, or immunomodulatory agent.

34. The agent of claim 33, wherein said cytotoxic agent is an alkylating agent, an antibiotic, an antineoplastic agent, an antiproliferative agent, an antimetabolite, a tubulin inhibitor, a topoisomerase I or II inhibitor, a hormonal agonist or antagonist, an immunomodulator, a DNA minor groove binder, or a radioactive agent.

35. The agent of claim 34, wherein said antineoplastic agent is selected from the group consisting of cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, caleachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and derivatives thereof.

36. The agent of claim 33, wherein said agent is capable of binding to and killing a tumor cell or inhibiting tumor cell proliferation.

37. The agent of claim 11 admixed with a pharmaceutically acceptable carrier, excipient, or diluent.

38. The agent of claim 11, wherein said agent exhibits an *in vivo* half-life of between 6 hours and 7 days.

39. The agent of claim 11, wherein said agent exhibits an *in vivo* half-life greater than 8 hours.

40. An agent comprising:

i) a human serum albumin (HSA) linker comprising the amino acid sequence set forth in any one of SEQ ID NOS:11-15, or a fragment or variant thereof; and

ii) at least a first binding moiety.

41. The agent of claim 40, wherein said first binding moiety is bonded to the amino terminus of said HSA linker.

42. The agent of claim 40, wherein said first binding moiety is bonded to the carboxy terminus of said HSA linker.

43. The agent of claim 40, wherein said first binding moiety is covalently bonded to said HSA linker.

44. The agent of claim 40 further comprising a second binding moiety.

45. The agent of claim 44, wherein said second binding moiety is bonded to the amino terminus of said HSA linker.

46. The agent of claim 44, wherein said second binding moiety is bonded to the carboxy terminus of said HSA linker.

47. The agent of claim 44, wherein said second binding moiety is covalently bonded to said HSA linker.

48. The agent of claim 44, wherein said first binding moiety or second binding moiety is selected from the group consisting of antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, cell surface receptors or ligands, aptamers, and biologically-active fragments thereof.

49. The agent of claim 44, wherein said first or second binding moiety specifically binds a protein selected from the group consisting of insulin-like growth factor 1 receptor (IGF1R), IGF2R, insulin-like growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF),

heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, α4 integrin, P-selectin, sphingosine-1-phosphate receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-related apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, receptors alpha and beta, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), $Lewis^Y$, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and fragments thereof.

50. The agent of claim 49, wherein said EGFR is erythroblastic leukemia viral oncogene homolog (ErbB) receptor.

51. The agent of claim 44, wherein said first or second binding moiety is alpha-fetoprotein (AFP) or an interferon, or a biologically-active fragment thereof.

52. The agent of claim 44, wherein said first or second binding moiety is selected from the group consisting of natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, panitumumab, and anakinra.

53. The agent of claim 44, wherein said first or second binding moiety is a single-chain Fv molecule.

54. The agent of claim 53, wherein said single-chain Fv molecule is human or humanized.

55. The agent of claim 40 further comprising a diagnostic or therapeutic agent.

56. The agent of claim 55, wherein said diagnostic agent is a detectable label.

57. The agent of claim 56, wherein said detectable label is a radioactive, fluorescent, or heavy metal label.

58. The agent of claim 55, wherein said therapeutic agent is a cytotoxic, cytostatic, or immunomodulatory agent.

59. The agent of claim 58, wherein said cytotoxic agent is an alkylating agent, an antibiotic, an antineoplastic agent, an antiproliferative agent, an antimetabolite, a tubulin inhibitor, a topoisomerase I or II inhibitor, a hormonal agonist or antagonist, an immunomodulator, a DNA minor groove binder, or a radioactive agent.

60. The agent of claim 59, wherein said antineoplastic agent is selected from the group consisting of cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, caleachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and derivatives thereof.

61. The agent of claim 58, wherein said agent is capable of binding to and killing a tumor cell or inhibiting tumor cell proliferation.

62. The agent of claim 40 admixed with a pharmaceutically acceptable carrier, excipient, or diluent.

63. The agent of claim 40, wherein said agent exhibits an *in vivo* half-life of between 6 hours and 7 days.

64. The agent of claim 40, wherein said agent exhibits an *in vivo* half-life greater than 8 hours.

65. A method for treating a mammal having a disease or disorder, wherein said method comprises administering to said mammal the agent of claim 11 or 40.

66. The method of claim 65, wherein said disease or disorder is associated with cellular signaling through a cell surface receptor.

67. The method of claim 65, wherein said mammal is a human.

68. The method of claim 65, wherein said disease or disorder is a proliferative or autoimmune disease.

69. The method of claim 68, wherein said proliferative disease is melanoma, clear cell sarcoma, head and neck cancer, bladder cancer, breast cancer, colon cancer, ovarian cancer, endometrial cancer, gastric cancer, pancreatic cancer, renal cancer, prostate cancer, salivary gland cancer, lung cancer, liver cancer, skin cancer, or brain cancer.

70. The method of claim 68, wherein said autoimmune disease is multiple sclerosis, psoriasis, myasthenia gravis, uveitis, systemic lupus erythematosus, or rheumatoid arthritis.

71. A method for making a human serum albumin (HSA) agent comprising bonding a first binding moiety to the amino terminus and a second binding moiety to the carboxy terminus of the amino acid sequence set forth in any one of SEQ ID NOS:1, 3, or 6-15.

72. The method of claim 71, wherein said joining comprises forming a covalent bond between said first and said second binding moiety and said amino and carboxy termini, respectively.

73. The method of claim 71, wherein said first binding moiety or second binding moiety is selected from the group consisting of antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, cell surface receptors or ligands, aptamers, and biologically-active fragments thereof,.

74. The method of claim 71, wherein said first or second binding moiety specifically binds a protein selected from the group consisting of insulin-like growth factor 1 receptor (IGF1R), IGF2R, insulin-like growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, α4 integrin, P-selectin, sphingosine-1-phosphate receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-related apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, receptors alpha and beta, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), Lewis^{Y}, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and fragments thereof.

75. The method of claim 74, wherein said EGFR is erythroblastic leukemia viral oncogene homolog (ErbB) receptor.

76. The method of claim 71, wherein said first or second binding moiety is alpha-fetoprotein (AFP) or an interferon, or a biologically-active fragment thereof.

77. The method of claim 71, wherein said first or second binding moiety is selected from the group consisting of natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, panitumumab, and anakinra.

78. The method of claim 71, wherein said first or second binding moiety is a single-chain Fv molecule.

79. The method of claim 71, wherein said single-chain Fv molecule is human or humanized.

80. The method of claim 71, wherein said method further comprises conjugating one or more detectable labels to said agent.

81. The method of claim 80, wherein said detectable label is a radioactive, fluorescent, or heavy metal label.

82. The method of claim 71, wherein said method further comprises conjugating one ore more cytotoxic, cytostatic, or immunomodulatory agents to said agent.

83. The method of claim 82, wherein said cytotoxic agent is an alkylating agent, an antibiotic, an antineoplastic agent, an antiproliferative agent, an antimetabolite, a tubulin inhibitor, a topoisomerase I or II inhibitor, a hormonal agonist or antagonist, an immunomodulator, or a radioactive agent.

84. The method of claim 83, wherein said antineoplastic agent is selected from the group consisting of cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, caleachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and derivatives thereof.

85. The method of any one of claims 71-84, wherein said method further comprises admixing said agent with a pharmaceutically acceptable carrier.

86. A method for making a human serum albumin (HSA) agent comprising substituting one or more surface-exposed amino acid residues in the amino acid sequences set forth in any one of SEQ ID

NOS:1, 3, and 6-15 with a substitute amino acid capable of chemical modification, wherein said modification allows conjugation of a diagnostic or therapeutic agent.

87. The method of claim 86, wherein said substitute amino acid is cysteine and wherein said surface exposed amino acid residues are serine or threonine.

88. The method of claim 86, wherein said chemical modification results in a covalent bond between said substitute amino acid and said diagnostic or therapeutic agent.

89. The method of claim 86, wherein said surface-exposed amino acid residues are selected from the group consisting of threonine at position 496, serine at position 58, threonine at position 76, threonine at position 79, threonine at position 83, threonine at position 125, threonine at position 236, serine at position 270, serine at position 273, serine at position 304, serine at position 435, threonine at position 478, threonine at position 506, and threonine at position 508.

90. A method for making a human serum albumin (HSA) agent comprising substituting one or more of the amino acid residues in the amino acid sequences set forth in any one of SEQ ID NOS:1, 3, and 6-15 with an asparagine, serine, or threonine, wherein said substituting incorporates a glycosylation site on said HSA agent.

91. A method for making a human serum albumin (HSA) agent comprising substituting one or more of the asparagine, serine, or threonine residues in the amino acid sequences set forth in any one of SEQ ID NOS:1, 3, and 6-15 with any amino acid other than asparagine, serine, or threonine, wherein said substituting removes a glycosylation site from said HSA agent.

92. A kit comprising the HSA linker of claims 8-10.

93. A kit comprising the agent of claims 1, 2, 11, or 40.

94. An agent comprising an amino acid sequence having at least 90% sequence identity to any one of the amino acid sequences set forth in SEQ ID NOS:16-25.

95. The agent of claim 94, comprising an amino acid sequence having at least 95% sequence identity to any one of the amino acid sequences set forth in SEQ ID NOS:16-25.

96. The agent of claim 94, comprising any one of the amino acid sequence set forth in SEQ ID NOS:16-25.

97. The agent of claim 94, further comprising a detectable label or therapeutic agent.

98. The agent of claim 97, wherein said detectable label is a radioactive, fluorescent, bioluminescent, or heavy metal molecule, or epitope tag.

99. The agent of claim 98, wherein said fluorescent molecule is green fluorescent protein (GFP), enhanced GFP (eGFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), red fluorescent protein (RFP), or dsRed.

100. The agent of claim 98, wherein said bioluminescent molecule is luciferase.

101. The agent of claim 98, wherein said epitope tag is c-myc, hemagglutinin, or a histidine tag.

102. The agent of claim 97, wherein said therapeutic agent is a cytotoxic polypeptide selected from the group consisting of cytochrome c, caspase 1-10, granzyme A or B, tumor necrosis factor-alpha (TNF-α), TNF-β, Fas, Fas ligand, Fas-associated death doman-like IL-1β converting enzyme (FLICE), TRAIL/APO2L, TWEAK/APO3L, Bax, Bid, Bik, Bad, Bak, RICK, vascular apoptosis inducing proteins 1 and 2 (VAP1 and VAP2), pierisin, apoptosis-inducing protein (AIP), IL-1α propiece polypeptide, apoptin, apoptin-associated protein 1 (AAP-1), endostatin, angiostatin, and biologically-active fragments thereof.

103. The agent of claim 94, in combination with one or more therapeutic agents selected from the group consisting of cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, caleachimicin,

methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and derivatives thereof.

104. The agent of claim 1, 2, 16, or 44, wherein said first and second binding moieties specifically bind the same target molecule.

105. The agent of claim 1, 2, 16, or 44, wherein said first and second binding moieties specifically bind different target molecules.

106. The agent of claim 1, 2, 16, or 44, wherein said first and second binding moieties specifically bind different epitopes on the same target molecule.

107. The method of claim 65, wherein said first and second binding moieties specifically bind the same target molecule.

108. The method of claim 65, wherein said first and second binding moieties specifically bind different target molecules.

109. The method of claim 65, wherein said first and second binding moieties specifically bind different epitopes on the same target molecule.

110. The method of claim 71, wherein said first and second binding moieties specifically bind the same target molecule.

111. The method of claim 71, wherein said first and second binding moieties specifically bind different target molecules.

112. The method of claim 71, wherein said first and second binding moieties specifically bind different epitopes on the same target molecule.

113. A polypeptide linker comprising amino acid residues 25-44 and amino acid residues 494-513 of SEQ ID NO: 1.

114. The polypeptide linker of claim 113, comprising amino acid residues 25-70 and amino acid residues 450-513 of SEQ ID NO: 1.

115. The polypeptide linker of claim 114, comprising amino acid residues 15-100 and amino acid residues 400-520 of SEQ ID NO: 1.

116. The polypeptide linker of claim 115, comprising amino acid residues 10-200 and amino acid residues 300-575 of SEQ ID NO: 1.

117. The polypeptide linker of claim 116, comprising amino acid residues 5-250 and 275-580 of SEQ ID NO: 1.

118. The polypeptide linker of claim 113, further comprising at least a first binding moiety.

119. The polypeptide linker of claim 118 further comprising a first polypeptide connector that binds said first binding moiety to said polypeptide linker.

120. The polypeptide linker of claim 119, wherein said connector binds said first binding moiety to the amino-terminus of said polypeptide linker.

121. The polypeptide linker of claim 119, wherein said connector binds said first binding moiety to the carboxy terminus of said polypeptide linker.

122. The polypeptide linker of claim 119, wherein said connector covalently binds said first binding moiety to said polypeptide linker.

123. The polypeptide linker of claim 118 further comprising a second binding moiety.

124. The polypeptide linker of claim 123 further comprising a second polypeptide connector that binds said second binding moiety to said polypeptide linker.

125. The polypeptide linker of claim 124, wherein said connector binds said second binding moiety to the amino terminus of said polypeptide linker.

126. The polypeptide linker of claim 124, wherein said connector binds said second binding moiety to the carboxy terminus of said polypeptide linker.

127. The polypeptide linker of claim 124, wherein said connector covalently binds said second binding moiety to said polypeptide linker.

128. The polypeptide linker of claim 123 further comprising a first connector that covalently binds said first binding moiety to the amino terminus of said polypeptide linker and a second connector that covalently binds a second binding moiety to the carboxy terminus of said polypeptide linker.

129. The polypeptide linker of claim 128, wherein said first connector comprises the amino acid sequence AAS or AAQ and said second connector comprises the amino acid sequence set forth in SEQ ID NO:5.

130. The polypeptide linker of claim 123, wherein said first binding moiety or second binding moiety is selected from the group consisting of antibodies, single-chain Fv molecules, bispecific single chain Fv ($(scFv')_2$) molecules, domain antibodies, diabodies, triabodies, hormones, Fab fragments, $F(ab')_2$ molecules, tandem scFv (taFv) fragments, cell surface receptors or ligands, aptamers, and biologically-active fragments thereof,.

131. The polypeptide linker of claim 123, wherein said first or second binding moiety specifically binds a protein selected from the group consisting of insulin-like growth factor 1 receptor (IGF1R), IGF2R, insulin-like growth factor (IGF), mesenchymal epithelial transition factor receptor (c-met), hepatocyte growth factor (HGF), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heregulin, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor

(PDGFR), platelet-derived growth factor (PDGF), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR), tumor necrosis factor alpha (TNF-α), TNF-β, folate receptor (FOLR), folate, transferrin receptor (TfR), mesothelin, Fc receptor, c-kit receptor, c-kit, α4 integrin, P-selectin, sphingosine-1-phosphate receptor-1 (S1PR), hyaluronate receptor, leukocyte function antigen-1 (LFA-1), CD4, CD11, CD18, CD20, CD25, CD27, CD52, CD70, CD80, CD85, CD95 (Fas receptor), CD106 (vascular cell adhesion molecule 1 (VCAM1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD178 (Fas ligand), CD253 (TNF-related apoptosis-inducing ligand (TRAIL)), ICOS ligand, CCR2, CXCR3, CCR5, CXCL12 (stromal cell-derived factor 1 (SDF-1)), interleukin 1 (IL-1), CTLA-4, receptors alpha and beta, MART-1, gp100, MAGE-1, ephrin (Eph) receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), carcinoembryonic antigen (CEA), $Lewis^Y$, MUC-1, epithelial cell adhesion molecule (EpCAM), cancer antigen 125 (CA125), prostate specific membrane antigen (PSMA), TAG-72 antigen, and fragments thereof.

132. The polypeptide linker of claim 131, wherein said EGFR is erythroblastic leukemia viral oncogene homolog (ErbB) receptor.

133. The polypeptide linker of claim 123, wherein said first or second binding moiety is alpha-fetoprotein (AFP) or an interferon, or a biologically-active fragment thereof.

134. The polypeptide linker of claim 123, wherein said first or second binding moiety is selected from the group consisting of natalizumab, infliximab, adalimumab, rituximab, alemtuzumab, bevacizumab, daclizumab, efalizumab, golimumab, certolizumab, trastuzumab, abatacept, etanercept, pertuzumab, cetuximab, panitumumab, and anakinra.

135. The polypeptide linker of claim 123, wherein said first or second binding moiety is a single-chain Fv molecule.

136. The polypeptide linker of claim 135, wherein said single-chain Fv molecule is human or humanized.

137. The polypeptide linker of claim 118 further comprising a diagnostic or therapeutic agent.

138. The polypeptide linker of claim 137, wherein said diagnostic agent is a detectable label.

139. The polypeptide linker of claim 138, wherein said detectable label is a radioactive, fluorescent, or heavy metal label.

140. The polypeptide linker of claim 137, wherein said therapeutic agent is a cytotoxic, cytostatic, or immunomodulatory agent.

141. The polypeptide linker of claim 140, wherein said cytotoxic agent is an alkylating agent, an antibiotic, an antineoplastic agent, an antiproliferative agent, an antimetabolite, a tubulin inhibitor, a topoisomerase I or II inhibitor, a hormonal agonist or antagonist, an immunomodulator, a DNA minor groove binder, or a radioactive agent.

142. The polypeptide linker of claim 141, wherein said antineoplastic agent is selected from the group consisting of cyclophosphamide, camptothecin, homocamptothecin, colchicine, combrestatin, combrestatin, rhizoxin, dolistatin, ansamitocin p3, maytansinoid, auristatin, calcachimicin, methotrexate, 5-fluorouracil (5-FU), doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, tamoxifen, raloxifene, letrozole, epirubicin, bevacizumab, pertuzumab, trastuzumab, and derivatives thereof.

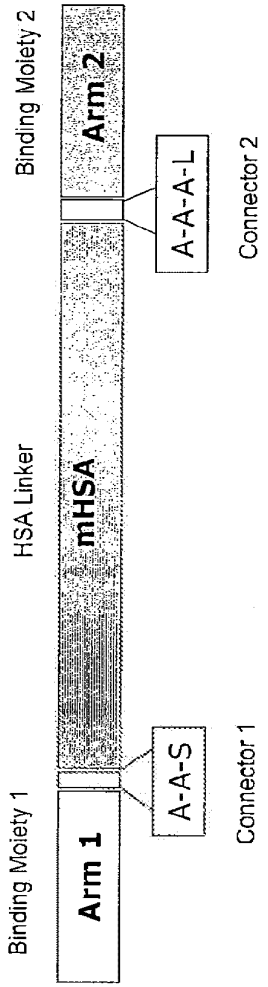
143. The polypeptide linker of claim 140, wherein said agent is capable of binding to and killing a tumor cell or inhibiting tumor cell proliferation.

144. The polypeptide linker of claim 118 admixed with a pharmaceutically acceptable carrier, excipient, or diluent.

145. The polypeptide linker of claim 118, wherein said agent exhibits an *in vivo* half-life of between 6 hours and 7 days.

146. The polypeptide linker of claim 118, wherein said agent exhibits an *in vivo* half-life greater than 8 hours.

Figure 1


Binding Moiety 2
Arm 2
A-A-A-L
Connector 2
HSA Linker
mHSA
Binding Moiety 1
Arm 1
A-A-S
Connector 1

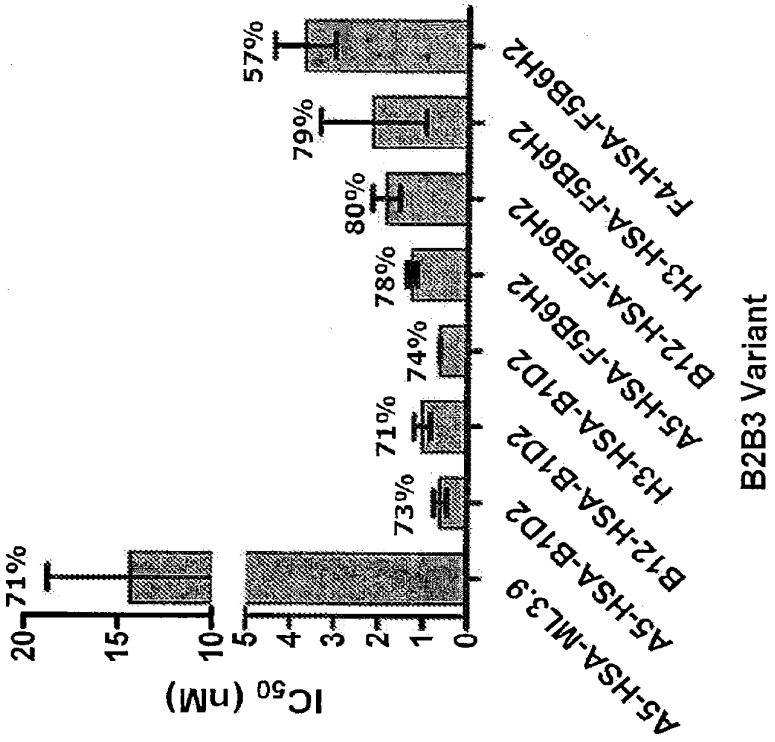

A5-HSA-ML3.9
A5-HSA-B1D2
B12-HSA-B1D2
H3-HSA-B1D2
A5-HSA-F5B6H2
B12-HSA-F5B6H2
H3-HSA-F5B6H2
F4-HSA-F5B6H2
71%
73%
71%
74%
78%
80%
79%
57%
IC50 (nM)
0
1
2
3
4
5
10
15
20
B2B3 Variant

Figure 2

Figure 3

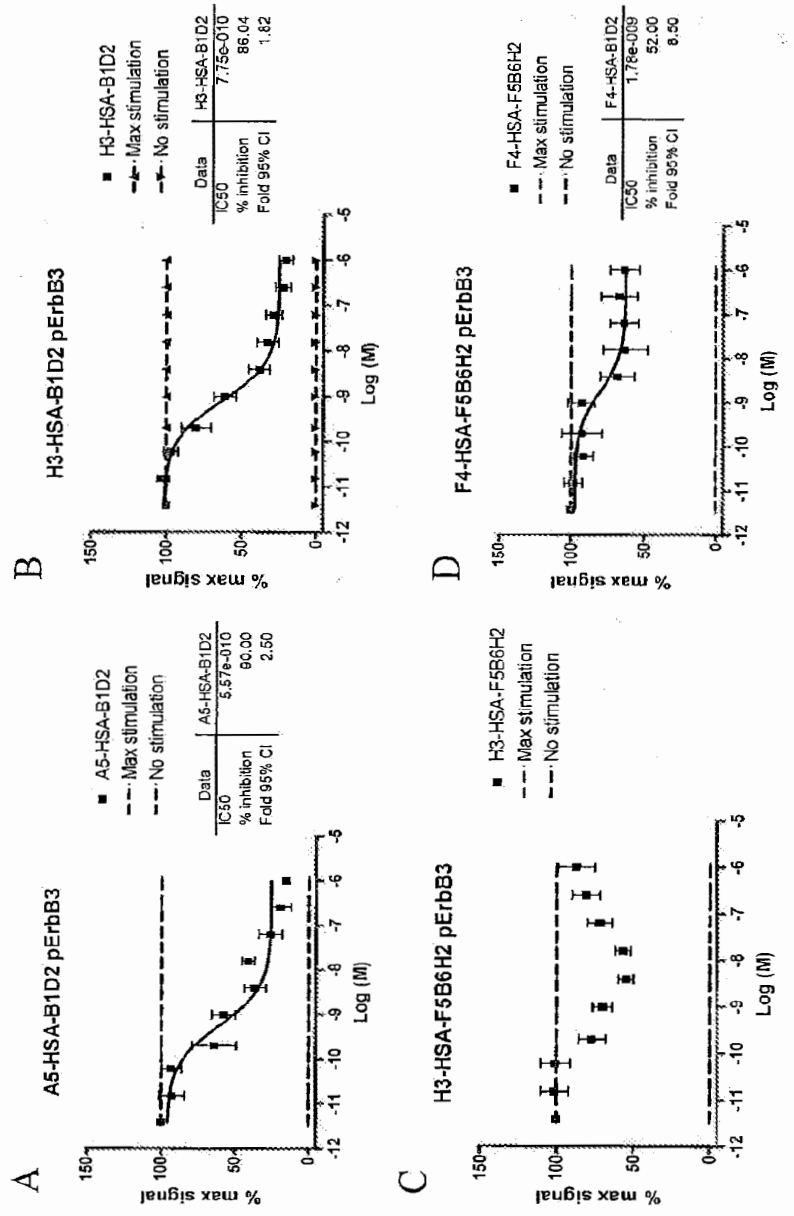

A
A5-HSA-B1D2 pErbB3
A5-HSA-B1D2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
A5-HSA-B1D2
5.57e-010
90.00
2.50
% max signal
Log (M)
B
H3-HSA-B1D2 pErbB3
H3-HSA-B1D2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
H3-HSA-B1D2
7.75e-010
86.04
1.82
% max signal
Log (M)
C
H3-HSA-F5B6H2 pErbB3
H3-HSA-F5B6H2
Max stimulation
No stimulation
% max signal
Log (M)
D
F4-HSA-F5B6H2 pErbB3
F4-HSA-F5B6H2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
F4-HSA-B1D2
1.78e-009
52.00
8.50
% max signal
Log (M)

Figure 4

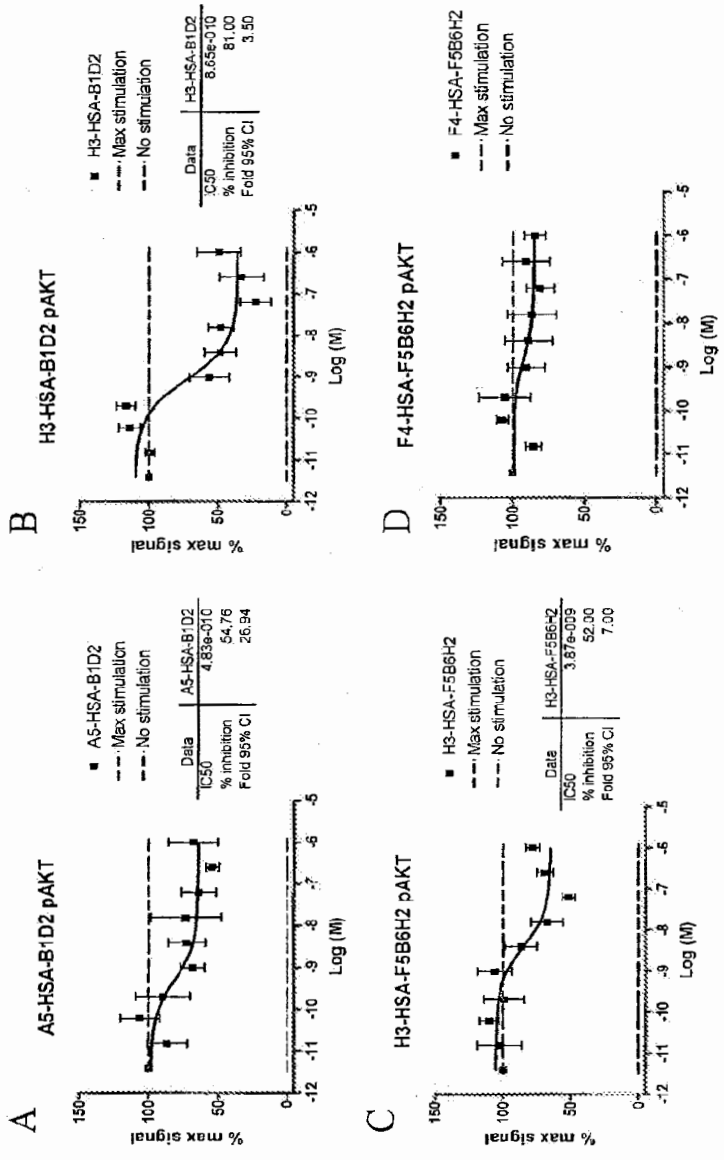
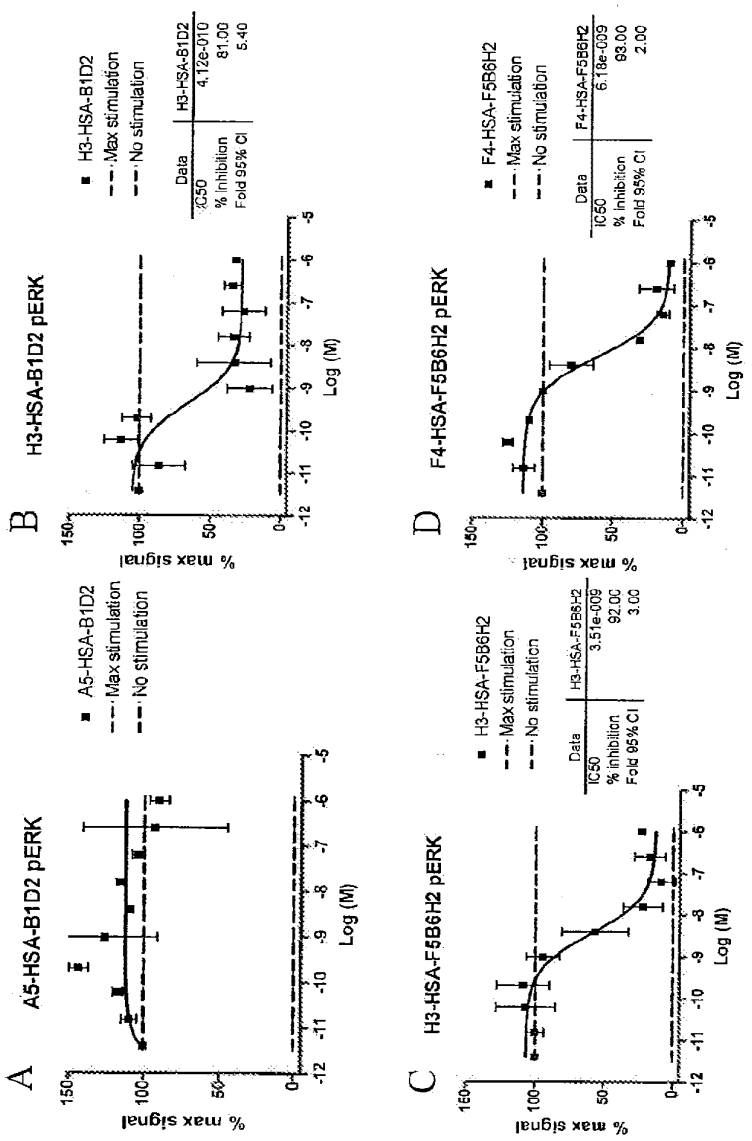

A
A5-HSA-B1D2 pAKT
A5-HSA-B1D2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
A5-HSA-B1D2
4.83e-010
54.76
26.94
% max signal
Log (M)
B
H3-HSA-B1D2 pAKT
H3-HSA-B1D2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
H3-HSA-B1D2
8.65e-010
81.00
3.50
% max signal
Log (M)
C
H3-HSA-F5B6H2 pAKT
H3-HSA-F5B6H2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
H3-HSA-F5B6H2
3.87e-009
52.00
7.00
% max signal
Log (M)
D
F4-HSA-F5B6H2 pAKT
F4-HSA-F5B6H2
Max stimulation
No stimulation
% max signal
Log (M)

Figure 5


A
A5-HSA-B1D2 pERK
A5-HSA-B1D2
Max stimulation
No stimulation
% max signal
Log (M)
B
H3-HSA-B1D2 pERK
H3-HSA-B1D2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
H3-HSA-B1D2
4.12e-010
81.00
5.40
C
H3-HSA-F5B6H2 pERK
H3-HSA-F5B6H2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
H3-HSA-F5B6H2
3.51e-009
92.00
3.00
D
F4-HSA-F5B6H2 pERK
F4-HSA-F5B6H2
Max stimulation
No stimulation
Data
IC50
% inhibition
Fold 95% CI
F4-HSA-F5B6H2
6.18e-009
93.00
2.00

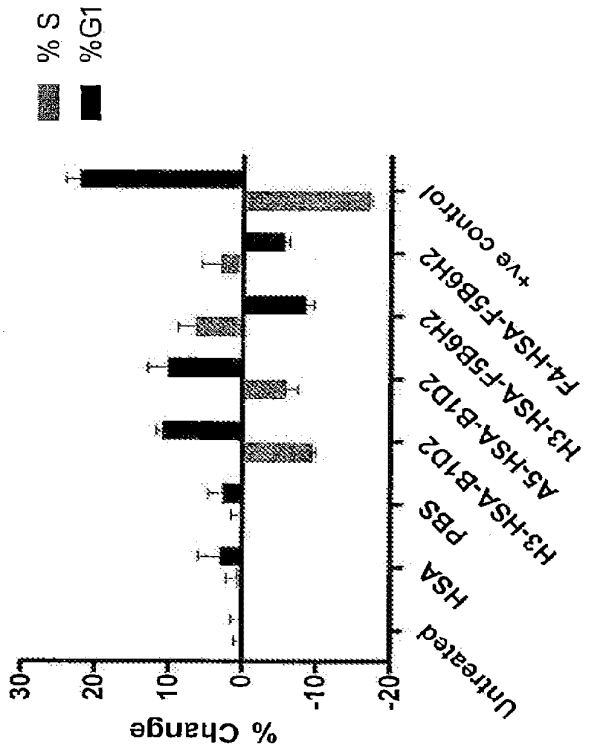
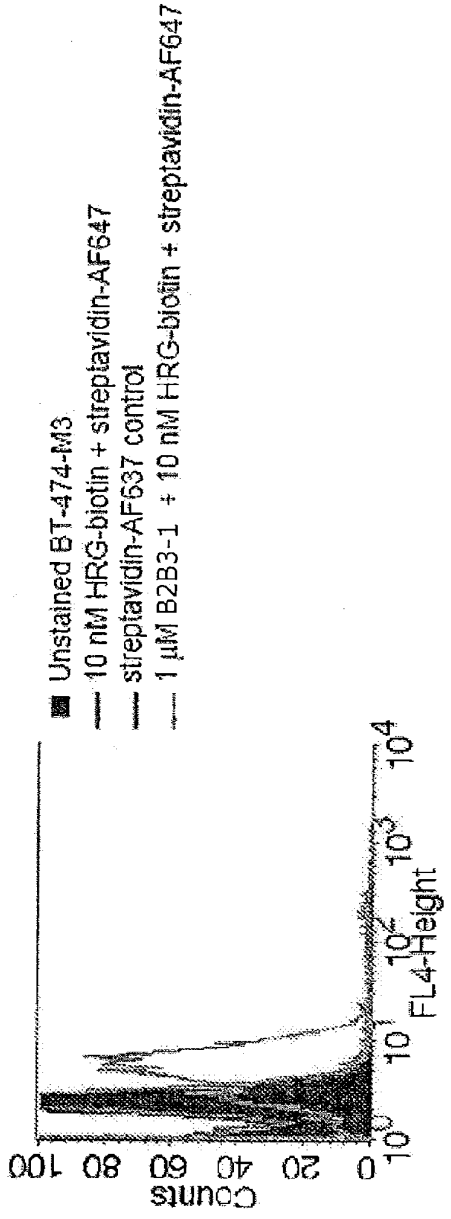

% S
%G1
% Change
30
20
10
0
-10
-20
Untreated
HSA
PBS
H3-HSA-B1D2
A5-HSA-B1D2
H3-HSA-F5B6H2
F4-HSA-F5B6H2
+ve control

Figure 6

Figure 7


Unstained BT-474-M3
10 nM HRG-biotin + streptavidin-AF647
streptavidin-AF637 control
1 µM B2B3-1 + 10 nM HRG-biotin + streptavidin-AF647
Counts
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4
FL4-Height

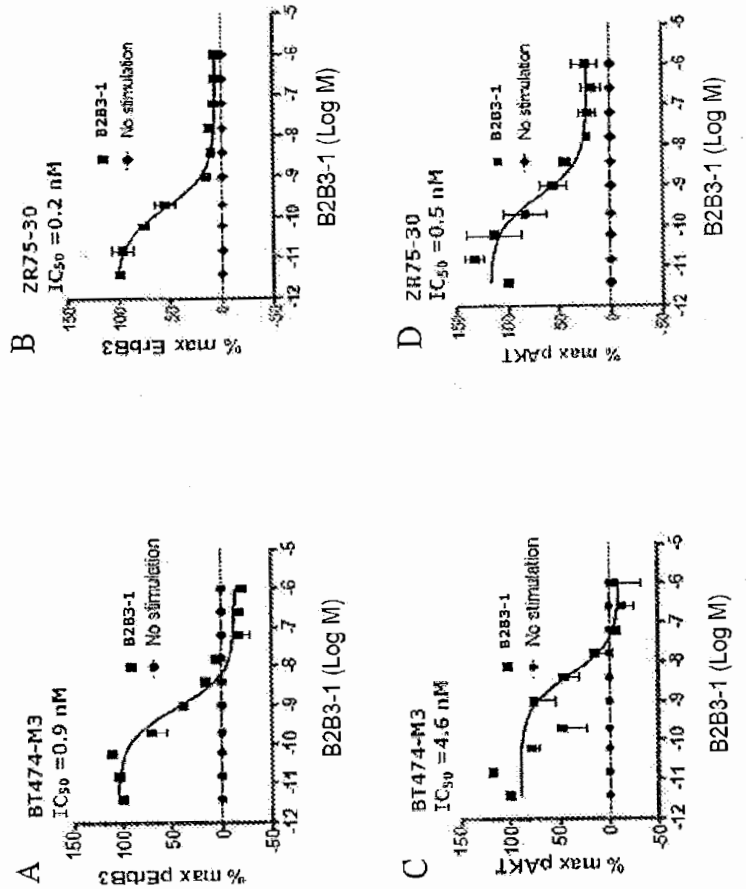

A
BT474-M3
IC50 =0.9 nM
B2B3-1
No stimulation
% max pErbB3
B2B3-1 (Log M)
B
ZR75-30
IC50 =0.2 nM
B2B3-1
No stimulation
% max ErbB3
B2B3-1 (Log M)
C
BT474-M3
IC50 =4.6 nM
B2B3-1
No stimulation
% max pAKT
B2B3-1 (Log M)
D
ZR75-30
IC50 =0.5 nM
B2B3-1
No stimulation
% max pAKT
B2B3-1 (Log M)

Figure 8

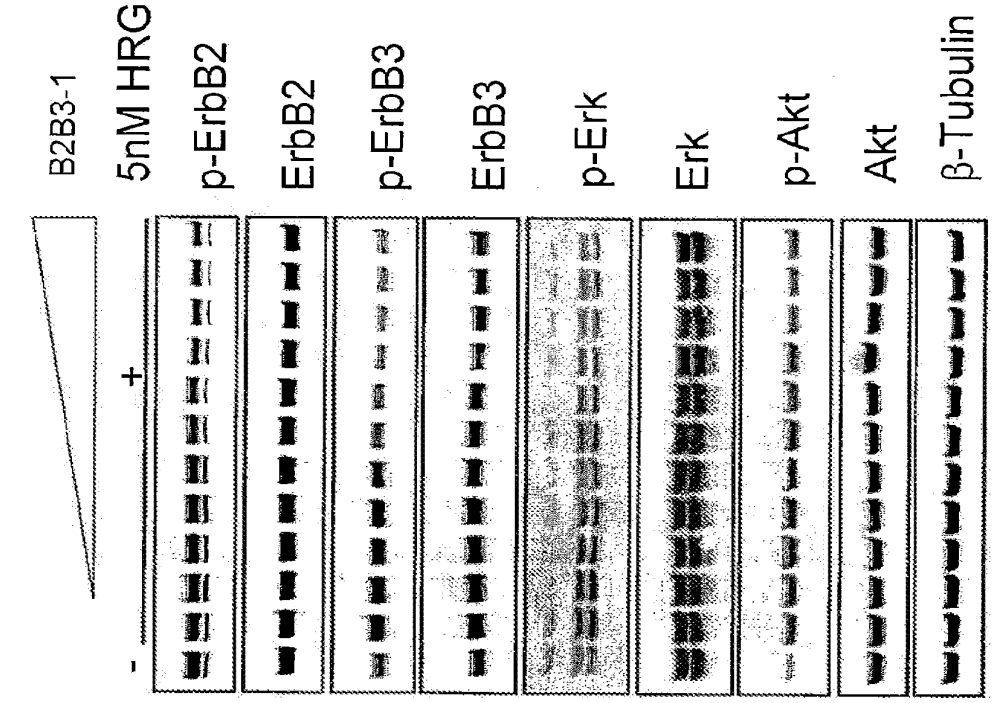

B2B3-1
5nM HRG
-
+
p-ErbB2
ErbB2
p-ErbB3
ErbB3
p-Erk
Erk
p-Akt
Akt
β-Tubulin

Figure 9

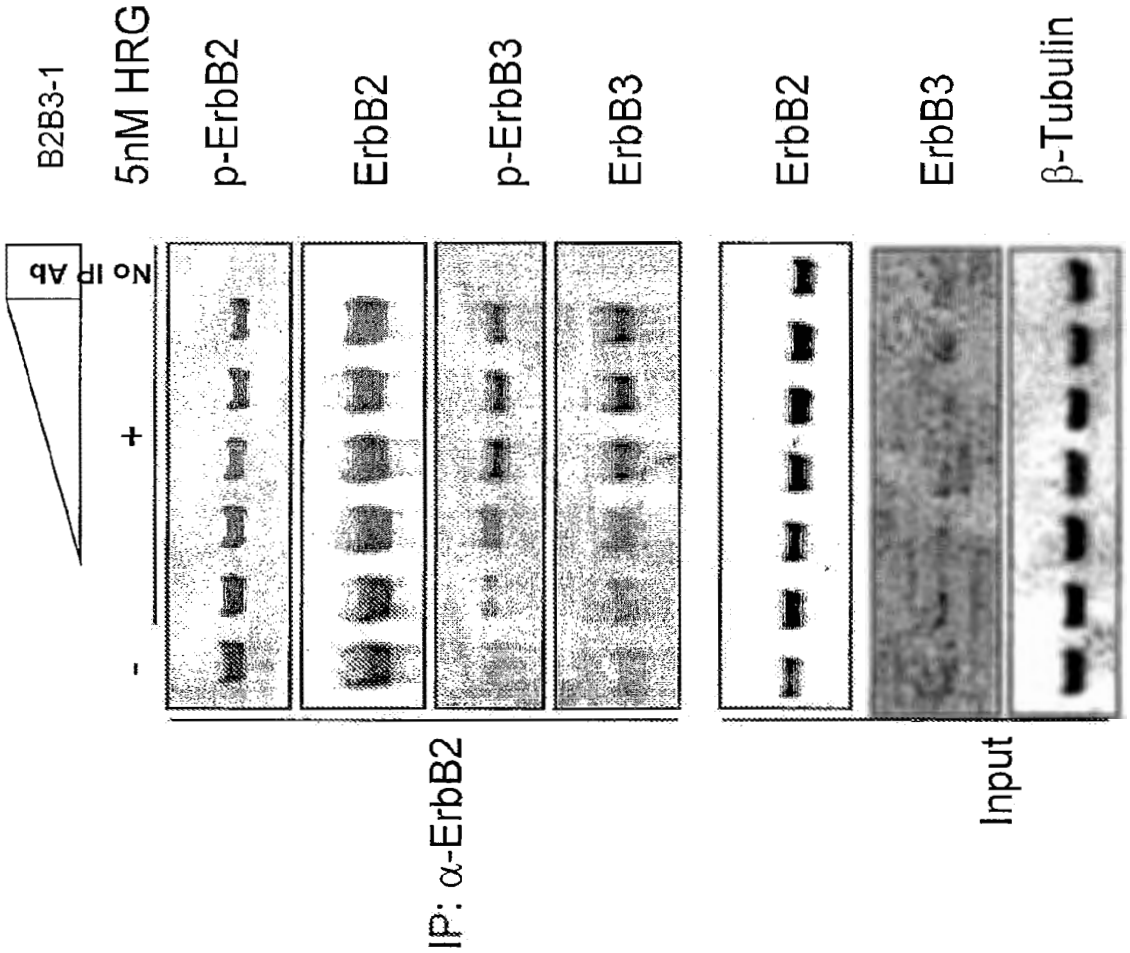

B2B3-1
5nM HRG
No IP Ab
-
+
p-ErbB2
ErbB2
p-ErbB3
ErbB3
ErbB2
ErbB3
β-Tubulin
IP: α-ErbB2
Input

Figure 10

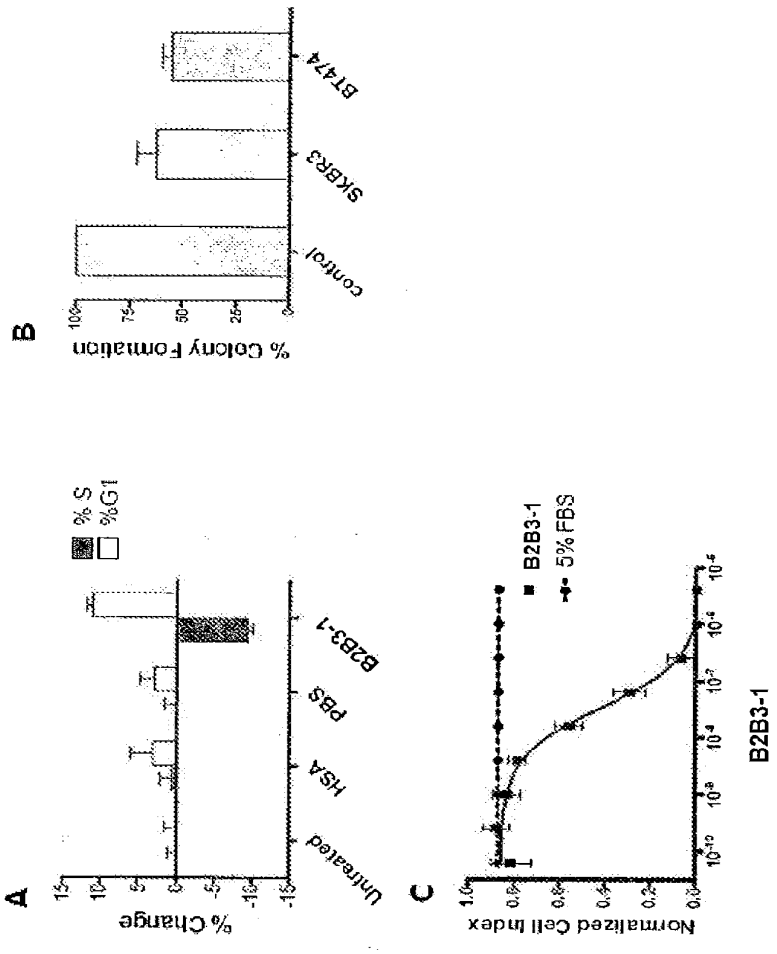

A
% S
%G1
% Change
15
10
5
0
-5
-10
-15
Untreated
HSA
PBS
B2B3-1
B
% Colony Formation
100
75
50
25
0
control
SKBR3
BT474
C
B2B3-1
5% FBS
Normalized Cell Index
1.0
0.8
0.6
0.4
0.2
0.0
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
10^{-1}
B2B3-1

Figure 11

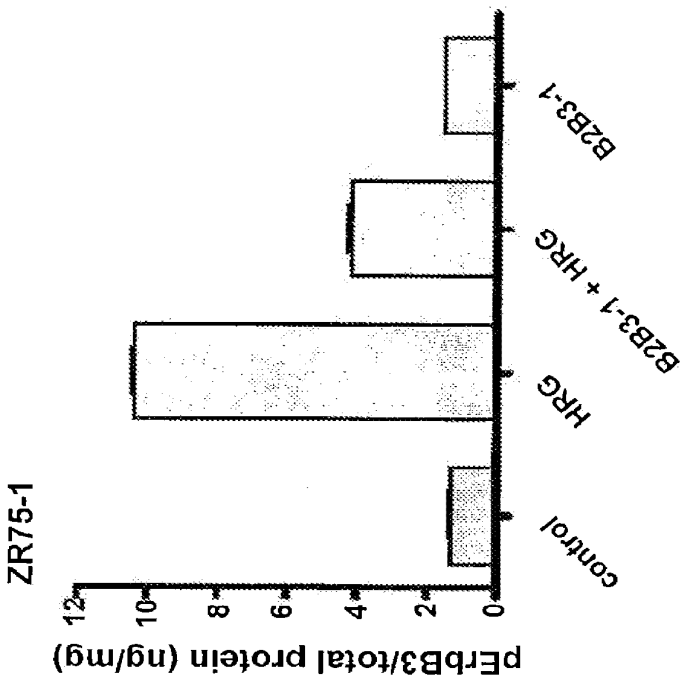
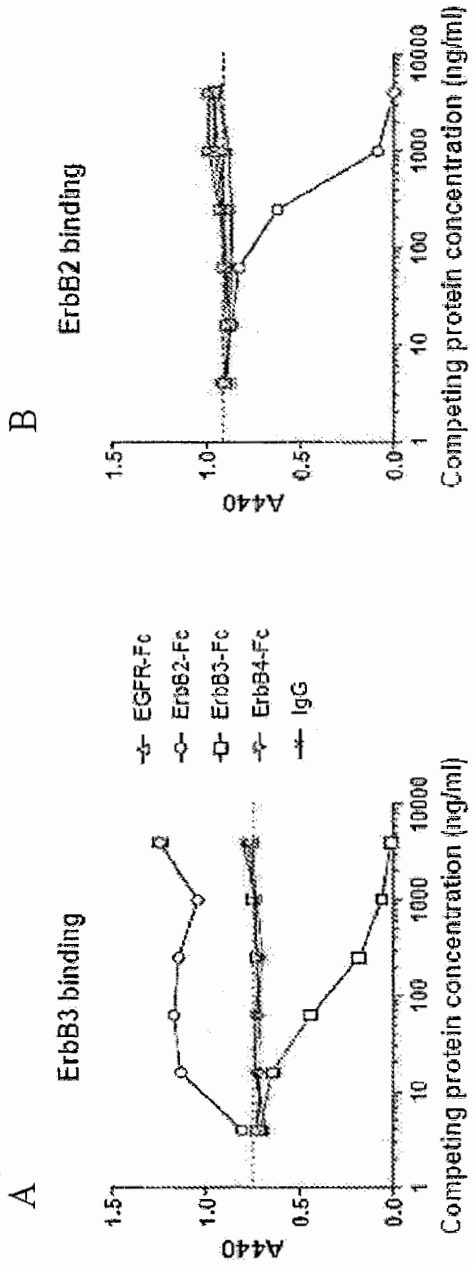

pErbB3/total protein (ng/mg)
0
2
4
6
8
10
12
ZR75-1
control
HRG
B2B3-1 + HRG
B2B3-1

Figure 12

Figure 13


A
ErbB3 binding
EGFR-Fc
ErbB2-Fc
ErbB3-Fc
ErbB4-Fc
IgG
A440
1.5
1.0
0.5
0.0
1
10
100
1000
10000
Competing protein concentration (ng/ml)
B
ErbB2 binding
A440
1.5
1.0
0.5
0.0
1
10
100
1000
10000
Competing protein concentration (ng/ml)

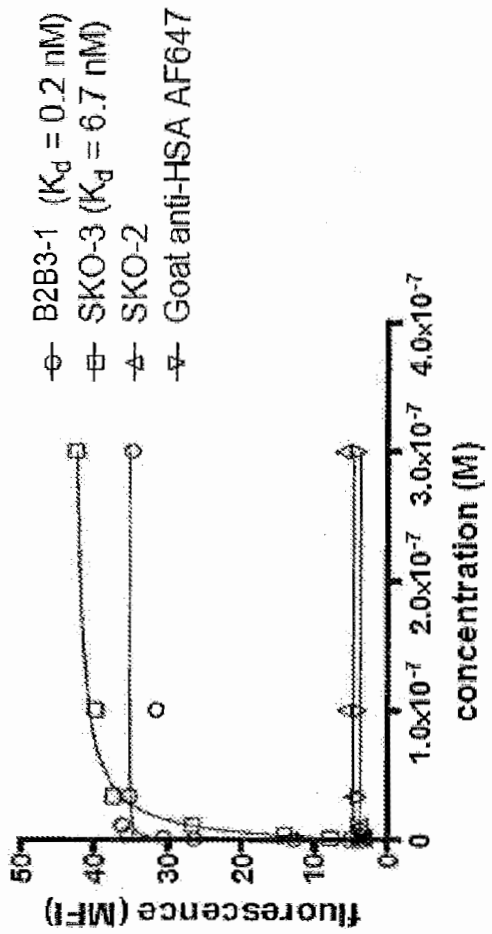
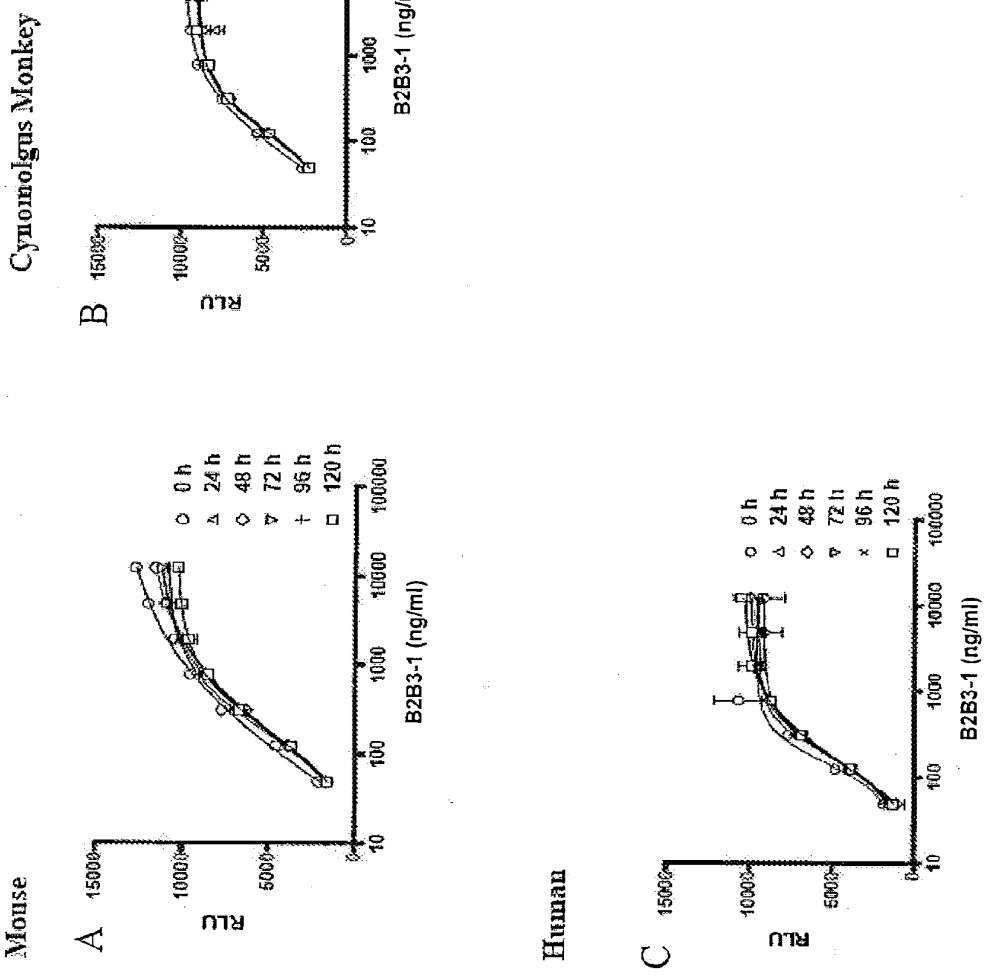

B2B3-1 (K_d = 0.2 nM)
SKO-3 (K_d = 6.7 nM)
SKO-2
Goat anti-HSA AF647
fluorescence (MFI)
50
40
30
20
10
0
0
1.0x10⁻⁷
2.0x10⁻⁷
3.0x10⁻⁷
4.0x10⁻⁷
concentration (M)

Figure 14

Figure 15


Mouse
A
RLU
B2B3-1 (ng/ml)
0 h
24 h
48 h
72 h
96 h
120 h
Cynomolgus Monkey
B
Human
C

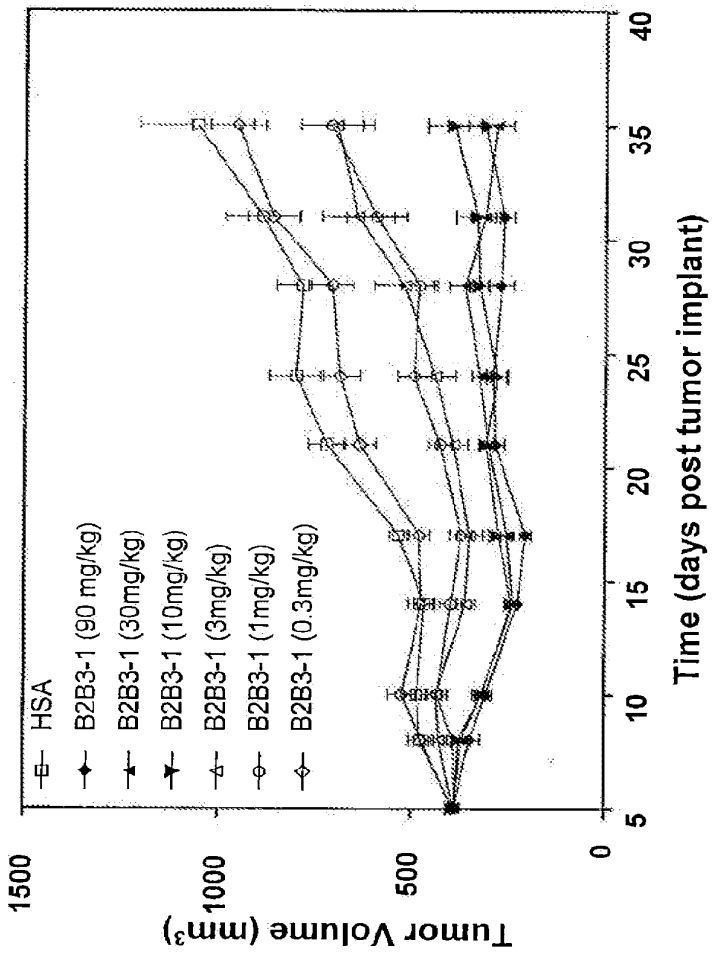
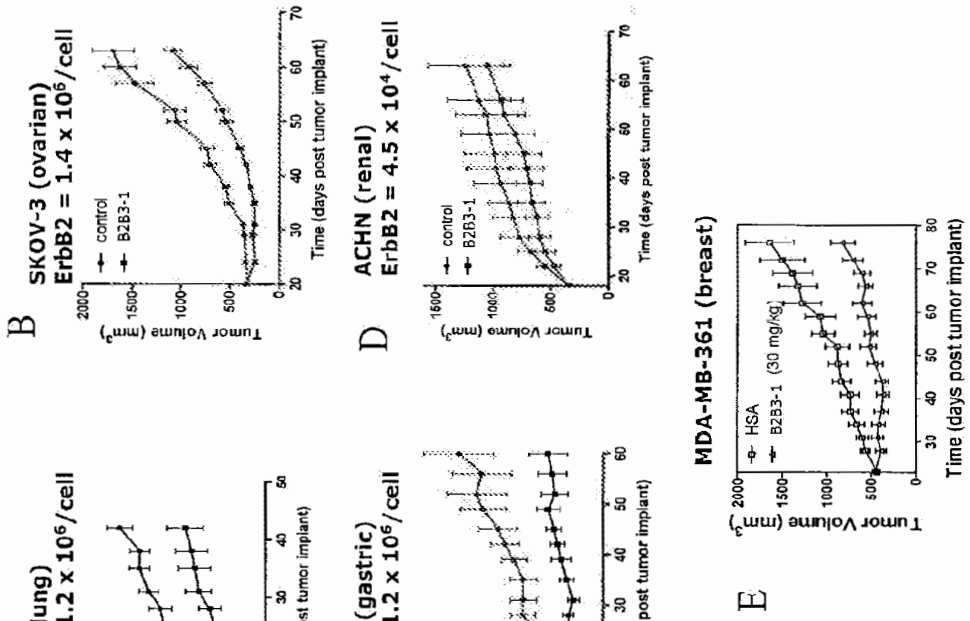

Tumor Volume (mm³)
1500
1000
500
0
5
10
15
20
25
30
35
40
Time (days post tumor implant)
HSA
B2B3-1 (90 mg/kg)
B2B3-1 (30mg/kg)
B2B3-1 (10mg/kg)
B2B3-1 (3mg/kg)
B2B3-1 (1mg/kg)
B2B3-1 (0.3mg/kg)

Figure 16

Figure 17


A
CALU-3 (lung)
ErbB2 = 1.2 x 10^6/cell
control
B2B3-1
Tumor Volume (mm^3)
Time (days post tumor implant)
B
SKOV-3 (ovarian)
ErbB2 = 1.4 x 10^6/cell
control
B2B3-1
Tumor Volume (mm^3)
Time (days post tumor implant)
C
NCI-N87 (gastric)
ErbB2 = 1.2 x 10^6/cell
control
B2B3-1
Tumor Volume (mm^3)
Time (days post tumor implant)
D
ACHN (renal)
ErbB2 = 4.5 x 10^4/cell
control
B2B3-1
Tumor Volume (mm^3)
Time (days post tumor implant)
E
MDA-MB-361 (breast)
HSA
B2B3-1 (30 mg/kg)
Tumor Volume (mm^3)
Time (days post tumor implant)

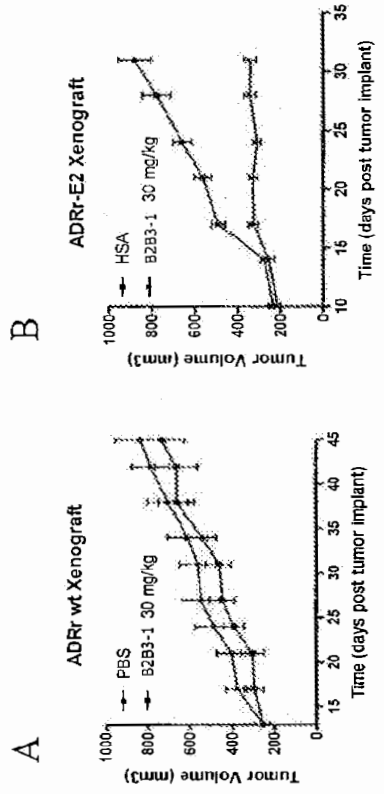

A
ADRr wt Xenograft
PBS
B2B3-1 30 mg/kg
Tumor Volume (mm3)
Time (days post tumor implant)
B
ADRr-E2 Xenograft
HSA
B2B3-1 30 mg/kg
Tumor Volume (mm3)
Time (days post tumor implant)

Figure 18

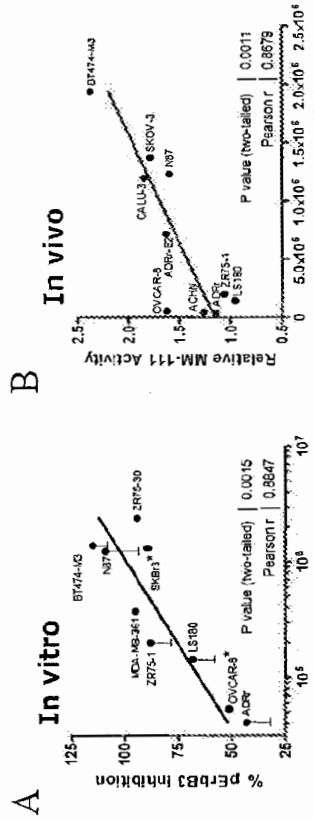
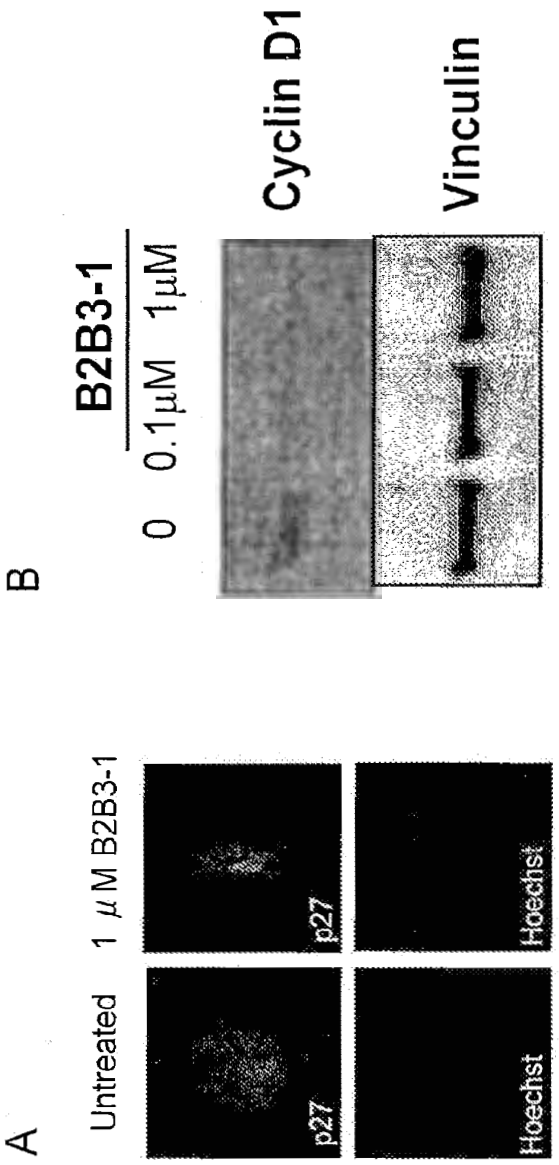

A
In vitro
% pErbB3 Inhibition
125
100
75
50
25
BT474-HG
N87
ZR75-30
MDA-MB-361
SKBr3
ZR75-1
LS180
OVCAR-5
ADR
P value (two-tailed) | 0.0015
Pearson r | 0.8647
10^5
10^6
10^7
ErbB2 Receptors/Cell
B
In vivo
Relative NM-111 Activity
2.5
2.0
1.5
1.0
0.5
0
BT474-HG
SKOV-3
CALU-3
N87
OVCAR-3
ADR-E
ACHN
ADR
ZR75-1
LS180
P value (two-tailed) | 0.0011
Pearson r | 0.8679
5.0×10^5
1.0×10^6
1.5×10^6
2.0×10^6
2.5×10^6
ErbB2 Receptors/Cell

Figure 19

Figure 20


A
Untreated
1 μM B2B3-1
p27
p27
Hoechst
Hoechst
B
B2B3-1
0
0.1μM
1μM
Cyclin D1
Vinculin

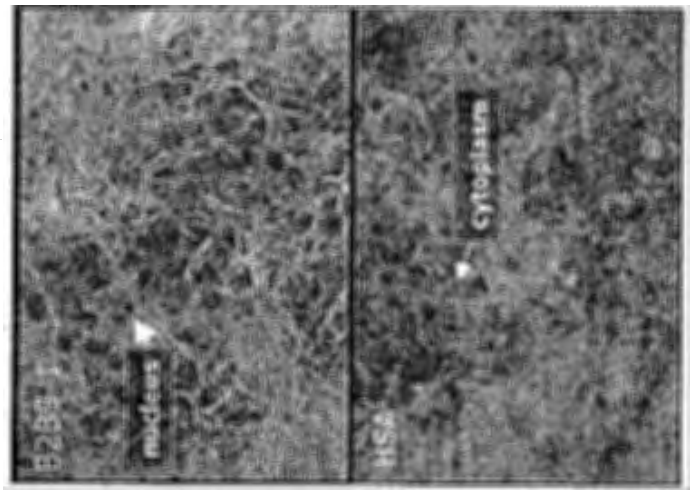


Figure 21

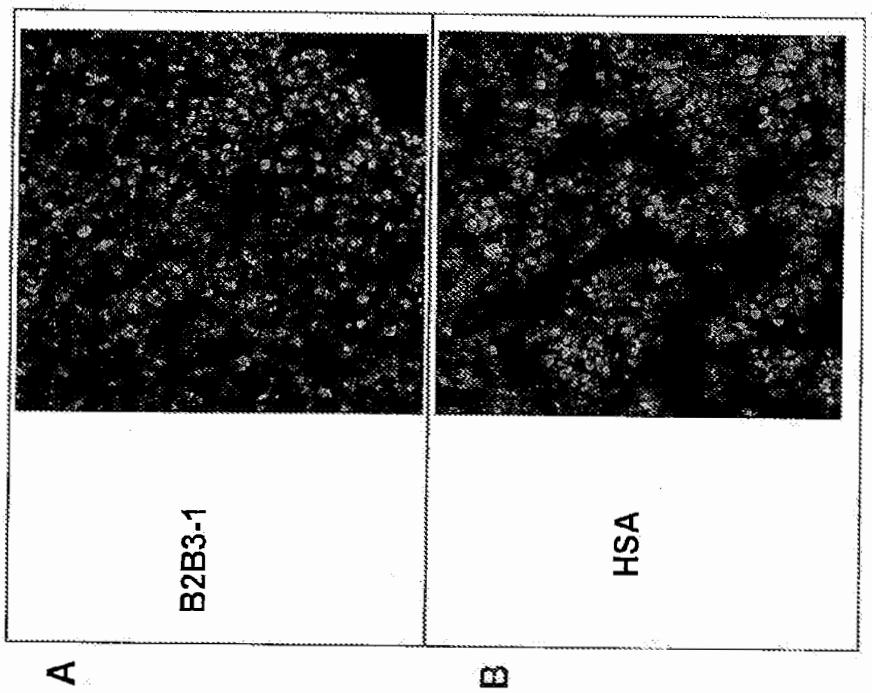


Figure 22

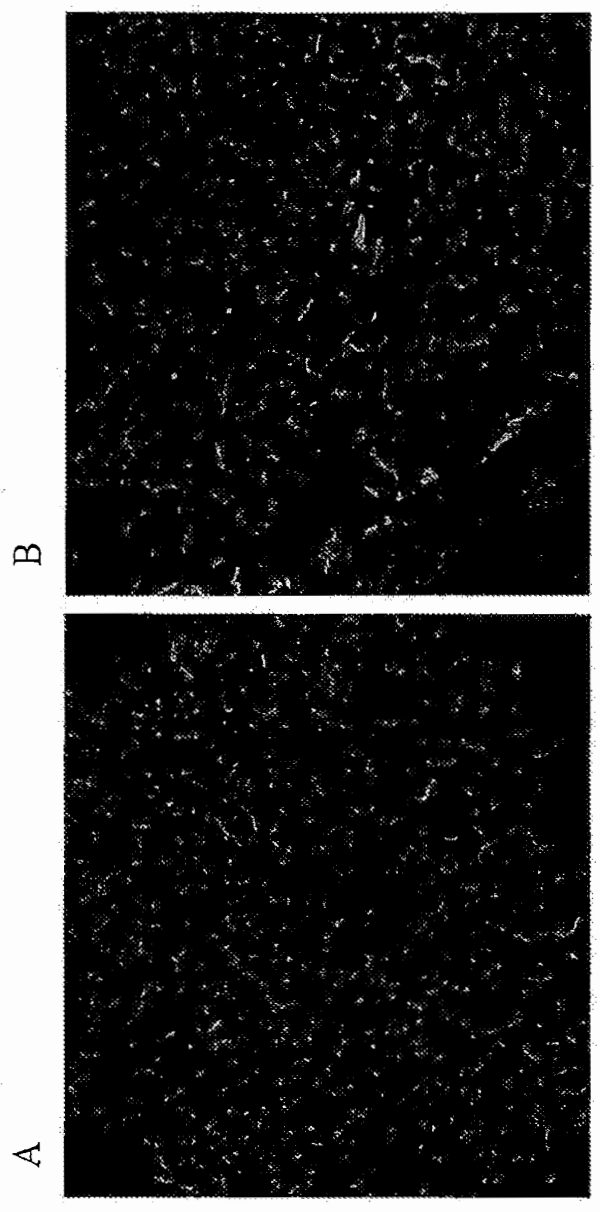


Figure 23

Figure 24

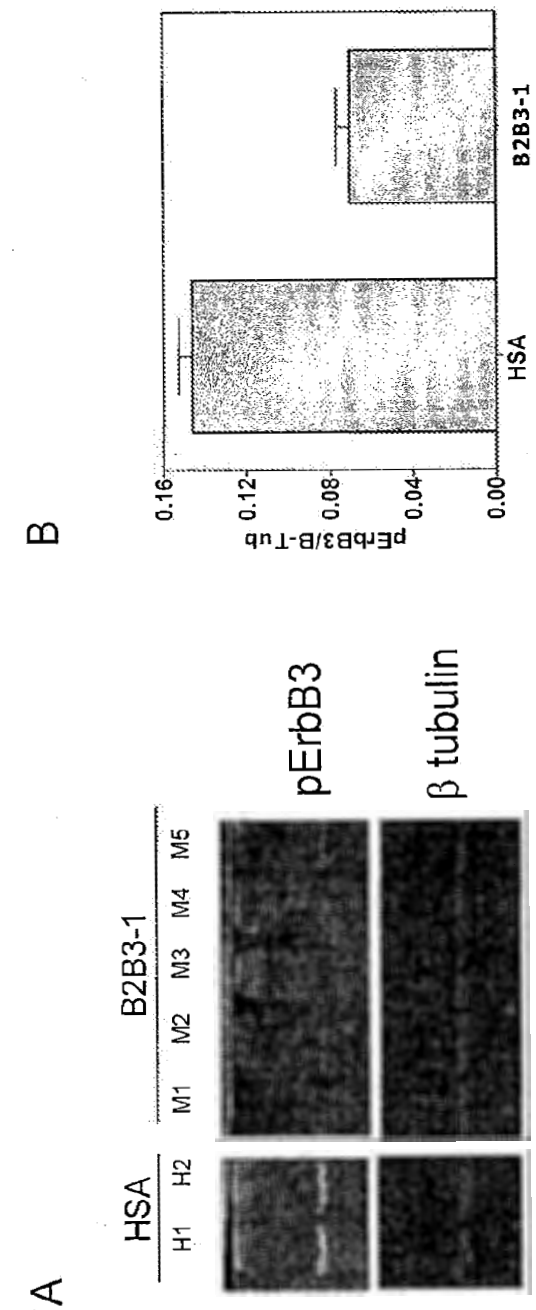
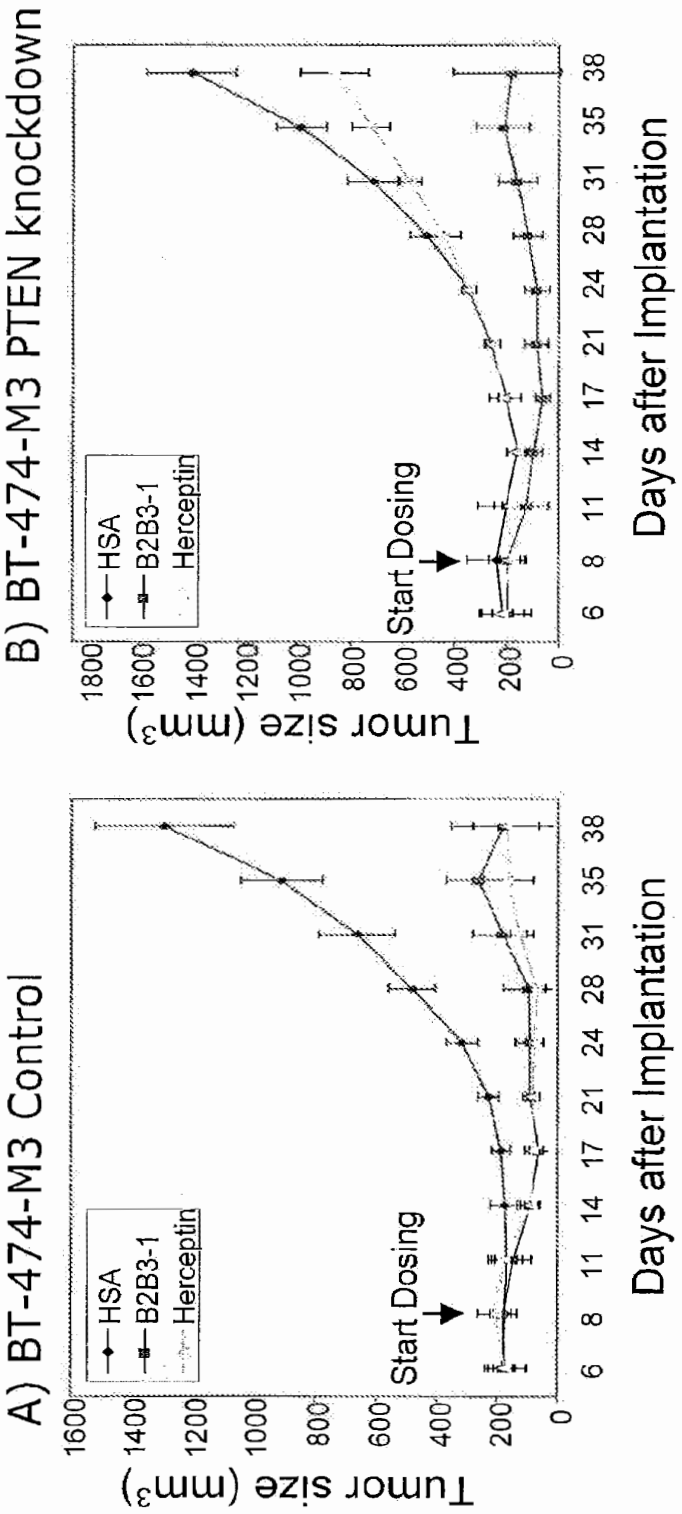

A
HSA
H1
H2
B2B3-1
M1
M2
M3
M4
M5
pErbB3
β tubulin
B
pErbB3/B-Tub
0.16
0.12
0.08
0.04
0.00
HSA
B2B3-1

Figure 25


A) BT-474-M3 Control
B) BT-474-M3 PTEN knockdown
Tumor size (mm3)
Days after Implantation
HSA
B2B3-1
Herceptin
Start Dosing
0
200
400
600
800
1000
1200
1400
1600
1800
6
8
11
14
17
21
24
28
31
35
38

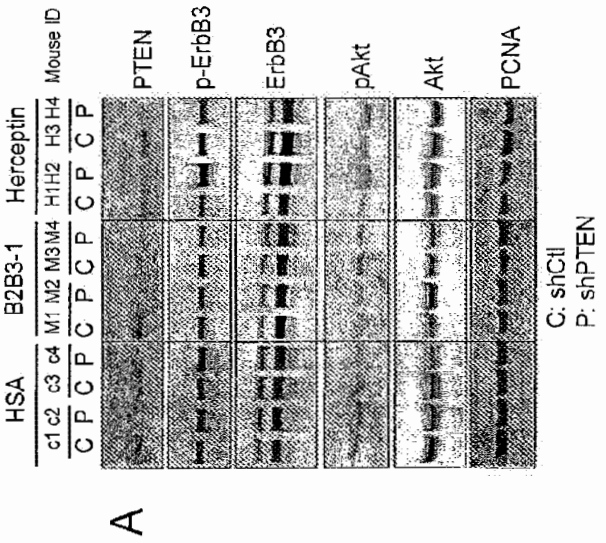
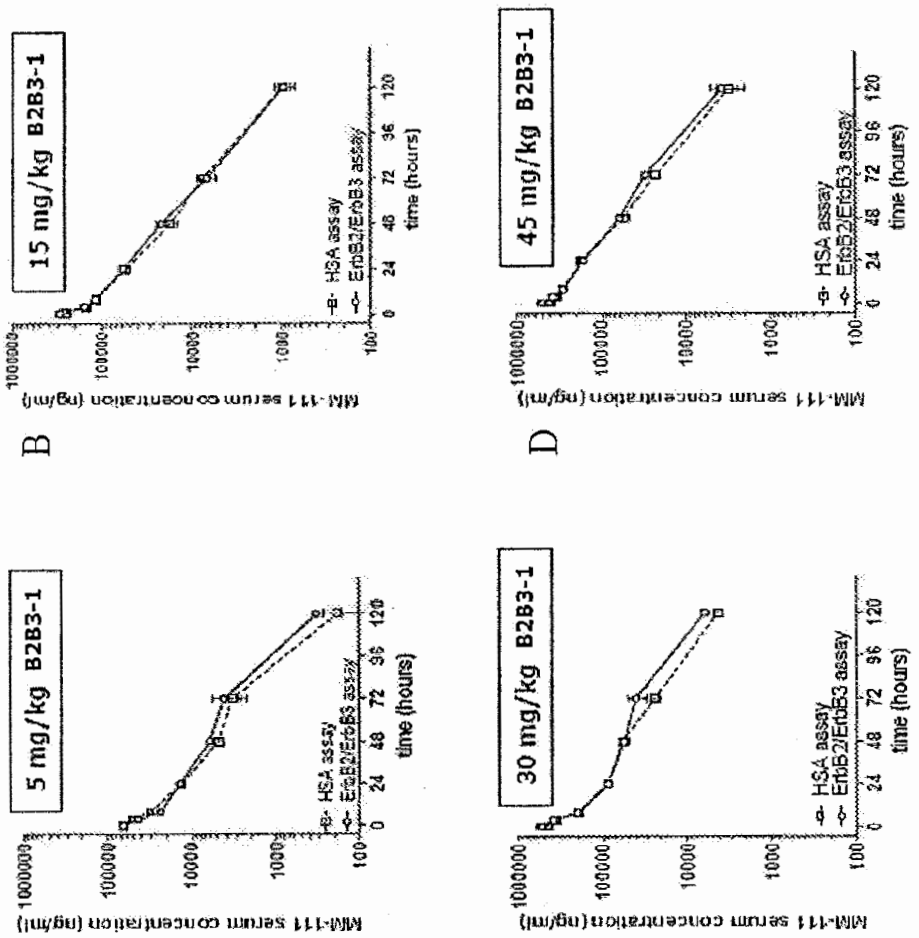

A
HSA
B2B3-1
Herceptin
Mouse ID
c1 c2 c3 c4 M1 M2 M3 M4 H1 H2 H3 H4
C P C P C P C P C P C P C P C P
PTEN
p-ErbB3
ErbB3
pAkt
Akt
PCNA
C: shCtl
P: shPTEN
B
HSA
B2B3-1
Trastuzumab
pAKT/tAKT
2.0
1.5
1.0
0.5
0.0
BT-474-M3 control
BT-474-M3 PTEN knock-ou

Figure 26

Figure 27


A
5 mg/kg B2B3-1
B
15 mg/kg B2B3-1
C
30 mg/kg B2B3-1
D
45 mg/kg B2B3-1
MM-111 serum concentration (ng/ml)
time (hours)
HSA assay
ErbB2/ErbB3 assay

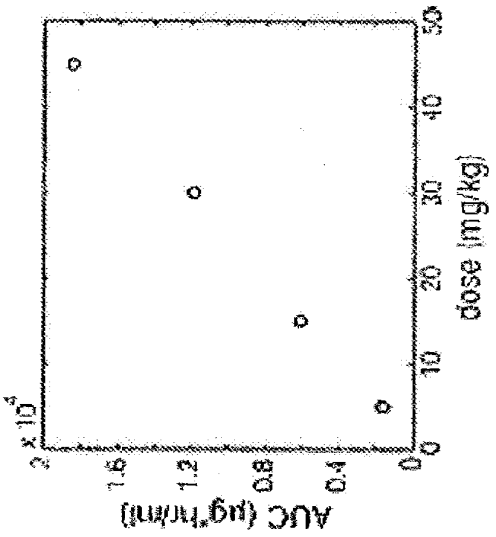


Figure 28

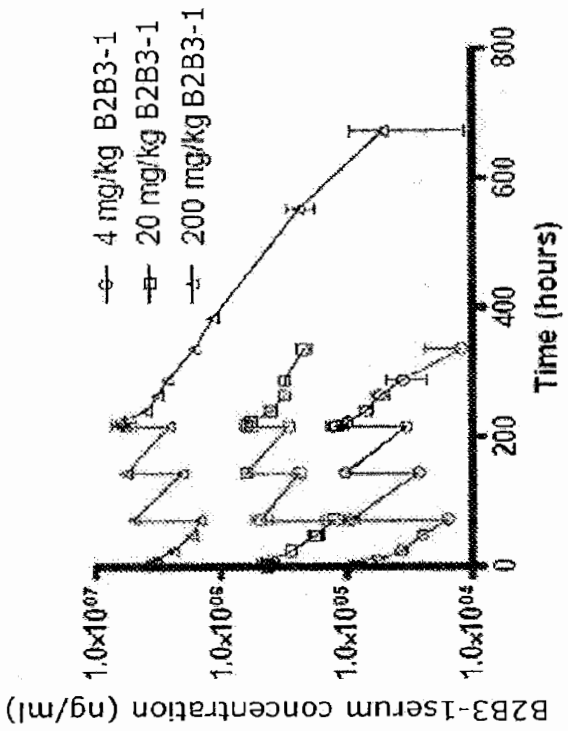

4 mg/kg B2B3-1
20 mg/kg B2B3-1
200 mg/kg B2B3-1
B2B3-1serum concentration (ng/ml)
1.0x10^07
1.0x10^06
1.0x10^05
1.0x10^04
0
200
400
600
800
Time (hours)

Figure 29

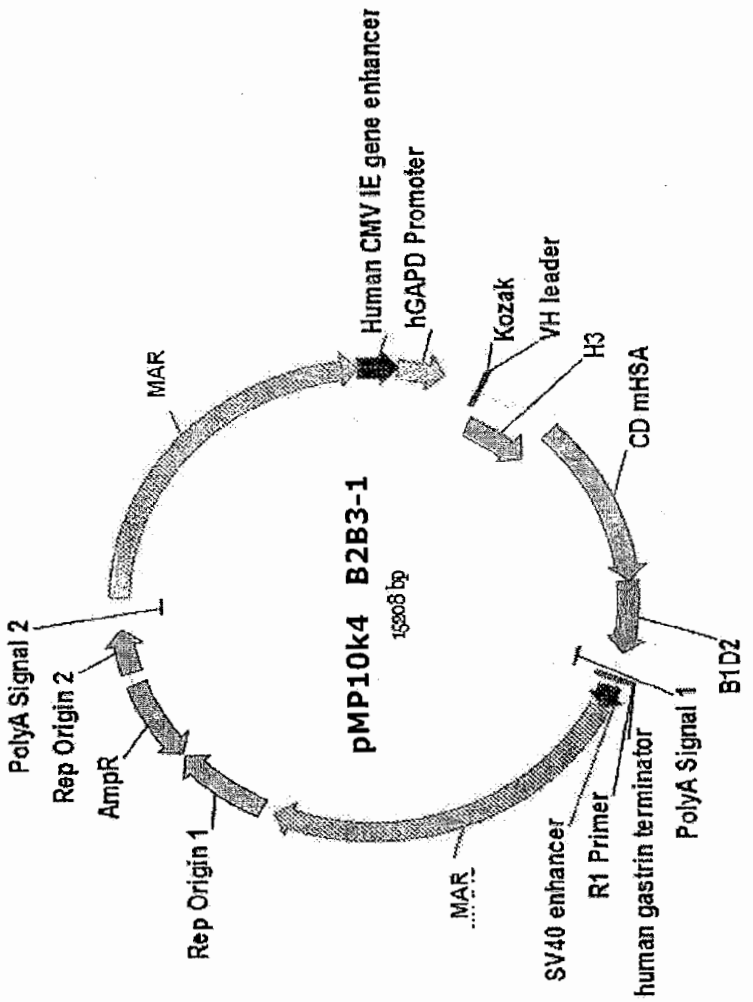

pMP10k4 B2B3-1
15908 bp
Human CMV IE gene enhancer
hGAPD Promoter
Kozak
VH leader
H3
CD mHSA
B1D2
PolyA Signal 1
human gastrin terminator
R1 Primer
SV40 enhancer
MAR
Rep Origin 1
AmpR
Rep Origin 2
PolyA Signal 2
MAR

Figure 30

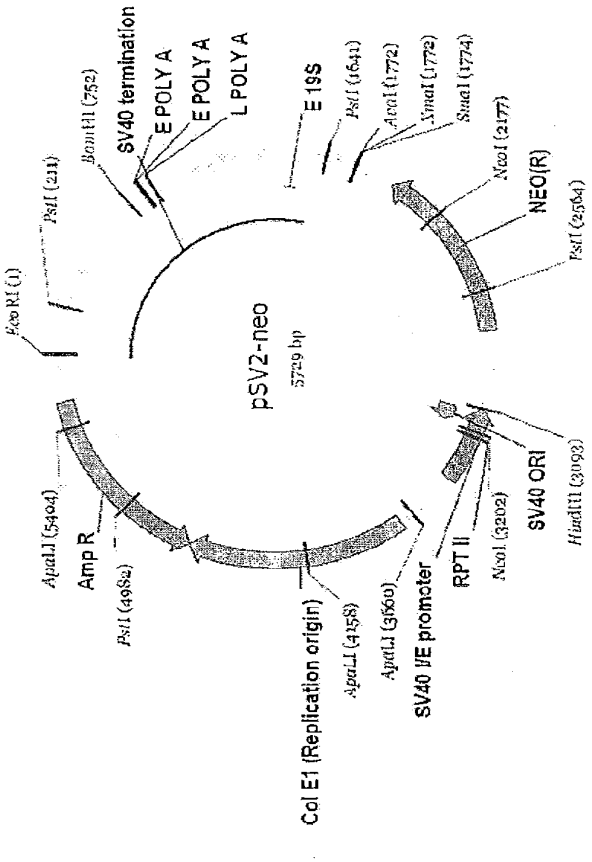

pSV2-neo
5729 bp
EcoRI (1)
PstI (211)
BamHI (752)
SV40 termination
E POLY A
E POLY A
L POLY A
E 19S
PstI (1641)
AccI (1772)
XmaI (1773)
StuaI (1774)
NcoI (2177)
NEO(R)
PstI (2584)
HindIII (3093)
SV40 ORI
NcoI (3202)
RPT II
SV40 I/E promoter
ApaLI (3660)
ApaLI (4158)
Col E1 (Replication origin)
PstI (4982)
Amp R
ApaLI (5404)

Figure 31

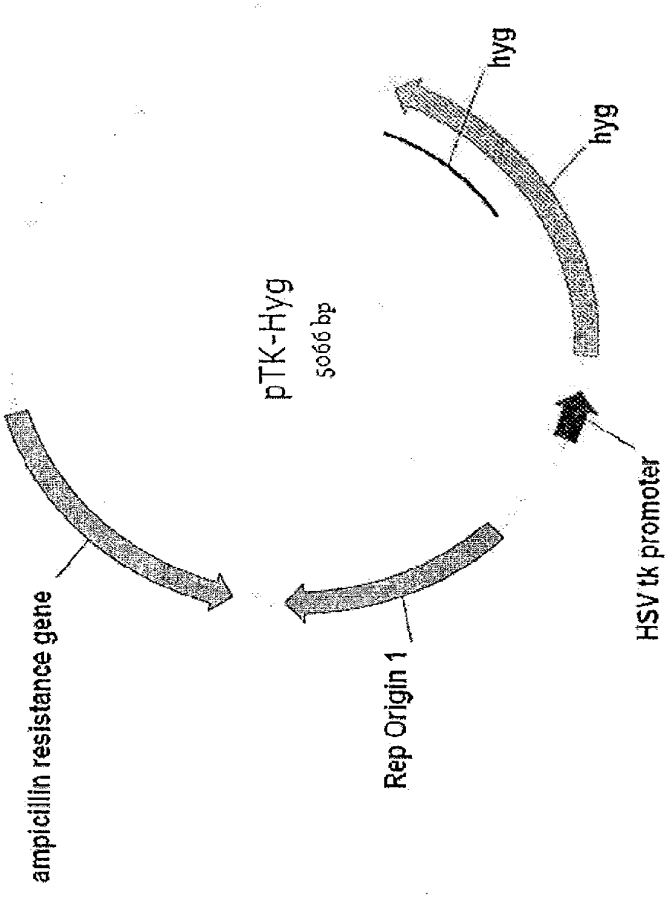

pTK-Hyg
5066 bp
hyg
hyg
HSV tk promoter
Rep Origin 1
ampicillin resistance gene

Figure 32

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61/492,762	2 June 2011 (02.06.2011)	US

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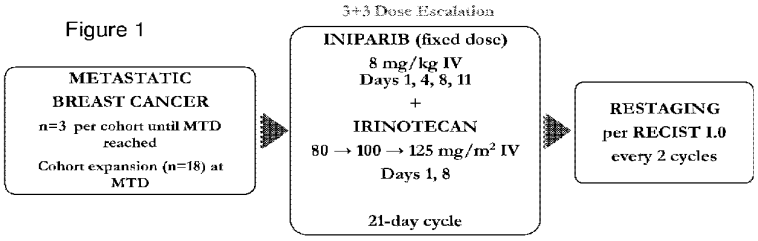
(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) **Title**: METHODS OF TREATING METASTATIC BREAST CANCER WITH 4-IODO-3-NITROBENZAMIDE AND IRINOTECAN

Figure 1



(57) **Abstract**: Provided herein are methods, compositions and kits for the treatment of locally advanced or metastatic breast cancer or breast cancer brain metastases. The method comprises administration of 4-iodo-3-nitrobenzamide, a metabolite or salt thereof in combination with irinotecan. The method of treating locally advanced or metastatic breast cancer comprises at least one 21 day treatment cycle.

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METHODS OF TREATING METASTATIC BREAST CANCER WITH 4-IODO-3-NITROBENZAMIDE AND IRINOTECAN

RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. provisional applications U.S. Serial No. 61/365,698, filed July 19, 2010, U.S. Serial No. 61/391,048, filed October 7, 2010, U.S. Serial No. 61/420,745, filed December 7, 2010, U.S. Serial No. 61/481,629, filed May 2, 2011, U.S. Serial No. 61/486,660, filed May 16, 2011, and U.S. Serial No. 61/492,762, filed June 2, 2011, the contents of each of which are incorporated herein by reference in their entirety.

TECHINICAL FIELD

[0002] The present invention relates to methods and compositions for the treatment of breast cancer comprising the administration of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof in combination with irinotecan.

BACKGROUND

[0003] Cancer is a group of diseases characterized by aberrant control of cell growth. The annual incidence of cancer is estimated to be in excess of 1.3 million in the United States alone. While surgery, radiation, chemotherapy, and hormones are used to treat cancer, it remains the second leading cause of death in the U.S. It is estimated that over 560,000 Americans will die from cancer each year.

[0004] Cancer cells simultaneously activate several pathways that positively and negatively regulate cell growth and cell death. This trait suggests that the modulation of cell death and survival signals could provide new strategies for improving the efficacy of current chemotherapeutic treatments.

[0005] Breast cancer is generally treated with a combination of surgery to remove the cancerous lesion and adjuvant therapy – radiation, chemotherapy or both – to attack any cancer cells that may be left after the surgery. Breast cancer can be classified broadly by the presence or absence of hormone receptors (HRs). Hormone receptor positive (HR+) cancer is characterized by the expression of one or both female hormone receptors – estrogen receptor (ER) or progesterone receptor (PR).

[0006] Treatment with anthracycline is limited by lifetime dosing limits based on cardiotoxicity concerns. Treatment with gemcitabine and carboplatin is an established combination chemotherapy for metastatic breast cancer patients – whether taxane-naïve or taxane-pretreated. Platinum agents have demonstrated promising antitumor activity in basal-like locally advanced breast cancers. DNA damaging agents have promising antitumor efficacy

against basal-like breast cancer because of defects in DNA repair pathways inherent in these breast cancers.

[0007] Despite the availability of antimetabolites such as gemcitabine and platinum complex agents such as carboplatin, there is no accepted standard of care for ER negative breast cancer. In particular, triple negative metastatic breast cancer (*i.e.*, breast cancer that is ER negative, ands/or PR negative, and/or human epidermal growth factor receptor 2 (HER2) negative) is refractory to standard treatments and is entirely refractory to SERM chemotherapy. There is thus a need for an effective treatment for cancer in general, and especially for triple negative metastatic breast cancer.

[0008] Metastatic breast cancer is a complex multi-step process involving the expansion of cancerous cells from the breast to other areas of the body. It is a serious complication of breast cancer, as metastatic disease in breast cancer is often fatal, with treatments mainly limited to palliation.

[0009] Although there are limited therapeutic options for cancer treatment, variants of cancers, including metastatic breast cancer, triple negative breast cancer, are especially difficult because they can be refractory to standard chemotherapeutic or hormonal treatment. There is thus a need for an effective treatment for cancer in general, and cancer variants in particular.

SUMMARY OF THE INVENTION

[0010] Provided herein are methods of treating breast cancer (*e.g.*, locally advanced or metastatic breast cancer) in a patient, comprising administering to the patient an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof. In some embodiments, the method comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle. In some embodiments, the breast cancer is metastatic breast cancer. In some embodiments, the metastasis comprises brain metastases (e.g., brain metastasis measuring at least about 0.5 centimeter). In some embodiments, the breast cancer is locally advanced breast cancer. In some embodiments, the breast cancer is progressing locally advanced breast cancer.

[0011] In some embodiments, the breast cancer (*e.g.*, locally advanced or metastatic breast cancer) is hormone receptor-negative ("HR-negative") breast cancer. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is negative for at least one of: estrogen receptor ("ER"), progesterone receptor ("PR") or human epidermal growth factor receptor 2 ("HER2"). In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is negative for at least one

of: ER, PR or HER2; and the breast cancer (*e.g.*, metastatic breast cancer) is positive for at least one of ER, PR or HER2. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is HR-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is an ER-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative and HER2-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative and PR-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative and both HER2-positive and PR-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is a PR-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is PR-negative and ER-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is PR-negative and HER2-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is PR-negative and both ER-positive and HER2-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is a HER2-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is HER2-negative and ER-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is HER2-negative and PR-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is HER2-negative and both ER-positive and PR-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative and PR-negative. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative, PR-negative and HER-2 positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative and HER2-negative. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative, HER2-negative and PR-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is PR-negative and HER2-negative. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is PR-negative, HER2-negative and ER-positive. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative, PR-negative and HER2-negative. In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is ER-negative, PR-negative and HER2-nonoverexpressing.

[0012] In some embodiments, there is provided a method of treating locally advanced or metastatic breast cancer in a patient, comprising administering to the patient having locally advanced or metastatic breast cancer an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof, wherein the method comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is

administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle. In some embodiments, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg on days 1, 4, 8, and 11 of the 21-day cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the 21-day cycle.

[0013] In some embodiments, the patient has breast adenocarcinoma. In some embodiments, the breast cancer is locoregional breast cancer. In some embodiments, the breast cancer is progressing locoregional breast cancer. In some embodiments, the patient has distant metastasis. In some embodiments, the patient has systemic metastasis. In some embodiments, the breast cancer is refractory to standard therapy. In some embodiments, the patient has received prior chemotherapy treatment comprising at least one regimen selected from the group consisting of an anthracycline, an anthraquinone, and a taxane. In some embodiments, the patient is refractory to at least one regimen selected from the group consisting of an anthracycline, an anthraquinone, and a taxane. In some embodiments, the patient has lesion of at least 2.0 centimeter.

[0014] Also provided are methods of treating a patient with breast cancer brain metastasis comprising administering to the patient an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan or a pharmaceutically acceptable salt thereof, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, 4-iodo-3-nitrobenzamide or a pharmaceutically acceptable salt thereof (*e.g.*, 4-iodo-3-nitrobenzamide) is administered to the patient. In some embodiments, irinotecan is administered to the patient. In some embodiments, there is provided a method of treating metastatic breast cancer brain metastasis in a patient comprising administering to the patient having the metastatic breast cancer brain metastasis an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, the brain metastasis is at least about or larger than about 0.5 centimeter (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter in longest dimension). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new and/or progressive brain metastasis following radiation therapy (e.g., central nervous system ("CNS") radiation therapy or intracranial radiation therapy). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new and/or progressive brain metastasis following radiation therapy (e.g., central nervous system ("CNS") radiation therapy or intracranial radiation therapy) for breast cancer brain metastases. In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than

about 0.5 centimeter) is new and/or progressive brain metastasis after prior radiation therapy (e.g., after prior central nervous system radiation therapy or after prior intracranial radiation therapy). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new and/or progressive brain metastasis after prior radiation therapy (e.g., after prior central nervous system radiation therapy or after prior intracranial radiation therapy) for breast cancer brain metastases. In some embodiments, the brain metastasis is new brain metastasis (e.g., new brain metastasis measuring at least about or larger than about 0.5 centimeter) after the radiation therapy. In some embodiments, the brain metastasis is progressive brain metastasis (e.g., progressive brain metastasis measuring at least about or larger than about 0.5 centimeter) after the radiation therapy. In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is asymptomatic and the patient is CNS-radiation therapy naïve patient. In some embodiments, the patient has no prior radiation therapy (e.g., prior intracranial radiation therapy). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new brain metastasis and the patient has no prior intracranial radiation therapy and/or the intracranial radiation therapy is not emergently indicated for the patient. In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new brain metastasis (e.g., brain metastasis is found within 2 weeks of initiation of a therapy such as a protocol-based therapy) and the patient is intracranial radiation-naïve patient for whom intracranial radiation therapy is not emergently indicated. In some embodiments, the patient does not have leptomeningeal disease (e.g., the patient does not have diffuse leptomeningeal disease). In some embodiments, the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 5.6 mg/kg on days 1, 4, 8, 11 of the treatment cycle, and wherein irinotecan is administered at about 125 mg/m^2 on days 1 and 8 of the cycle. In some embodiments, the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 11.2 mg/kg on days 1 and 8 of the treatment cycle, and wherein irinotecan is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

[0015] In some embodiments, 4-iodo-3-nitrobenzamide or the metabolite thereof or the pharmaceutically acceptable salt thereof is administered intravenously. In some embodiments, irinotecan or the pharmaceutically acceptable salt thereof is administered intravenously.

[0016] In some embodiments, the method further comprises surgery, radiation therapy, chemotherapy, gene therapy, DNA therapy, viral therapy, RNA therapy, adjuvant therapy,

neoadjuvant therapy, immunotherapy, nanotherapy or a combination thereof. In some embodiments, the radiation therapy comprises administering to the patient gamma irradiation.

[0017] In some embodiments, the effective amount produces at least one therapeutic effect selected from the group consisting of reduction in size of a breast tumor, reduction in metastasis, complete remission, partial remission, stable disease, increase in overall response rate, or a pathologic complete response. In some embodiments, the treatment produces complete response, partial response, or stable disease.

[0018] Also provided herein are uses of 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, in combination with irinotecan, a pharmaceutically acceptable salt or solvate thereof for the manufacture of a medicament for the treatment or prevention of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) described herein. Also provided herein are uses of 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof for the manufacture of a medicament for the treatment or prevention of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) in combination with irinotecan, a pharmaceutically acceptable salt or solvate thereof described herein. In certain embodiments, the medicament is provided for the treatment of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis). Also provided herein are uses of -iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, in combination with irinotecan, a pharmaceutically acceptable salt or solvate thereof, for treatment of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) in a patient. Also provided herein are synergistic compositions used for treating breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) in a patient comprising a) 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, and b) irinotecan, or pharmaceutically acceptable salt or solvate thereof, to said patient. The uses described herein may be in accordance with any of the methods described herein.

[0019] Also provided herein are kits for the treatment or prevention in a patient with breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain

metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis), comprising 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, in combination with irinotecan, or a pharmaceutically acceptable salt or solvate thereof. In some embodiments, the kit comprises instructions for using 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof in combination with irinotecan, or a pharmaceutically acceptable salt or solvate thereof for the treatment or prevention in a patient with breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) according to any of the methods described herein. Also provided herein are kits comprising 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, and a label or packaging insert containing information and/or instructions related to use of 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof in combination with irinotecan, or a pharmaceutically acceptable salt or solvate thereof for the treatment or prevention in a patient with breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) according to any of the methods described herein. A kit described herein may comprise packaging. The dosage or dosing regimen for 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof can be any dosage or dosing regimen described herein. The dosage or dosing regimen for irinotecan, a pharmaceutically acceptable salt or solvate thereof can be any dosage or dosing regimen described herein.

[0020] For example, in some embodiments, there is provided a kit for treating a patient with breast cancer brain metastasis comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, the kit further comprises instructions for using effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof to treat the patient with breast cancer brain metastasis. In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) instructions for using an effective amount of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, in combination with irinotecan or a pharmaceutically acceptable salt thereof to treat a patient with breast cancer brain metastasis, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a

pharmaceutically acceptable salt thereof, (b) irinotecan or a pharmaceutically acceptable salt thereof, and (c) instructions for using 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and irinotecan or a pharmaceutically acceptable salt thereof to treat locally advanced or metastatic breast cancer in a patient, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle. In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) instructions for using 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, in combination with irinotecan or a pharmaceutically acceptable salt thereof to treat locally advanced or metastatic breast cancer in a patient, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle.

[0021] Also provided are articles of manufacture comprising the compositions described herein in suitable packaging. Suitable packaging for compositions described herein are known in the art, and include, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed. Also provided are unit dosage forms comprising the compositions described herein. These unit dosage forms can be stored in a suitable packaging in single or multiple unit dosages and may also be further sterilized and sealed.

[0022] It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention. These and other aspects of the invention will become apparent to one of skill in the art.

INCORPORATION BY REFERENCE

[0023] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

[0024] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative

embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0025] FIGURE 1 shows the treatment scheme (3+3 dose escalation) for treating patients with metastatic breast cancer with 4-iodo-3-nitrobenzamide and irinotecan.

[0026] FIGURE 2 shows best % change in tumor size from baseline in triple negative breast cancer ("TNBC") and non-TNBC patients treated with 4-iodo-3-nitrobenzamide and irinotecan. The bars represent best single measurement of target lesion from each patient.

[0027] FIGURE 3 shows immunohistochemistry ("IHC") staining for BRCA1 on tumor samples measured with AQUA™ technology.

DETAILED DESCRIPTION

Definitions

[0028] As used herein, "treatment" or "treating" or its grammatical equivalents as used herein includes achieving beneficial or desired results including, *e.g.*, a therapeutic benefit, a prophylactic benefit, and/or clinical results. For purposes of this invention, beneficial or desired results include, but are not limited to, one or more of the following: alleviating one or more symptoms resulting from the disease, diminishing the extent of the disease, stabilizing the disease (*e.g.*, preventing or delaying the worsening of the disease), preventing or delaying the spread (*e.g.*, metastasis) of the disease, preventing or delaying the recurrence of the disease, delay or slowing the progression of the disease, eradication of the underlying disease, ameliorating the disease state, providing a remission (partial or total) of the disease, decreasing the dose of one or more other medications required to treat the disease, delaying the progression of the disease, increasing the quality of life, and/or prolonging survival. Also encompassed by "treatment" is a reduction of pathological consequence of cancer. The methods of the invention contemplate any one or more of these aspects of treatment.

[0029] For example, in an individual with breast cancer (*e.g.*, metastatic breast cancer), a benefit (*e.g.*, a therapeutic benefit) includes eradication or amelioration of the underlying breast cancer (*e.g.*, metastatic breast cancer), *e.g.*, slowing of progression of the breast cancer (*e.g.*, metastatic breast cancer). Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder (*e.g.*, breast cancer) such that an improvement is observed in the individual, notwithstanding the fact that the individual may still be afflicted with the underlying disorder (*e.g.*, breast cancer). For a benefit such as prophylactic benefit, a method of the invention may be performed on, or a composition of the invention administered to an individual at risk of developing breast cancer, or to an individual reporting one or more of the physiological symptoms of breast cancer, even though a diagnosis of breast cancer may not have been made. In

some embodiments, the individual being treated has been diagnosed with a breast cancer described herein.

[0030] The term "individual" or "patient" refers to a mammal and includes, but is not limited to, human, bovine, horse, feline, canine, rodent, or primate. Preferably, the individual is a human. An individual may be a patient.

[0031] As used herein, an "at risk" individual is an individual who is at risk of developing cancer. An individual "at risk" may or may not have detectable disease, and may or may not have displayed detectable disease prior to the treatment methods described herein. "At risk" denotes that an individual has one or more so-called risk factors, which are measurable parameters that correlate with development of cancer. An individual having one or more of these risk factors has a higher probability of developing cancer than an individual without these risk factor(s).

[0032] "Adjuvant setting" refers to a clinical setting in which an individual has had a history of cancer, and generally (but not necessarily) been responsive to therapy, which includes, but is not limited to, surgery (*e.g.*, surgery resection), radiotherapy, and chemotherapy. However, because of their history of cancer, these individuals are considered at risk of development of the disease. Treatment or administration in the "adjuvant setting" refers to a subsequent mode of treatment. The degree of risk (*e.g.*, when an individual in the adjuvant setting is considered as "high risk" or "low risk") depends upon several factors, most usually the extent of disease when first treated.

[0033] "Neoadjuvant setting" refers to a clinical setting in which the method is carried out before the primary/definitive therapy.

[0034] As used herein, "delaying" the development of cancer means to defer, hinder, slow, retard, stabilize, and/or postpone development of the disease. This delay can be of varying lengths of time, depending on the history of the disease and/or individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop the disease. A method that "delays" development of cancer is a method that reduces probability of disease development in a given time frame and/or reduces the extent of the disease in a given time frame, when compared to not using the method. Such comparisons are typically based on clinical studies, using a statistically significant number of subjects. Cancer development can be detectable using standard methods, including, but not limited to, computerized axial tomography (CAT Scan), Magnetic Resonance Imaging (MRI), abdominal ultrasound, clotting tests, arteriography, or biopsy. Development may also refer to cancer progression that may be initially undetectable and includes occurrence, recurrence, and onset.

[0035] As used herein "surgery" refers to any therapeutic or diagnostic procedure that involves methodical action of the hand or of the hand with an instrument, on the body of a human or other mammal, to produce a curative, remedial, or diagnostic effect.

[0036] "Radiation therapy" refers to exposing an individual to high-energy radiation, including without limitation x-rays, gamma rays, and neutrons. This type of therapy includes without limitation external-beam therapy, internal radiation therapy, implant radiation, brachytherapy, systemic radiation therapy, and radiotherapy.

[0037] "Chemotherapy" refers to the administration of one or more anti-cancer drugs such as, antineoplastic chemotherapeutic agents, chemopreventative agents, and/or other agents to an individual with breast cancer (*e.g.*, metastatic breast cancer) by various methods, including intravenous, oral, intramuscular, intraperitoneal, intravesical, subcutaneous, transdermal, buccal, or inhalation or in the form of a suppository. Unless clearly dictated otherwise by context, "chemotherapy" as used herein is not intended to refer to the administration of 4-iodo-3-nitrobenzamide and irinotecan. Chemotherapy may be given prior to surgery to shrink a large tumor prior to a surgical procedure to remove it, prior to radiation therapy, or after surgery and/or radiation therapy to prevent the growth of any remaining breast cancer cells in the body. Chemotherapy may also occur during the course of radiation therapy.

[0038] The terms "effective amount" or "pharmaceutically effective amount" refer to a sufficient amount of an agent to provide the desired biological, therapeutic, and/or prophylactic result. That result can be reduction, amelioration, palliation, lessening, delaying, and/or alleviation of one or more of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. In reference to cancer, an effective amount comprises an amount sufficient to cause a tumor to shrink and/or to decrease the growth rate of the tumor (such as to suppress tumor growth) or to prevent or delay other unwanted cell proliferation. In some embodiments, an effective amount is an amount sufficient to delay development. In some embodiments, an effective amount is an amount sufficient to prevent or delay recurrence. An effective amount can be administered in one or more administrations. The effective amount of the drug or composition may: (i) reduce the number of cancer cells; (ii) reduce tumor size; (iii) inhibit, retard, slow to some extent and preferably stop cancer cell infiltration into peripheral organs; (iv) inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; (v) inhibit tumor growth; (vi) prevent or delay occurrence and/or recurrence of tumor; and/or (vii) relieve to some extent one or more of the symptoms associated with the cancer. For example, an "effective amount" for therapeutic uses is the amount of a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt or solvate thereof or b) irinotecan, or a pharmaceutically acceptable salt or solvate thereof, provided herein, or a composition comprising a) 4-iodo-3-

nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and b) irinotecan required to provide a clinically significant decrease in the breast tumor or slowing of progression of the breast cancer (*e.g.*, metastatic breast cancer).

[0039] "Metabolite" refers to a compound produced through any *in vitro* or *in vivo* metabolic process which results in a product that is different in structure than that of the starting compound. In other words, the term "metabolite" includes the metabolite compounds of 4-iodo-3-nitrobenzamide, for example, 4-iodo-3-aminobenzoic acid ("IABA") and 4-iodo-3-aminobenzamide ("IABM"). A metabolite can include a varying number or types of substituents that are present at any position relative to a precursor compound. In addition, the terms "metabolite" and "metabolite compound" are used interchangeably herein.

[0040] As used herein, by "pharmaceutically acceptable" or "pharmacologically compatible" is meant a material that is not biologically or otherwise undesirable, *e.g.*, the material may be incorporated into a pharmaceutical composition administered to an individual without causing any significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained. Pharmaceutically acceptable carriers or excipients have preferably met the required standards of toxicological and manufacturing testing and/or are included on the Inactive Ingredient Guide prepared by the U.S. Food and Drug administration.

[0041] An "adverse event" or "AE," unless specified otherwise, refers to any untoward medical occurrence in a patient receiving a marketed pharmaceutical product or in a patient who is participating on a clinical trial who is receiving an investigational or non-investigational pharmaceutical agent. The AE does not necessarily have a causal relationship with the patient's treatment. Therefore, an AE can be any unfavorable and unintended sign, symptom, or disease temporally associated with the use of a medicinal product, whether or not considered to be related to the medicinal product. An AE includes, but is not limited to: an exacerbation of a pre-existing illness; an increase in frequency or intensity of a pre-existing episodic event or condition; a condition detected or diagnosed after study drug administration even though it may have been present prior to the start of the study; and continuously persistent disease or symptoms that were present at baseline and worsen following the start of the study. An AE generally does not include: medical or surgical procedures (*e.g.*, surgery, endoscopy, tooth extraction, or transfusion); however, the condition that leads to the procedure is an adverse event; pre-existing diseases, conditions, or laboratory abnormalities present or detected at the start of the study that do not worsen; hospitalizations or procedures that are done for elective purposes not related to an untoward medical occurrence (*e.g.*, hospitalizations for cosmetic or elective surgery or social/convenience admissions); the disease being studied or signs/symptoms associated with the

disease unless more severe than expected for the patient's condition; and overdose of study drug without any clinical signs or symptoms.

[0042] A "serious adverse event" or (SAE), unless specified otherwise, refers to any untoward medical occurrence at any dose including, but not limited to, that: a) is fatal; b) is life-threatening (defined as an immediate risk of death from the event as it occurred); c) results in persistent or significant disability or incapacity; d) requires in-patient hospitalization or prolongs an existing hospitalization (exception: Hospitalization for elective treatment of a pre-existing condition that did not worsen during the study is not considered an adverse event; complications that occur during hospitalization are AEs and if a complication prolongs hospitalization, then the event is serious); e) is a congenital anomaly/birth defect in the offspring of an individual who received medication; or f) conditions not included in the above definitions that may jeopardize the individual or may require intervention to prevent one of the outcomes listed above unless clearly related to the individual's underlying disease. "Lack of efficacy" (progressive disease) is not considered an AE or SAE. The signs and symptoms or clinical sequelae resulting from lack of efficacy should be reported if they fulfill the AE or SAE definitions.

[0043] The following definitions may be used to evaluate response based on target lesions: unless specified otherwise, "complete response" or "CR" (also known as "complete remission") refers to disappearance of all target lesions; "partial response" or "PR" (also known as "partial remission") refers to at least a 30% decrease in the sum of the longest diameters (SLD) of target lesions, taking as reference the baseline SLD; "stable disease" or "SD" refers to neither sufficient shrinkage of target lesions to qualify for PR, nor sufficient increase to qualify for PD, taking as reference the nadir SLD since the treatment started; and "progressive disease" or "PD" refers to at least a 20% increase in the SLD of target lesions, taking as reference the nadir SLD recorded since the treatment started, or, the presence of one or more new lesions.

[0044] The following definitions of response assessments may be used to evaluate a non-target lesion: unless specified otherwise, "complete response" or "CR" refers to disappearance of all non-target lesions; "stable disease" or "SD" refers to the persistence of one or more non-target lesions not qualifying for CR or PD; and "progressive disease" or "PD" refers to the "unequivocal progression" of existing non-target lesion(s) or appearance of one or more new lesion(s) is considered progressive disease (if PD for the subject is to be assessed for a time point based solely on the progression of non-target lesion(s), then additional criteria are required to be fulfilled).

[0045] "Progression free survival" (PFS) may indicate the length of time during and after treatment that the cancer does not grow. Progression-free survival may include the amount of

time patients have experienced a complete response or a partial response, as well as the amount of time patients have experienced stable disease.

[0046] As used herein, "sample" refers to a composition which contains a molecule which is to be characterized and/or identified, for example, based on physical, biochemical, chemical, physiological, and/or genetic characteristics.

[0047] "Cells," as used herein, is understood to refer not only to the particular subject cell, but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0048] "HER2-negative" used herein means "HER2 non-overexpressing" as understood by one skilled in the art.

[0049] Reference to "about" a value or parameter herein includes (and describes) variations that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X".

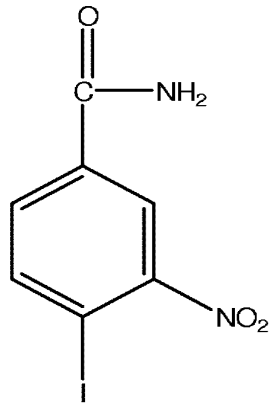
[0050] As used herein and in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise.

[0051] It is understood that aspects and variations of the invention described herein include "consisting" and/or "consisting essentially of" aspects and variations.

[0052] As is apparent to one skilled in the art, an individual assessed, selected for, and/or receiving treatment is an individual in need of such activities.

4-iodo-3-nitrobenzamide or a metabolite thereof

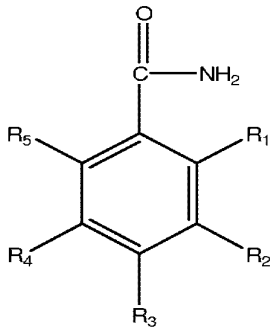
[0053] 4-iodo-3-nitrobenzamide, also known as iniparib or "BA," has the formula:



4-iodo-3-nitrobenzamide
(BA)

[0054] Methods of making 4-iodo-3-nitrobenzamide are known to the field, such as the methods disclosed in U.S. Patent No. 5,464,871, which is hereby incorporated by reference in its entirety, particularly with respect to the synthetic methods disclosed therein.

[0055] Provided herein are precursor compounds of Formula (Ia)

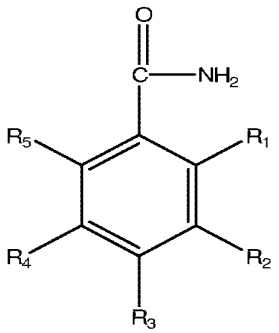


(Ia)

Formula Ia

wherein R_1, R_2, R_3, R_4, and R_5 are, independently selected from the group consisting of hydrogen, hydroxy, amino, nitro, nitroso, iodo, (C_1 -C_6) alkyl, (C_1 -C_6) alkoxy, (C_3 -C_7) cycloalkyl, and phenyl, wherein at least two of the five R_1, R_2, R_3, R_4, and R_5 substituents are always hydrogen, at least one of the five substituents is always nitro, and at least one substituent positioned adjacent to a nitro is always iodo, and pharmaceutically acceptable salts, solvates, isomers, tautomers, metabolites, analogs, or pro-drugs thereof. R_1, R_2, R_3, R_4, and R_5 can also be a halide such as chloro, fluoro, or bromo substituents. In some embodiments, at least one of the R_1, R_2, R_3, R_4, and R_5 substituents is always nitro or nitroso and at least one substituent positioned adjacent to the nitro or nitroso is always iodo. In some embodiments, the compound of formula Ia is a compound of formula IA or a metabolite or pharmaceutically acceptable salt, solvate, isomer, or tautomer thereof. In some embodiments, at least one of the R_1, R_2, R_3, R_4, and R_5 substituents is always nitro or nitroso and at least one substituent positioned adjacent to the nitro or nitroso is always iodo. In some embodiments, the compound of formula Ia is a compound of formula IA or pharmaceutically acceptable salt, solvate, isomer, or tautomer thereof.

[0056] Also provided herein are metabolites with the Formula (IIa):

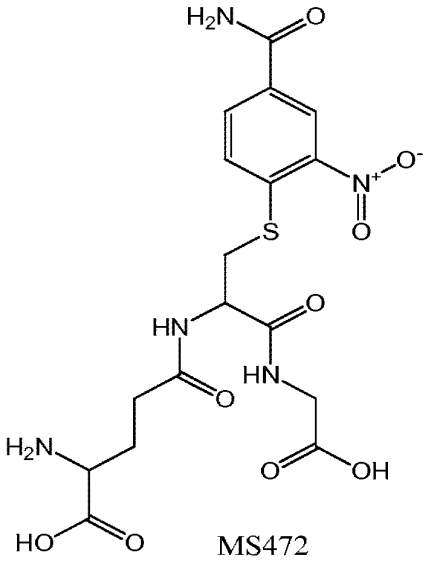


(IIa)

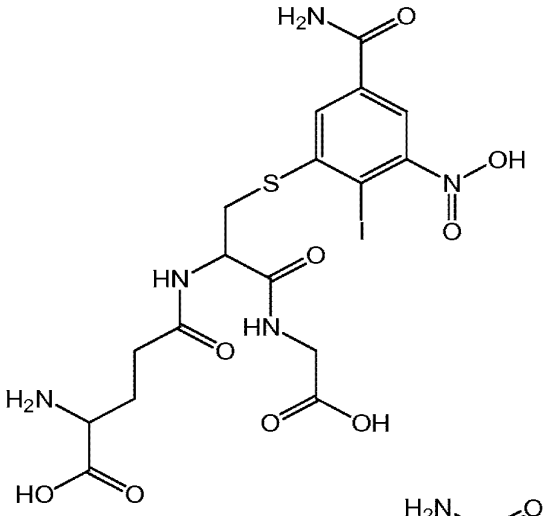
wherein either: (1) at least one of R_1, R_2, R_3, R_4, and R_5 substituent is always a sulfur-containing substituent, and the remaining substituents R_1, R_2, R_3, R_4, and R_5 are independently selected from the group consisting of hydrogen, hydroxy, amino, nitro, iodo, bromo, fluoro, chloro, (C_1 -C_6) alkyl, (C_1 -C_6) alkoxy, (C_3 -C_7) cycloalkyl, and phenyl, wherein at least two of the five R_1, R_2, R_3, R_4, and R_5 substituents are always hydrogen; or (2) at least one of R_1, R_2, R_3, R_4, and R_5 substituents is not a sulfur-containing substituent and at least one of the five substituents R_1, R_2, R_3, R_4, and R_5 is always iodo, and wherein said iodo is always adjacent to a R_1, R_2, R_3, R_4, or R_5 group that is either a nitro, a nitroso, a hydroxyamino, hydroxy or an amino group; and pharmaceutically acceptable salts, solvates, isomers, tautomers, metabolites, analogs, or pro-drugs thereof. In some embodiments, the compounds of (2) are such that the iodo group is always adjacent to a R_1, R_2, R_3, R_4 or R_5 group that is a nitroso, hydroxyamino, hydroxy or amino group. In some embodiments, the compounds of (2) are such that the iodo group is always adjacent to a R_1, R_2, R_3, R_4 or R_5 group that is a nitroso, hydroxyamino, or amino group.

[0057] Any of the compounds with structure formula Ia or IIa may be used for a treatment described herein. In some embodiments, the compound with structure formula Ia or IIa is 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof.

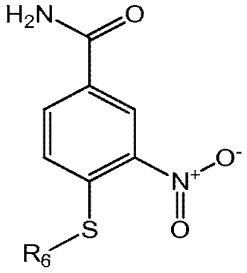
[0058] Provided herein are metabolite compounds, each represented by a chemical formula:



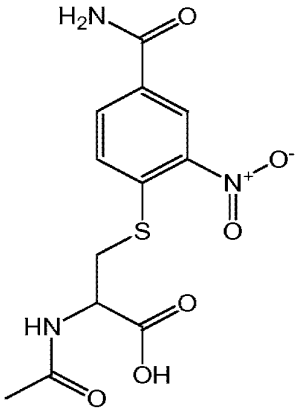
MS472



MS601

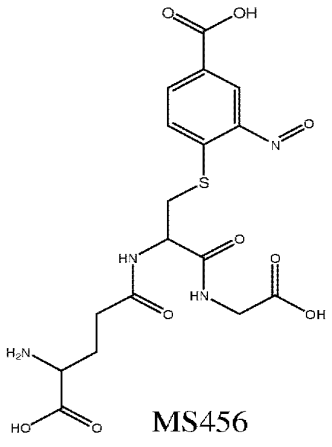


MS213

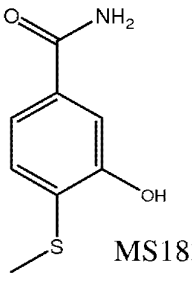


MS328

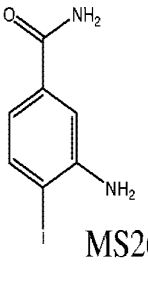
R_6 is selected from the group consisting of hydrogen, alkyl (C_1-C_8), alkoxy (C_1-C_8), isoquinolinones, indoles, thiazole, oxazole, oxadiazole, thiophene, or phenyl.



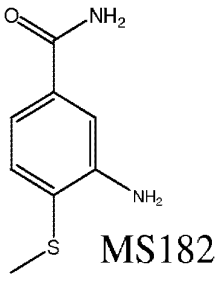
MS456



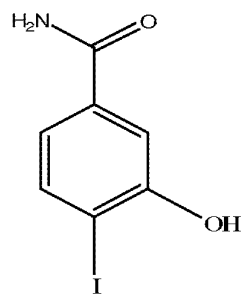
MS183



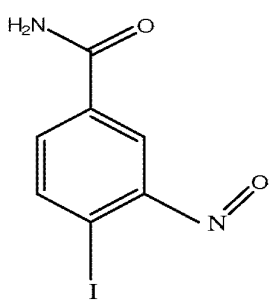
MS261



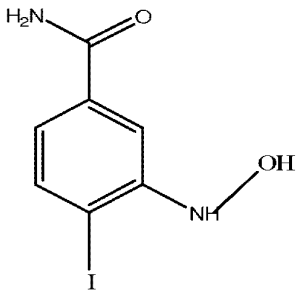
MS182



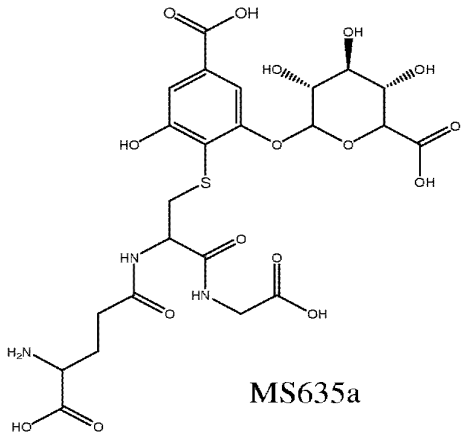
MS263



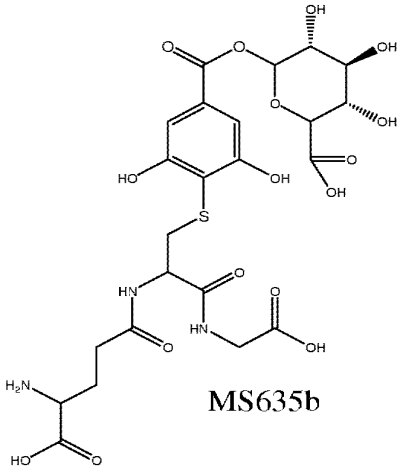
MS276



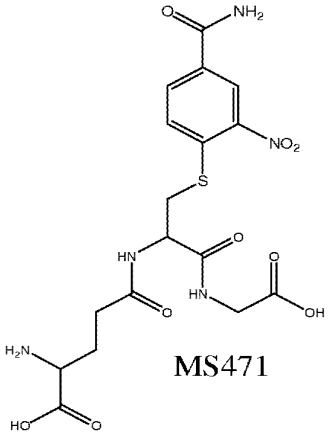
MS278



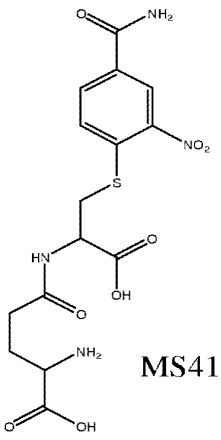
MS635a



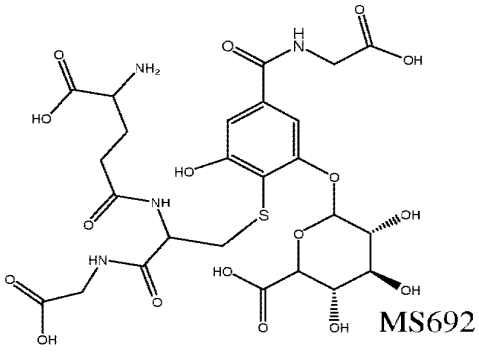
MS635b



MS471



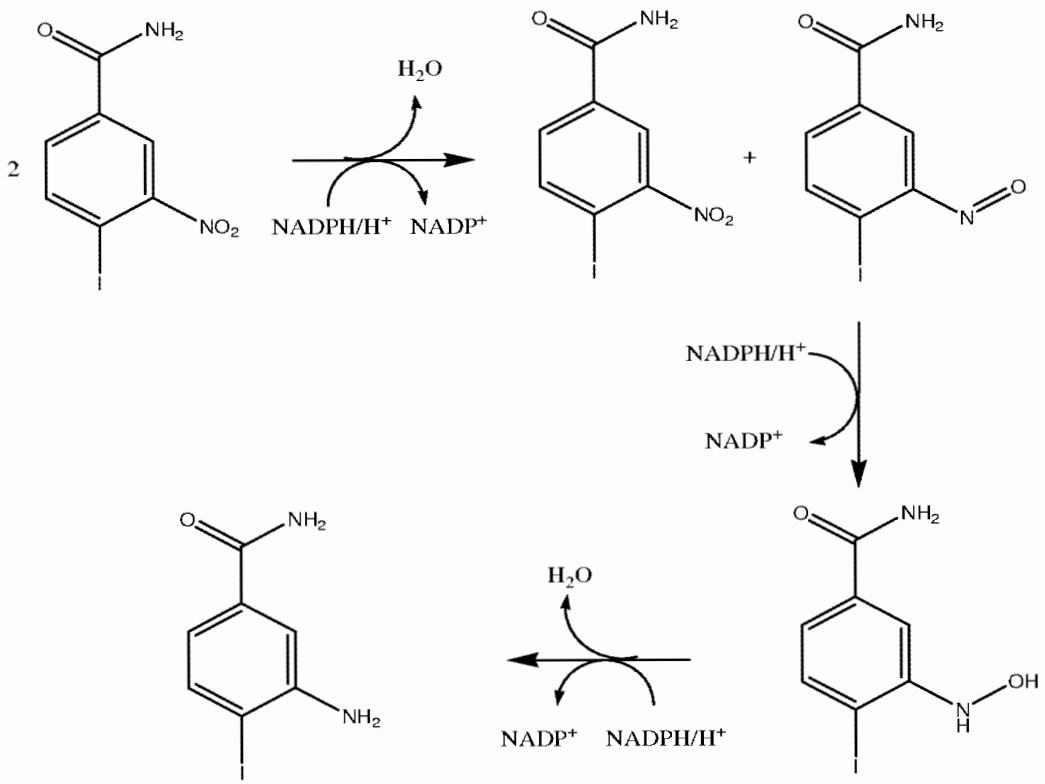
MS414



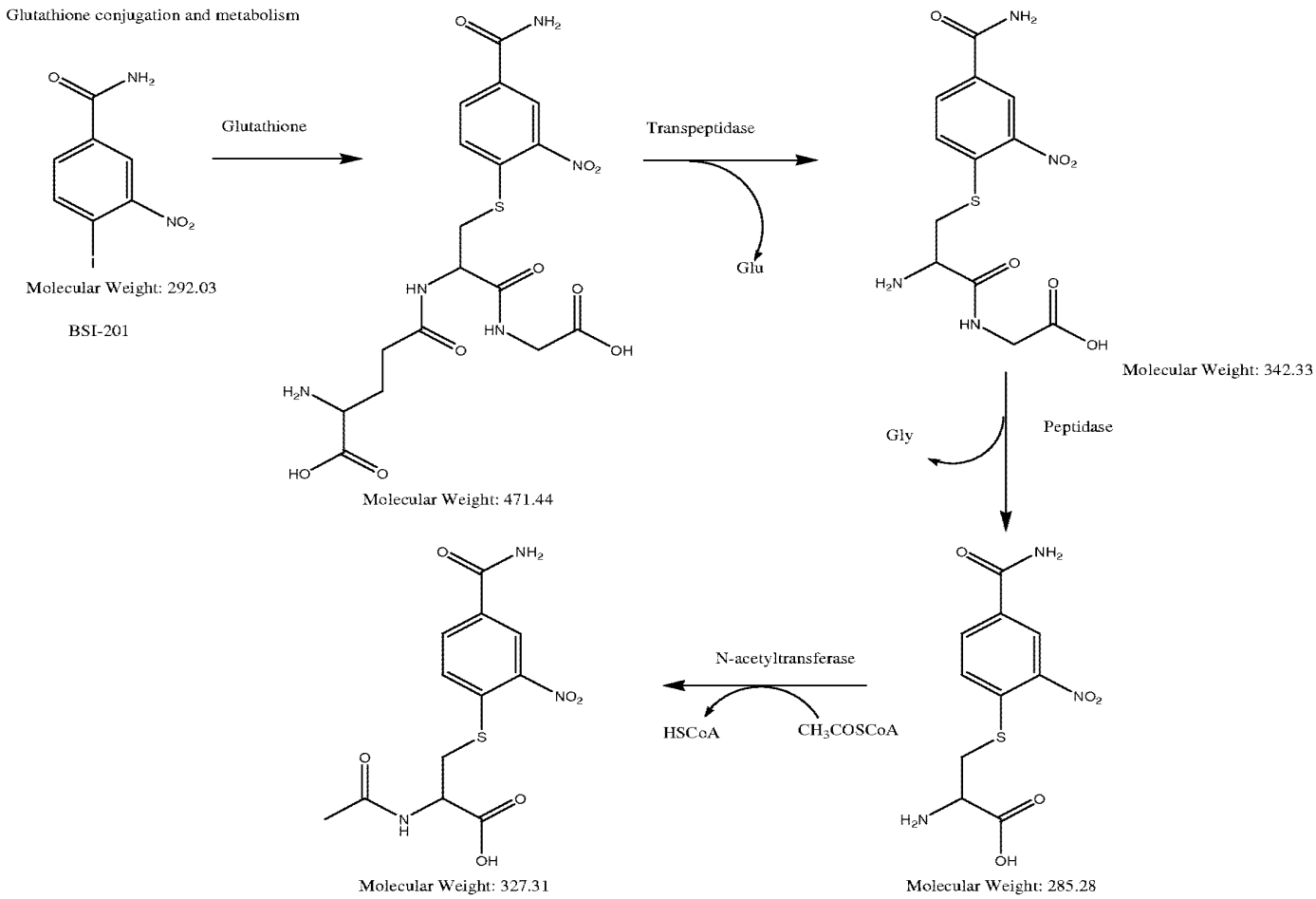
MS692

[0059] While not being limited to any one particular mechanism, the following provides an example for MS292 metabolism via a nitroreductase or glutathione conjugation mechanism:

Nitroreductase mechanism



[0060] 4-iodo-3-nitrobenzamide glutathione conjugation and metabolism:



[0061] Any one of the metabolites of 4-iodo-3-nitrobenzamide described herein may be used in any one of the methods provided herein. Metabolites of 4-iodo-3-nitrobenzamide include, for example, 4-iodo-3-aminobenzoic acid ("IABA"), 4-iodo-3-aminobenzamide ("IABM"), 4-iodo-3-nitrosobenzamide ("BNO"), and 4-iodo-3-hydroxyaminobenzamide ("BNHOH"). Metabolites and methods of making metabolites are disclosed in U.S. Publication No. 2008/0103104 and U.S. Patent No. 5,877,185, which are hereby incorporated by reference in their entirety, and in particular with respect to the metabolites and methods of making metabolites.

[0062] In some embodiments, 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered. In some embodiments, 4-iodo-3-nitrobenzamide or a pharmaceutically acceptable salt thereof is administered. In some embodiments, a metabolite of 4-iodo-3-nitrobenzamide is administered. In some embodiments, the metabolite of 4-iodo-3-nitrobenzamide is 4-iodo-3-aminobenzoic acid or 4-iodo-3-aminobenzamide.

[0063] The dosage range for the metabolites described herein used for treating breast cancer described herein may be in the range of about 0.0004 to about 0.5 mmol/kg (millimoles of

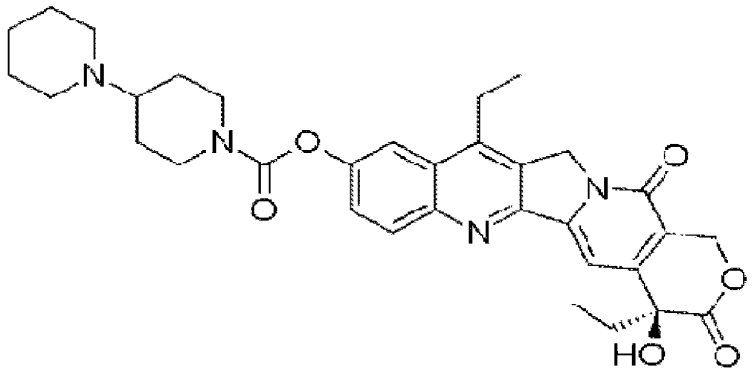
metabolite per kilogram of patient's body weight), which dosage corresponds, on a molar basis, to a range of about 0.1 to about 100 mg/kg of 4-iodo-3-nitrobenzamide. Other effective ranges of dosages for metabolites are 0.0024-0.5 mmol/kg and 0.0048-0.25 mmol/kg. Such doses may be administered on a daily, every-other-daily, twice-weekly, weekly, bi-weekly, monthly or other suitable schedule. Essentially the same modes of administration may be employed for the metabolites as for 4-iodo-3-nitrobenzamide—e.g., oral, i.v., i.p., etc.

[0064] In some embodiments, 4-iodo-3-nitrobenzamide or a pharmaceutically acceptable salt thereof is administered. In some embodiments, a metabolite of 4-iodo-3-nitrobenzamide or a pharmaceutically acceptable salt of a metabolite of 4-iodo-3-nitrobenzamide is administered. The term "pharmaceutically acceptable salt" means those salts which retain the biological effectiveness and properties of the compounds used herein, and which are not biologically or otherwise undesirable. For example, a pharmaceutically acceptable salt does not interfere with the beneficial effect of the compound described herein in treating breast cancer.

[0065] Typical salts are those of the inorganic ions, such as, for example, sodium, potassium, calcium and magnesium ions. Such salts include salts with inorganic or organic acids, such as hydrochloric acid, hydrobromic acid, phosphoric acid, nitric acid, sulfuric acid, methanesulfonic acid, p-toluenesulfonic acid, acetic acid, fumaric acid, succinic acid, lactic acid, mandelic acid, malic acid, citric acid, tartaric acid or maleic acid. In addition, where compounds contain a carboxy group or other acidic group, it may be converted into a pharmaceutically acceptable addition salt with inorganic or organic bases. Examples of suitable bases include sodium hydroxide, potassium hydroxide, ammonia, cyclohexylamine, dicyclohexyl-amine, ethanolamine, diethanolamine and triethanolamine. In some embodiments, 4-iodo-3-nitrobenzamide is formulated in 25% (w/v) hydroxypropyl-β-cyclodextrin and 10 mM phosphate buffer for intravenous administration as described in U.S. Patent Publication No. 2010/0160442, which is incorporated herein by reference.

Irinotecan

[0066] Irinotecan is a topoisomerase 1 inhibitor. Irinotecan ((S)-4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo1*H*-pyrano[3',4':6,7]-indolizino[1,2-b]quinolin-9-yl-[1,4'bipiperidine]-1'-carboxylate), also known as CPT-11, has the following structure:



[0067] Irinotecan is available, for example, from Pfizer under the trade name Camptosar®. In some embodiments, irinotecan or a pharmaceutically acceptable salt thereof is used in combination with 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, for treating breast cancer (*e.g.*, locally advanced or metastatic breast cancer). Methods of making irinotecan are known to one skilled in the art.

[0068] In some embodiments, 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof potentiates the effect of irinotecan. In some embodiments, the amount of irinotecan administered to treat the breast cancer (*e.g.*, metastatic breast cancer) is lowered when irinotecan is administered in combination with 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate, compared to the amount of irinotecan administered to treat the breast cancer (*e.g.*, metastatic breast cancer) when irinotecan is administered not in combination with 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate. In some embodiments, the toxicity is reduced when irinotecan is administered in combination with 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate, compared to the toxicity when irinotecan is administered not in combination with 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate.

[0069] While not wishing to be bound by theory, it is thought that combined treatment of 4-iodo-3-nitrobenzamide with irinotecan described herein may permit efficacious dosing of irinotecan at a lower, and hence less toxic, dose. In some embodiments, the effective dose of irinotecan used with 4-iodo-3-nitrobenzamide may be about 10 to about 90%, about 10 to about 80%, about 10 to about 60%, about 10 to about 50%, less than about 90%, less than about 80%, less than about 60%, less than about 50% or less than about 40% of an effective dose of irinotecan used without administration of 4-iodo-3-nitrobenzamide.

[0070] The dosage of irinotecan used in the present invention may vary depending upon the patient's age, height, weight, overall health, etc. In some embodiments, the dosage of irinotecan is in the range of about 10 mg/m^2 to about 1000 mg/m^2, about 25 mg/m^2 to about 500 mg/m^2,

about 50 mg/m^2 to about 200 mg/m^2, about 75 mg/m^2 to about 200 mg/m^2, about 75 mg/m^2 to about 150 mg/m^2, or about 80 mg/m^2 to about 125 mg/m^2. In some embodiments, the dosage of irinotecan is greater than or at least about any of 25 mg/m^2, 50 mg/m^2, 75 mg/m^2, 80 mg/m^2, 100 mg/m^2, 125 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 250 mg/m^2, or 300 mg/m^2. In some embodiments, the dosage of irinotecan is about any of 50 mg/m^2, 75 mg/m^2, 80 mg/m^2, 100 mg/m^2, 125 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 250 mg/m^2, or 300 mg/m^2. Irinotecan may be administered intravenously, *e.g.* by IV infusion over about 10 to about 500 minutes, about 10 to about 300 minutes, about 30 to about 180 minutes, about 45 to about 120 minutes, about 60 minutes (*i.e.* about 1 hour), or about 90 minutes. In some embodiments, irinotecan may alternatively be administered orally.

Combination Therapy

[0071] In some embodiments, a method provided herein may further comprise another anti-cancer therapy including but not limited to surgery, radiation therapy (e.g., X ray), chemotherapy (such as anti-tumor agent), gene therapy, immunotherapy, DNA therapy, viral therapy, adjuvant therapy, immunotherapy, neoadjuvant therapy, RNA therapy, nanotherapy, or a combination thereof. In some embodiments, the radiation therapy comprises administering to the subject or patient gamma irradiation.

[0072] Provided herein are 4-iodo-3-nitrobenzamide or a pharmaceutically acceptable salt or solvate, or a metabolite thereof described herein and irinotecan or a pharmaceutically acceptable salt thereof, in combination with one or more additional therapy or therapies used in the treatment of the breast cancer (*e.g.*, metastatic breast cancer). The additional therapy may be radiation therapy, surgery (*e.g.*, lumpectomy and a mastectomy), chemotherapy, gene therapy, DNA therapy, viral therapy, RNA therapy, immunotherapy, bone marrow transplantation, nanotherapy, monoclonal antibody therapy, or a combination of the foregoing. The additional therapy may be in the form of adjuvant or neoadjuvant therapy. In some embodiments, the additional therapy is the administration of small molecule enzymatic inhibitor or anti-metastatic agent. In some embodiments, the additional therapy is the administration of side-effect limiting agents (*e.g.*, agents intended to lessen the occurrence and/or severity of side effects of treatment, such as anti-nausea agents, etc.). In some embodiments, the additional therapy is radiation therapy. In some embodiments, the additional therapy is surgery. In some embodiments, the additional therapy is a combination of radiation therapy and surgery. In some embodiments, the additional therapy is gamma irradiation. In some embodiments, the additional therapy is therapy targeting PI3k/mTOR pathway, HSP90 inhibitor, tubulin inhibitor, apoptosis inhibitor, and/or chemopreventative agent.

[0073] Where the combination therapy further comprises a non-drug treatment, the non-drug treatment may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of the therapeutic agents and non-drug treatment is achieved. For example, in appropriate cases, the beneficial effect is still achieved when the non-drug treatment is temporally removed from the administration of the therapeutic agents, by a significant period of time.

[0074] Combination therapy may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of selected additional therapy(ies) is achieved. The additional therapy may be administered before, after, or at the same time as the administration of 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and irinotecan. "At the same time" means that the additional therapy is administered approximately at the same time as the administration of the 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and irinotecan, *e.g.,* within several hours before or after the administration of one or both of 4-iodo-3-nitrobenazmide or irinotecan.

[0075] Combination therapy may be conducted as a sequential administration or a concurrent administration. Sequential administration in this context means that the additional therapy(ies) and the administration of 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and irinotecan are administered with a time separation of more than about 15 minutes, such as more than about any of 20, 30, 40, 50, 60, or more minutes. Either the additional therapy(ies) may be administered first, or the administration of 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and irinotecan may be administered first. The additional therapy(ies), 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof), and irinotecan are contained in separate compositions, which may be contained in the same or different packages or kits. Concurrent administration in this context means that the administration of the additional therapy(ies) and the administration of 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and irinotecan overlap with each other.

[0076] It will be appreciated that when using a combination of 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and irinotecan and an additional chemotherapeutic or other agent, the one ore more of the 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof), irinotecan and the other pharmacologically active agent may be in the same pharmaceutically acceptable carrier and therefore administered simultaneously.

[0077] In some cases, the beneficial effect is achieved when the additional therapy is temporally removed from the administration of the 4-iodo-3-nitrobenazmide (or pharmaceutically

acceptable salt or solvate thereof, or metabolite thereof) and irinotecan, by a significant period of time (*e.g.*, about 12 hours, about 24 hours, about 36 hours, about 48 hours, etc.), or, for example, spaced apart by at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, etc. For example, administration on different days of a treatment cycle, such as the treatment cycles described herein. The interval between administration of the 4-iodo-3-nitrobenzamide, irinotecan and/or additional agents or therapies may vary within a treatment cycle (*e.g.*, administration is not always spaced apart by 1 day, but may be intervals of 1 days followed by an interval of 3 days, etc.). Similarly, at certain times during the treatment cycle, 4-iodo-3-nitrobenzamide, irinotecan and/or additional agents or therapies may be administered at the same time, and at other points during the treatment administered at different times.

Anti-tumor Agents

[0078] In some embodiments, a method provided herein may further comprise at least one anti-tumor agent. For example, a method provided herein comprising administering (a) 4-iodo-3-nitrobenzamide or metabolite thereof or a pharmaceutically acceptable salt thereof, (b) irinotecan or a pharmaceutically acceptable salt thereof may further comprise at least one anti-tumor agent.

[0079] Anti-tumor agents that may be used in the present invention include but are not limited to antitumor alkylating agents, antitumor antimetabolites, antitumor antibiotics, plant-derived antitumor agents, antitumor platinum-complex compounds, antitumor camptothecin derivatives, antitumor tyrosine kinase inhibitors, anti-tumor viral agent, monoclonal antibodies, interferons, biological response modifiers, and other agents that exhibit anti-tumor activities, or a pharmaceutically acceptable salt thereof.

[0080] In some embodiments, the anti-tumor agent is an alkylating agent. The term "alkylating agent" herein generally refers to an agent giving an alkyl group in the alkylation reaction in which a hydrogen atom of an organic compound is substituted with an alkyl group. Examples of anti-tumor alkylating agents include but are not limited to nitrogen mustard N-oxide, cyclophosphamide, ifosfamide, melphalan, busulfan, mitobronitol, carboquone, thiotepa, ranimustine, nimustine, temozolomide or carmustine.

[0081] In some embodiments, the anti-tumor agent is an antimetabolite. The term "antimetabolite" used herein includes, in a broad sense, substances which disturb normal metabolism and substances which inhibit the electron transfer system to prevent the production of energy-rich intermediates, due to their structural or functional similarities to metabolites that are important for living organisms (such as vitamins, coenzymes, amino acids and saccharides). Examples of antimetabolites that have anti-tumor activities include but are not limited to methotrexate, 6-mercaptopurine riboside, mercaptopurine, 5-fluorouracil, tegafur, doxifluridine,

carmofur, cytarabine, cytarabine ocfosfate, enocitabine, S-1, gemcitabine, fludarabine or pemetrexed disodium.

[0082] In some embodiments, the anti-tumor agent is an antitumor antibiotic. Examples of antitumor antibiotics include but are not limited to actinomycin D, doxorubicin, daunorubicin, neocarzinostatin, bleomycin, peplomycin, mitomycin C, aclarubicin, pirarubicin, epirubicin, zinostatin, stimalamer, idarubicin, sirolimus or valrubicin.

[0083] In some embodiments, the anti-tumor agent is a plant-derived antitumor agent. Examples of plant-derived antitumor agents include but are not limited to vincristine, vinblastine, vindesine, etoposide, sobuzoxane, docetaxel, paclitaxel and vinorelbine.

[0084] In some embodiments, the anti-tumor agent is a camptothecin derivative that exhibits anti-tumor activities. Examples of anti-tumor camptothecin derivatives include but are not limited to camptothecin, 10-hydroxycamptothecin, topotecan, irinotecan or 9-aminocamptothecin. Irinotecan may be metabolized in vivo and exhibit antitumor effect as SN-38. The action mechanism and the activity of the camptothecin derivatives are believed to be virtually the same as those of camptothecin (e.g., Nitta et al., Gan to Kagaku Ryoho, 14, 850-857 (1987)).

[0085] In some embodiments, the anti-tumor agent is an organoplatinum compound or a platinum coordination compound having antitumor activity. The terms "organoplatinum compound," "platinum compound," or "platinum complex" and the like as used herein refer to a platinum-containing compound which provides platinum in ion form. Organoplatinum compounds include but are not limited to cisplatin; cis-diamminediaquoplatinum (II)-ion; chloro(diethylenetriamine)-platinum (II) chloride; dichloro(ethylenediamine)-platinum (II); diammine(1,1-cyclobutanedicarboxylato) platinum (II) (carboplatin); spiroplatin; iproplatin; diammine(2-ethylmalonato)platinum (II); ethylenediaminemalonatoplatinum (II); aqua(1,2-diaminodicyclohexane)sulfatoplatinum (II); aqua(1,2-diaminodicyclohexane)malonatoplatinum (II); (1,2-diaminocyclohexane)malonatoplatinum (II); (4-carboxyphthalato)(1,2-diaminocyclohexane) platinum (II); (1,2-diaminocyclohexane)-(isocitrato)platinum (II); (1,2-diaminocyclohexane)oxalatoplatinum (II); ormaplatin; tetraplatin; carboplatin, nedaplatin and oxaliplatin. Further, other antitumor organoplatinum compounds mentioned in the specification are known and are commercially available and/or producible by a person having ordinary skill in the art by conventional techniques.

[0086] In some embodiments, the anti-tumor agent is an antitumor tyrosine kinase inhibitor. The term "tyrosine kinase inhibitor" herein refers to a chemical substance inhibiting "tyrosine kinase" which transfers a λ-phosphate group of ATP to a hydroxyl group of a specific tyrosine in protein. Examples of anti-tumor tyrosine kinase inhibitors include but are not limited to gefitinib, imatinib, erlotinib, Sutent, Nexavar, Recentin, ABT-869, and Axitinib.

[0087] In some embodiments, the anti-tumor agent is an antibody or a binding portion of an antibody that exhibits anti-tumor activity. In some embodiments, the anti-tumor agent is a monoclonal antibody. Examples thereof include but are not limited to abciximab, adalimumab, alemtuzumab, basiliximab, bevacizumab, cetuximab, daclizumab, eculizumab, efalizumab, ibritumomab, tiuxetan, infliximab, muromonab-CD3, natalizumab, omalizumab, palivizumab, panitumumab, ranibizumab, gemtuzumab ozogamicin, rituximab, tositumomab, trastuzumab, or any antibody fragments specific for antigens.

[0088] In some embodiments, the anti-tumor agent is an interferon. Such interferon has antitumor activity, and it is a glycoprotein which is produced and secreted by most animal cells upon viral infection. It has not only the effect of inhibiting viral growth but also various immune effector mechanisms including inhibition of growth of cells (in particular, tumor cells) and enhancement of the natural killer cell activity, thus being designated as one type of cytokine. Examples of anti-tumor interferons include but are not limited to interferon α, interferon α-2a, interferon α-2b, interferon β, interferon γ-1a and interferon γ-n1.

[0089] In some embodiments, the anti-tumor agent is a biological response modifier. It is generally the generic term for substances or drugs for modifying the defense mechanisms of living organisms or biological responses such as survival, growth or differentiation of tissue cells in order to direct them to be useful for an individual against tumor, infection or other diseases. Examples of the biological response modifier include but are not limited to krestin, lentinan, sizofiran, picibanil and ubenimex.

[0090] In some embodiments, the anti-tumor agents include but are not limited to mitoxantrone, L-asparaginase, procarbazine, dacarbazine, hydroxycarbamide, pentostatin, tretinoin, alefacept, darbepoetin alfa, anastrozole, exemestane, bicalutamide, leuprorelin, flutamide, fulvestrant, pegaptanib octasodium, denileukin diftitox, aldesleukin, thyrotropin alfa, arsenic trioxide, bortezomib, capecitabine, and goserelin.

[0091] The above-described terms "antitumor alkylating agent", "antitumor antimetabolite", "antitumor antibiotic", "plant-derived antitumor agent", "antitumor platinum coordination compound", "antitumor camptothecin derivative", "antitumor tyrosine kinase inhibitor", "monoclonal antibody", "interferon", "biological response modifier" and "other antitumor agent" are all known and are either commercially available or producible by a person skilled in the art by methods known per se or by well-known or conventional methods. The process for preparation of gefitinib is described, for example, in U.S. Pat. No. 5,770,599; the process for preparation of cetuximab is described, for example, in WO 96/40210; the process for preparation of bevacizumab is described, for example, in WO 94/10202; the process for preparation of oxaliplatin is described, for example, in U.S. Pat. Nos. 5,420,319 and 5,959,133; the process for

preparation of gemcitabine is described, for example, in U.S. Pat. Nos. 5,434,254 and 5,223,608; and the process for preparation of camptothecin is described in U.S. Pat. Nos. 5,162,532, 5,247,089, 5,191,082, 5,200,524, 5,243,050 and 5,321,140; the process for preparation of irinotecan is described, for example, in U.S. Pat. No. 4,604,463; the process for preparation of topotecan is described, for example, in U.S. Pat. No. 5,734,056; the process for preparation of temozolomide is described, for example, in JP-B No. 4-5029; and the process for preparation of rituximab is described, for example, in JP-W No. 2-503143.

[0092] The above-mentioned antitumor alkylating agents are commercially available, as exemplified by the following: nitrogen mustard N-oxide from Mitsubishi Pharma Corp. as Nitrorin (tradename); cyclophosphamide from Shionogi & Co., Ltd. as Endoxan (tradename); ifosfamide from Shionogi & Co., Ltd. as Ifomide (tradename); melphalan from GlaxoSmithKline Corp. as Alkeran (tradename); busulfan from Takeda Pharmaceutical Co., Ltd. as Mablin (tradename); mitobronitol from Kyorin Pharmaceutical Co., Ltd. as Myebrol (tradename); carboquone from Sankyo Co., Ltd. as Esquinon (tradename); thiotepa from Sumitomo Pharmaceutical Co., Ltd. as Tespamin (tradename); ranimustine from Mitsubishi Pharma Corp. as Cymerin (tradename); nimustine from Sankyo Co., Ltd. as Nidran (tradename); temozolomide from Schering Corp. as Temodar (tradename); and carmustine from Guilford Pharmaceuticals Inc. as Gliadel Wafer (tradename).

[0093] The above-mentioned antitumor antimetabolites are commercially available, as exemplified by the following: methotrexate from Takeda Pharmaceutical Co., Ltd. as Methotrexate (tradename); 6-mercaptopurine riboside from Aventis Corp. as Thioinosine (tradename); mercaptopurine from Takeda Pharmaceutical Co., Ltd. as Leukerin (tradename); 5-fluorouracil from Kyowa Hakko Kogyo Co., Ltd. as 5-FU (tradename); tegafur from Taiho Pharmaceutical Co., Ltd. as Futraful (tradename); doxyfluridine from Nippon Roche Co., Ltd. as Furutulon (tradename); carmofur from Yamanouchi Pharmaceutical Co., Ltd. as Yamafur (tradename); cytarabine from Nippon Shinyaku Co., Ltd. as Cylocide (tradename); cytarabine ocfosfate from Nippon Kayaku Co., Ltd. as Strasid (tradename); enocitabine from Asahi Kasei Corp. as Sanrabin (tradename); S-1 from Taiho Pharmaceutical Co., Ltd. as TS-1 (tradename); gemcitabine from Eli Lilly & Co. as Gemzar (tradename); fludarabine from Nippon Schering Co., Ltd. as Fludara (tradename); and pemetrexed disodium from Eli Lilly & Co. as Alimta (tradename).

[0094] The above-mentioned antitumor antibiotics are commercially available, as exemplified by the following: actinomycin D from Banyu Pharmaceutical Co., Ltd. as Cosmegen (tradename); doxorubicin from Kyowa Hakko Kogyo Co., Ltd. as adriacin (tradename); daunorubicin from Meiji Seika Kaisha Ltd. as Daunomycin; neocarzinostatin from Yamanouchi Pharmaceutical Co.,

Ltd. as Neocarzinostatin (tradename); bleomycin from Nippon Kayaku Co., Ltd. as Bleo (tradename); pepromycin from Nippon Kayaku Co, Ltd. as Pepro (tradename); mitomycin C from Kyowa Hakko Kogyo Co., Ltd. as Mitomycin (tradename); aclarubicin from Yamanouchi Pharmaceutical Co., Ltd. as Aclacinon (tradename); pirarubicin from Nippon Kayaku Co., Ltd. as Pinorubicin (tradename); epirubicin from Pharmacia Corp. as Pharmorubicin (tradename); zinostatin stimalamer from Yamanouchi Pharmaceutical Co., Ltd. as Smancs (tradename); idarubicin from Pharmacia Corp. as Idamycin (tradename); sirolimus from Wyeth Corp. as Rapamune (tradename); and valrubicin from Anthra Pharmaceuticals Inc. as Valstar (tradename).

[0095] The above-mentioned plant-derived antitumor agents are commercially available, as exemplified by the following: vincristine from Shionogi & Co., Ltd. as Oncovin (tradename); vinblastine from Kyorin Pharmaceutical Co., Ltd. as Vinblastine (tradename); vindesine from Shionogi & Co., Ltd. as Fildesin (tradename); etoposide from Nippon Kayaku Co., Ltd. as Lastet (tradename); sobuzoxane from Zenyaku Kogyo Co., Ltd. as Perazolin (tradename); docetaxel from Aventis Corp. as Taxsotere (tradename); paclitaxel from Bristol-Myers Squibb Co. as Taxol (tradename); and vinorelbine from Kyowa Hakko Kogyo Co., Ltd. as Navelbine (tradename).

[0096] The above-mentioned antitumor platinum coordination compounds are commercially available, as exemplified by the following: cisplatin from Nippon Kayaku Co., Ltd. as Randa (tradename); carboplatin from Bristol-Myers Squibb Co. as Paraplatin (tradename); nedaplatin from Shionogi & Co., Ltd. as Aqupla (tradename); and oxaliplatin from Sanofi-Synthelabo Co. as Eloxatin (tradename).

[0097] The above-mentioned antitumor camptothecin derivatives are commercially available, as exemplified by the following: irinotecan from Yakult Honsha Co., Ltd. as Campto (tradename); topotecan from GlaxoSmithKline Corp. as Hycamtin (tradename); and camptothecin from Aldrich Chemical Co., Inc., U.S.A.

[0098] The above-mentioned antitumor tyrosine kinase inhibitors are commercially available, as exemplified by the following: gefitinib from AstraZeneca Corp. as Iressa (tradename); imatinib from Novartis AG as Gleevec (tradename); and erlotinib from OSI Pharmaceuticals Inc. as Tarceva (tradename).

[0099] The above-mentioned monoclonal antibodies are commercially available, as exemplified by the following: cetuximab from Bristol-Myers Squibb Co. as Erbitux (tradename); bevacizumab from Genentech, Inc. as Avastin (tradename); rituximab from Biogen Idec Inc. as Rituxan (tradename); alemtuzumab from Berlex Inc. as Campath (tradename); and trastuzumab from Chugai Pharmaceutical Co., Ltd. as Herceptin (tradename).

[0100] The above-mentioned interferons are commercially available, as exemplified by the following: interferon α from Sumitomo Pharmaceutical Co., Ltd. as Sumiferon (tradename);

interferon α-2a from Takeda Pharmaceutical Co., Ltd. as Canferon-A (tradename); interferon α-2b from Schering-Plough Corp. as Intron A (tradename); interferon β from Mochida Pharmaceutical Co., Ltd. as IFN.beta. (tradename); interferon γ-1a from Shionogi & Co., Ltd. as Imunomax-γ (tradename); and interferon γ-n1 from Otsuka Pharmaceutical Co., Ltd. as Ogamma (tradename).

[0101] The above-mentioned biological response modifiers are commercially available, as exemplified by the following: krestin from Sankyo Co., Ltd. as krestin (tradename); lentinan from Aventis Corp. as Lentinan (tradename); sizofiran from Kaken Seiyaku Co., Ltd. as Sonifiran (tradename); picibanil from Chugai Pharmaceutical Co., Ltd. as Picibanil (tradename); and ubenimex from Nippon Kayaku Co., Ltd. as Bestatin (tradename).

[0102] The above-mentioned other antitumor agents are commercially available, as exemplified by the following: mitoxantrone from Wyeth Lederle Japan, Ltd. as Novantrone (tradename); L-asparaginase from Kyowa Hakko Kogyo Co., Ltd. as Leunase (tradename); procarbazine from Nippon Roche Co., Ltd. as Natulan (tradename); dacarbazine from Kyowa Hakko Kogyo Co., Ltd. as Dacarbazine (tradename); hydroxycarbamide from Bristol-Myers Squibb Co. as Hydrea (tradename); pentostatin from Kagaku Oyobi Kessei Ryoho Kenkyusho as Coforin (tradename); tretinoin from Nippon Roche Co., Ltd. As Vesanoid (tradename); alefacept from Biogen Idec Inc. as Amevive (tradename); darbepoetin alfa from Amgen Inc. as Aranesp (tradename); anastrozole from AstraZeneca Corp. as Arimidex (tradename); exemestane from Pfizer Inc. as Aromasin (tradename); bicalutamide from AstraZeneca Corp. as Casodex (tradename); leuprorelin from Takeda Pharmaceutical Co., Ltd. as Leuplin (tradename); flutamide from Schering-Plough Corp. as Eulexin (tradename); fulvestrant from AstraZeneca Corp. as Faslodex (tradename); pegaptanib octasodium from Gilead Sciences, Inc. as Macugen (tradename); denileukin diftitox from Ligand Pharmaceuticals Inc. as Ontak (tradename); aldesleukin from Chiron Corp. as Proleukin (tradename); thyrotropin alfa from Genzyme Corp. as Thyrogen (tradename); arsenic trioxide from Cell Therapeutics, Inc. as Trisenox (tradename); bortezomib from Millennium Pharmaceuticals, Inc. as Velcade (tradename); capecitabine from Hoffmann-La Roche, Ltd. as Xeloda (tradename); and goserelin from AstraZeneca Corp. as Zoladex (tradename). The term "antitumor agent" as used in the specification includes the above-described antitumor alkylating agent, antitumor antimetabolite, antitumor antibiotic, plant-derived antitumor agent, antitumor platinum coordination compound, antitumor camptothecin derivative, antitumor tyrosine kinase inhibitor, monoclonal antibody, interferon, biological response modifier, and other antitumor agents.

[0103] Other anti-tumor agents or anti-neoplastic agents can also be used. Such suitable anti-tumor agents or anti-neoplastic agents include, but are not limited to, 13-cis-Retinoic Acid, 2-

CdA, 2-Chlorodeoxyadenosine, 5-Azacitidine, 5-Fluorouracil, 5-FU, 6-Mercaptopurine, 6-MP, 6-TG, 6-Thioguanine, Abraxane, Accutane, Actinomycin-D, Adriamycin, Adrucil, Agrylin, Ala-Cort, Aldesleukin, Alemtuzumab, ALIMTA, Alitretinoin, Alkaban-AQ, Alkeran, All-transretinoic Acid, Alpha Interferon, Altretamine, Amethopterin, Amifostine, Aminoglutethimide, Anagrelide, Anandron, Anastrozole, Arabinosylcytosine, Ara-C, Aranesp, Aredia, Arimidex, Aromasin, Arranon, Arsenic Trioxide, Asparaginase, ATRA, Avastin, Azacitidine, BCG, BCNU, Bendamustine, Bevacizumab, Bexarotene, BEXXAR, Bicalutamide, BiCNU, Blenoxane, Bleomycin, Bortezomib, Busulfan, Busulfex, C225, Calcium Leucovorin, Campath, Camptosar, Camptothecin-11, Capecitabine, Carac, Carboplatin, Carmustine, Carmustine Wafer, Casodex, CC-5013, CCI-779, CCNU, CDDP, CeeNU, Cerubidine, Cetuximab, Chlorambucil, Cisplatin, Citrovorum Factor, Cladribine, Cortisone, Cosmegen, CPT-11, Cyclophosphamide, Cytadren, Cytarabine, Cytarabine Liposomal, Cytosar-U, Cytoxan, Dacarbazine, Dacogen, Dactinomycin, Darbepoetin Alfa, Dasatinib, Daunomycin, Daunorubicin, Daunorubicin Hydrochloride, Daunorubicin Liposomal, DaunoXome, Decadron, Decitabine, Delta-Cortef, Deltasone, Denileukin Diftitox, DepoCyt™, Dexamethasone, Dexamethasone Acetate, Dexamethasone Sodium Phosphate, Dexasone, Dexrazoxane, DHAD, DIC, Diodex, Docetaxel, Doxil, Doxorubicin, Doxorubicin Liposomal, Droxia™, DTIC, DTIC-Dome, Duralone, Efudex, Eligard, Ellence, Eloxatin, Elspar, Emcyt, Epirubicin, Epoetin Alfa, Erbitux, Erlotinib, Erwinia L-asparaginase, Estramustine, Ethyol, Etopophos, Etoposide, Etoposide Phosphate, Eulexin, Evista, Exemestane, Fareston, Faslodex, Femara, Filgrastim, Floxuridine, Fludara, Fludarabine, Fluoroplex, Fluorouracil, Fluorouracil (cream), Fluoxymesterone, Flutamide, Folinic Acid, FUDR , Fulvestrant, G-CSF, Gefitinib, Gemcitabine, Gemtuzumab ozogamicin, Gemzar & Gemzar Side Effects - Chemotherapy Drugs, Gleevec, Gliadel Wafer, GM-CSF, Goserelin, Granulocyte - Colony Stimulating Factor, Granulocyte Macrophage Colony Stimulating Factor, Halotestin, Herceptin, Hexadrol, Hexalen, Hexamethylmelamine, HMM, Hycamtin, Hydrea, Hydrocort Acetate, Hydrocortisone, Hydrocortisone Sodium Phosphate, Hydrocortisone Sodium Succinate, Hydrocortone Phosphate, Hydroxyurea, Ibritumomab, Ibritumomab Tiuxetan, Idamycin, Idarubicin, Ifex , IFN-alpha, Ifosfamide, IL-11, IL-2, Imatinib mesylate, Imidazole Carboxamide, Interferon alfa, Interferon Alfa-2b (PEG Conjugate), Interleukin - 2, Interleukin-11, Intron A (interferon alfa-2b), Iressa, Irinotecan, Isotretinoin, Ixabepilone, Ixempra, Kidrolase (t), Lanacort, Lapatinib, L-asparaginase, LCR, Lenalidomide, Letrozole, Leucovorin, Leukeran, Leukine, Leuprolide, Leurocristine, Leustatin, Liposomal Ara-C, Liquid Pred, Lomustine, L-PAM, L-Sarcolysin, Lupron, Lupron Depot, Matulane, Maxidex, Mechlorethamine, Mechlorethamine Hydrochloride, Medralone, Medrol, Megace, Megestrol, Megestrol Acetate, Melphalan, Mercaptopurine, Mesna, Mesnex, Methotrexate, Methotrexate

Sodium, Methylprednisolone, Meticorten, Mitomycin, Mitomycin-C, Mitoxantrone, M-Prednisol, MTC, MTX, Mustargen, Mustine, Mutamycin, Myleran, Mylocel, Mylotarg, Navelbine, Nelarabine, Neosar, Neulasta, Neumega, Neupogen, Nexavar, Nilandron, Nilutamide, Nipent, Nitrogen Mustard, Novaldex, Novantrone, Octreotide, Octreotide acetate, Oncospar, Oncovin, Ontak, Onxal, Oprevelkin, Orapred, Orasone, Oxaliplatin, Paclitaxel, Paclitaxel Protein-bound, Pamidronate, Panitumumab, Panretin, Paraplatin, Pediapred, PEG Interferon, Pegaspargase, Pegfilgrastim, PEG-INTRON, PEG-L-asparaginase, PEMETREXED, Pentostatin, Phenylalanine Mustard, Platinol, Platinol-AQ, Prednisolone, Prednisone, Prelone, Procarbazine, PROCRIT, Proleukin, Prolifeprospan 20 with Carmustine Implant, Purinethol, Raloxifene, Revlimid, Rheumatrex, Rituxan, Rituximab, Roferon-A (Interferon Alfa-2a), Rubex, Rubidomycin hydrochloride, Sandostatin, Sandostatin LAR, Sargramostim, Solu-Cortef, Solu-Medrol, Sorafenib, SPRYCEL, STI-571, Streptozocin, SU11248, Sunitinib, Sutent, Tamoxifen, Tarceva, Targretin, Taxol, Taxotere, Temodar, Temozolomide, Temsirolimus, Teniposide, TESPA, Thalidomide, Thalomid, TheraCys, Thioguanine, Thioguanine Tabloid, Thiophosphoamide, Thioplex, Thiotepa, TICE, Toposar, Topotecan, Toremifene, Torisel, Tositumomab, Trastuzumab, Tretinoin, Trexall™, Trisenox, TSPA, TYKERB, VCR, Vectibix, Vectibix, Velban, Velcade, VePesid, Vesanoid, Viadur, Vidaza, Vinblastine, Vinblastine Sulfate, Vincasar Pfs, Vincristine, Vinorelbine, Vinorelbine tartrate, VLB, VM-26, Vorinostat, VP-16, Vumon, Xeloda, Zanosar, Zevalin, Zinecard, Zoladex, Zoledronic acid, Zolinza, Zometa.

[0104] In some embodiments, the anti-tumor agent is administered prior to, concomitant with or subsequent to administering the effective amount of 4-iodo-3-nitrobenzamide or irinotecan.

[0105] In some embodiments, a method provided herein further comprises surgery, radiation therapy, chemotherapy, gene therapy, DNA therapy, adjuvant therapy, neoadjuvant therapy, viral therapy, RNA therapy, immunotherapy, nanotherapy or a combination thereof.

[0106] Anti-tumor agents and therapies are further described below.

Alkylating agents

[0107] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with any of the alkylating agents described herein.

[0108] Alkylating agents are known to act through the alkylation of macromolecules such as the DNA of cancer cells, and are usually strong electrophiles. This activity can disrupt DNA synthesis and cell division. Examples of alkylating reagents suitable for use herein include

nitrogen mustards and their analogues and derivatives including, cyclophosphamide, ifosfamide, chlorambucil, estramustine, mechlorethamine hydrochloride, melphalan, and uracil mustard. Other examples of alkylating agents include alkyl sulfonates (*e.g.* busulfan), nitrosoureas (*e.g.* carmustine, lomustine, and streptozocin), triazenes (*e.g.* dacarbazine and temozolomide), ethylenimines/methylmelamines (*e.g.* altretamine and thiotepa), and methylhydrazine derivatives (*e.g.* procarbazine). Included in the alkylating agent group are the alkylating-like platinum-containing drugs comprising carboplatin, cisplatin, and oxaliplatin.

Topoisomerase inhibitors

[0109] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with a second topoisomerase inhibitor, for example, topotecan.

[0110] Topoisomerase inhibitors are agents designed to interfere with the action of topoisomerase enzymes (topoisomerase I and II), which are enzymes that control the changes in DNA structure by catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle. Topoisomerases have become popular targets for cancer chemotherapy treatments. It is thought that topoisomerase inhibitors block the ligation step of the cell cycle, generating single and double stranded breaks that harm the integrity of the genome. Introduction of these breaks subsequently lead to apoptosis and cell death. Topoisomerase inhibitors are often divided according to which type of enzyme it inhibits. Topoisomerase I, the type of topoisomerase most often found in eukaryotes, is targeted by topotecan, irinotecan, lurtotecan and exatecan, each of which is commercially available. Topotecan is available from GlaxoSmithKline under the trade name Hycamtim®. Irinotecan is available from Pfizer under the trade name Camptosar®. Lurtotecan may be obtained as a liposomal formulation from Gilead Sciences Inc.

[0111] Compounds that target type II topoisomerase are split into two main classes: topoisomerase poisons, which target the topoisomerase-DNA complex, and topoisomerase inhibitors, which disrupt catalytic turnover. Topo II poisons include but are not limited to eukaryotic type II topoisomerase inhibitors (topo II): amsacrine, etoposide, etoposide phosphate, teniposide and doxorubicin. These drugs are anti-cancer therapies. Examples of topoisomerase inhibitors include ICRF-193. These inhibitors target the N-terminal ATPase domain of topo II and prevent topo II from turning over. The structure of this compound bound to the ATPase domain has been solved by Classen (Proceedings of the National Academy of Science, 2004)

showing that the drug binds in a non-competitive manner and locks down the dimerization of the ATPase domain.

Antimetabolites

[0112] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with any of the antimetabolites described herein. Antimetabolites are drugs that interfere with normal cellular metabolic processes. Since cancer cells are rapidly replicating, interference with cellular metabolism affects cancer cells to a greater extent than host cells.

Platinum complexes

[0113] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with any of the platinum complexes described herein. Platinum complexes are pharmaceutical agents or pharmaceutical compositions used to treat cancer, which contain at least one platinum center complexed with at least one organic group.

Taxanes

[0114] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with any of the taxanes described herein.

[0115] Taxanes are drugs that are derived from the twigs, needles and bark of Pacific yew tress, *Taxus brevifolia.* In particular paclitaxel may be derived from 10-deacetylbaccatin through known synthetic methods. Taxanes such as paclitaxel and its derivative docetaxel have demonstrated antitumor activity in a variety of tumor types. The taxanes interfere with normal function of microtubule growth by hyperstabilizing their structure, thereby destroying the cell's ability to use its cytoskeleton in a normal manner. Specifically, the taxanes bind to the β subunit of tubulin, which is the building block of microtubules. The resulting taxane/tubulin complex cannot disassemble, which results in aberrant cell function and eventual cell death. Paclitaxel induces programmed cell death (apoptosis) in cancer cells by binding to an apoptosis-inhibiting protein called Bcl-2 (B-cell leukemia 2), thereby preventing Bcl-2 from inhibiting apoptosis.

Thus paclitaxel has proven to be an effective treatment for various cancers, as it down-regulates cell division by interrupting normal cytoskeletal rearrangement during cell division and it induces apoptosis via the anti-Bcl-2 mechanism.

Anti-angiogenic agents

[0116] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with any of the anti-angiogenic agents described herein.

[0117] An angiogenesis inhibitor is a substance that inhibits angiogenesis (the growth of new blood vessels). Every solid tumor (in contrast to leukemia) needs to generate blood vessels to keep it alive once it reaches a certain size. Tumors can grow only if they form new blood vessels. Usually, blood vessels are not built elsewhere in an adult body unless tissue repair is actively in process. The angiostatic agent endostatin and related chemicals can suppress the building of blood vessels, preventing the cancer from growing indefinitely. In tests with patients, the tumor became inactive and stayed that way even after the endostatin treatment was finished. The treatment has very few side effects but appears to have very limited selectivity. Other angiostatic agents such as thalidomide and natural plant-based substances are being actively investigated.

[0118] Known inhibitors include the drug bevacizumab (Avastin), which binds vascular endothelial growth factor (VEGF), inhibiting its binding to the receptors that promote angiogenesis. Other anti-angiogenic agents include but are not limited to carboxyamidotriazole, TNF-470, CM101, IFN-alpha, IL-12, platelet factor-4, suramin, SU5416, thrombospondin, angiostatic steroids + heparin, cartilage-derived angiogenesis inhibitory factor, matrix metalloproteinase inhibitors, angiostatin, endostatin, 2-methoxyestradiol, tecogalan, thrombospondin, prolactin, $\alpha_V\beta_3$ inhibitors and linomide.

Her-2 targeted therapy

[0119] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with Herceptin.

[0120] Herceptin (trastuzumab) is a targeted therapy for use in early-stage HER2-positive breast cancers. Herceptin is approved for the adjuvant treatment of HER2-overexpressing, node-positive or node-negative (ER/PR-negative or with one high-risk feature) breast cancer. Herceptin can be used several different ways: as part of a treatment regimen including doxorubicin,

cyclophosphamide, and either paclitaxel or docetaxel; with docetaxel and carboplatin; or as a single agent following multi-modality anthracycline-based therapy. Herceptin in combination with paclitaxel is approved for the first-line treatment of HER2-overexpressing metastatic breast cancer. Herceptin as a single agent is approved for treatment of HER2-overexpressing breast cancer in patients who have received one or more chemotherapy regimens for metastatic disease.

[0121] Lapatinib or lapatinib ditosylate is an orally active chemotherapeutic drug treatment for solid tumours such as breast cancer. During development it was known as small molecule GW572016. Patients who meet specific indication criteria may be prescribed lapatinib as part of combination therapy for breast cancer. Pharmacologically, lapatinib is a dual tyrosine kinase inhibitor that interrupts cancer-causing cellular signals. Lapatinib is used as a treatment for women's breast cancer in patients who have HER2-positive advanced breast cancer that has progressed after previous treatment with other chemotherapeutic agents, such as anthracycline, taxane-derived drugs, or trastuzumab (Herceptin, Genentech).

Hormone therapy

[0122] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with hormone therapy.

[0123] There are certain hormones that can attach to cancer cells and can affect their ability to multiply. The purpose of hormone therapy is to add, block or remove hormones. With breast cancer, the female hormones estrogen and progesterone can promote the growth of some breast cancer cells. So in these patients, hormone therapy is given to block the body's naturally occurring estrogen and fight the cancer's growth. There are two types of hormone therapy for breast cancer: drugs that inhibit estrogen and progesterone from promoting breast cancer cell growth and drugs or surgery to turn off the production of hormones from the ovaries.

[0124] Common hormone therapy drugs used for breast cancer include but are not limited to Tamoxifen, Fareston, Arimidex, Aromasin, Femara, and Zoladex.

Tamoxifen-Hormone antagonist

[0125] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with tamoxifen.

[0126] Tamoxifen (marketed as Nolvadex) decreases the chance that some early-stage breast cancers will recur and can prevent the development of cancer in the unaffected breast. Tamoxifen

also slows or stops the growth of cancer cells present in the body. In addition, tamoxifen may offer an alternative to watchful waiting or prophylactic (preventative) mastectomy to women at high risk for developing breast cancer. Tamoxifen is a type of drug called a selective estrogen-receptor modulator (SERM). At the breast, it functions as an anti-estrogen. Estrogen promotes the growth of breast cancer cells and tamoxifen blocks estrogen from attaching to estrogen receptors on these cells. By doing this, it is believed that the growth of the breast cancer cells will be halted. Tamoxifen is often given along with chemotherapy and other breast cancer treatments. It is considered an option in the following cases: Treatment of ductal carcinoma in situ (DCIS) along with breast-sparing surgery or mastectomy; Adjuvant treatment of lobular carcinoma in situ (LCIS) to reduce the risk of developing more advanced breast cancer; Adjuvant treatment of metastatic breast cancer in men and women whose cancers are estrogen-receptor positive; Treatment of recurrent breast cancer; To prevent breast cancer in women at high risk for developing breast cancer.

Steroidal and non-steroidal aromatase inhibitor

[0127] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with any of the aromatase inhibitors described herein.

[0128] Aromatase inhibitors (AI) are a class of drugs used in the treatment of breast cancer and ovarian cancer in postmenopausal women that block the aromatase enzyme. Aromatase inhibitors lower the amount of estrogen in post-menopausal women who have hormone-receptor-positive breast cancer. With less estrogen in the body, the hormone receptors receive fewer growth signals, and cancer growth can be slowed down or stopped.

[0129] Aromatase inhibitor medications include Arimidex (chemical name: anastrozole), Aromasin (chemical name: exemestane), and Femara (chemical name: letrozole). Each is taken by pill once a day, for up to five years. But for women with advanced (metastatic) disease, the medicine is continued as long as it is working well.

[0130] AIs are categorized into two types: irreversible steroidal inhibitors such as exemestane that form a permanent bond with the aromatase enzyme complex; and non-steroidal inhibitors (such as anastrozole, letrozole) that inhibit the enzyme by reversible competition.

[0131] Fulvestrant, also known as ICI 182,780, and "Faslodex" is a drug treatment of hormone receptor-positive metastatic breast cancer in postmenopausal women with disease progression following anti-estrogen therapy. It is an estrogen receptor antagonist with no agonist effects,

which works both by down-regulating and by degrading the estrogen receptor. It is administered as a once-monthly injection.

Targeted therapy

[0132] Also provided are methods that comprise administering to a patient with breast cancer (*e.g.*, locally advanced or metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) in combination with an inhibitor targeting a growth factor receptor including but not limited to epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF1R).

[0133] EGFR is overexpressed in the cells of certain types of human carcinomas including but not limited to lung and breast cancers. Highly proliferating, invasive breast cancer cells often express abnormally high levels of the EGFR, and this is known to control both cell division and migration. The interest in EGFR is further enhanced by the availability and FDA approval of specific EGFR tyrosine kinase inhibitors, for example, Gefitinib. Inhibition of EGFR is an important anti-cancer treatment. Examples of EGFR inhibitors include but are not limited to cetuximab, which is a chimeric monoclonal antibody given by intravenous injection for treatment of cancers including but not limited to metastatic colorectal cancer and head and neck cancer. Panitumimab is another example of EGFR inhibitor. It is a humanized monoclonal antibody against EGFR. Panitumimab has been shown to be beneficial and better than supportive care when used alone in patients with advanced colon cancer and is approved by the FDA for this use.

[0134] Activation of the type 1 insulin-like growth factor receptor (IGF1R) promotes proliferation and inhibits apoptosis in a variety of cell types. Transgenic mice expressing a constitutively active IGF1R or IGF-1 develop mammary tumors and increased levels of IGF1R have been detected in primary breast cancers (Yanochko et.al. *Breast Cancer Research* 2006). It has also been shown that the insulin-like growth factor 1 receptor (IGF1R) and HER2 display important signaling interactions in breast cancer. Specific inhibitors of one of these receptors may cross-inhibit the activity of the other. Targeting both receptors give the maximal inhibition of their downstream extracellular signal-regulated kinase 1/2 and AKT signaling pathways. Hence, such drug combinations may be clinically useful and may be beneficial even in tumors in which single drugs are inactive, as exemplified by the effect of the HER2/IGF1R inhibitor combination in HER2 nonoverexpressing MCF7 cells (Chakraborty AK, et.al, Cancer Res. 2008 Mar 1;68(5):1538-45). One example of an IGF1R inhibitor is CP-751871. CP-751871 is a human monoclonal antibody that selectively binds to IGF1R, preventing IGF1 from binding to the receptor and subsequent receptor autophosphorylation. Inhibition of IGF1R autophosphorylation may result in a reduction in receptor expression on tumor cells that express IGF1R, a reduction in

the anti-apoptotic effect of IGF, and inhibition of tumor growth. IGF1R is a receptor tyrosine kinase expressed on most tumor cells and is involved in mitogenesis, angiogenesis, and tumor cell survival.

PI3K/mTOR pathway

[0135] Phosphatidylinositol-3-kinase (PI3K) pathway deregulation is a common event in human cancer, either through inactivation of the tumor suppressor phosphatase and tensin homologue deleted from chromosome 10 or activating mutations of p110-α. These hotspot mutations result in oncogenic activity of the enzyme and contribute to therapeutic resistance to the anti-HER2 antibody trastuzumab. The PI3K pathway is, therefore, an attractive target for cancer therapy. NVP-BEZ235, a dual inhibitor of the PI3K and the downstream mammalian target of rapamycin (mTOR) has been shown to inhibit the activation of the downstream effectors Akt, S6 ribosomal protein, and 4EBP1 in breast cancer cells. NVP-BEZ235 inhibits the PI3K/mTOR axis and results in antiproliferative and antitumoral activity in cancer cells with both wild-type and mutated p110-α (Violeta Serra, et.al. *Cancer Research* 68, 8022-8030, October 1, 2008).

Hsp90 inhibitors

[0136] These drugs target heat shock protein 90 (hsp90). Hsp90 is one of a class of chaperone proteins, whose normal job is to help other proteins acquire and maintain the shape required for those proteins to do their jobs. Chaperone proteins work by being in physical contact with other proteins. Hsp90 can also enable cancer cells to survive and even thrive despite genetic defects which would normally cause such cells to die. Thus, blocking the function of HSP90 and related chaperone proteins may cause cancer cells to die, especially if blocking chaperone function is combined with other strategies to block cancer cell survival.

Tubulin inhibitors

[0137] Tubulins are the proteins that form microtubules, which are key components of the cellular cytoskeleton (structural network). Microtubules are necessary for cell division (mitosis), cell structure, transport, signaling and motility. Given their primary role in mitosis, microtubules have been an important target for anticancer drugs — often referred to as antimitotic drugs, tubulin inhibitors and microtubule targeting agents. These compounds bind to tubulin in microtubules and prevent cancer cell proliferation by interfering with the microtubule formation required for cell division. This interference blocks the cell cycle sequence, leading to apoptosis.

Apoptosis inhibitors

[0138] The inhibitors of apoptosis (IAP) are a family of functionally- and structurally-related proteins, originally characterized in Baculovirus, which serve as endogenous inhibitors of

apoptosis. The human IAP family consists of at least 6 members, and IAP homologs have been identified in numerous organisms. 10058-F4 is a c-Myc inhibitor that induces cell-cycle arrest and apoptosis. It is a cell-permeable thiazolidinone that specifically inhibits the c-Myc-Max interaction and prevents transactivation of c-Myc target gene expression. 10058-F4 inhibits tumor cell growth in a c-Myc-dependent manner both *in vitro* and *in vivo*. BI-6C9 is a tBid inhibitor and antiapoptotic. GNF-2 belongs to a new class of Bcr-abl inhibitors. GNF-2 appears to bind to the myristoyl binding pocket, an allosteric site distant from the active site, stabilizing the inactive form of the kinase. It inhibits Bcr-abl phosphorylation with an IC_{50} of 267 nM, but does not inhibit a panel of 63 other kinases, including native c-Abl, and shows complete lack of toxicity towards cells not expressing Bcr-Abl. GNF-2 shows great potential for a new class of inhibitor to study Bcr-abl activity and to treat resistant Chronic myelogenous leukemia (CML), which is caused the Bcr-Abl oncoprotein. Pifithrin-α is a reversible inhibitor of p53-mediated apoptosis and p53-dependent gene transcription such as cyclin G, p21/waf1, and mdm2 expression. Pifithrin-α enhances cell survival after genotoxic stress such as UV irradiation and treatment with cytotoxic compounds including doxorubicin, etopoxide, paclitaxel, and cytosine-β-D-arabinofuranoside. Pifithrin-α protects mice from lethal whole body γ-irradiation without an increase in cancer incidence.

Radiation Therapy

[0139] Radiation therapy (or radiotherapy) is the medical use of ionizing radiation as part of cancer treatment to control malignant cells. Radiotherapy may be used for curative or adjuvant cancer treatment. It is used as palliative treatment (where cure is not possible and the aim is for local disease control or symptomatic relief) or as therapeutic treatment (where the therapy has survival benefit and it can be curative). Radiotherapy is used for the treatment of malignant tumors and may be used as the primary therapy. It is also common to combine radiotherapy with surgery, chemotherapy, hormone therapy or some mixture of the three. Most common cancer types can be treated with radiotherapy in some way. The precise treatment intent (curative, adjuvant, neoadjuvant, therapeutic, or palliative) will depend on the tumour type, location, and stage, as well as the general health of the patient.

[0140] Radiation therapy is commonly applied to the cancerous tumor. The radiation fields may also include the draining of lymph nodes if they are clinically or radiologically involved with tumor, or if there is thought to be a risk of subclinical malignant spread. It is necessary to include a margin of normal tissue around the tumor to allow for uncertainties in daily set-up and internal tumor motion.

[0141] Radiation therapy works by damaging the DNA of cells. The damage is caused by a photon, electron, proton, neutron, or ion beam directly or indirectly ionizing the atoms which

make up the DNA chain. Indirect ionization happens as a result of the ionization of water, forming free radicals, notably hydroxyl radicals, which then damage the DNA. In the most common forms of radiation therapy, most of the radiation effect is through free radicals. Because cells have mechanisms for repairing DNA damage, breaking the DNA on both strands proves to be the most significant technique in modifying cell characteristics. Because cancer cells generally are undifferentiated and stem cell-like, they reproduce more, and have a diminished ability to repair sub-lethal damage compared to most healthy differentiated cells. The DNA damage is inherited through cell division, accumulating damage to the cancer cells, causing them to die or reproduce more slowly. Proton radiotherapy works by sending protons with varying kinetic energy to precisely stop at the tumor.

[0142] Gamma rays are also used to treat some types of cancer including breast cancer. In the procedure called gamma-knife surgery, multiple concentrated beams of gamma rays are directed on the growth in order to kill the cancerous cells. The beams are aimed from different angles to focus the radiation on the growth while minimizing damage to the surrounding tissues.

Gene Therapy Agents

[0143] Gene therapy agents insert copies of genes into a specific set of a patient's cells, and can target both cancer and non-cancer cells. The goal of gene therapy can be to replace altered genes with functional genes, to stimulate a patient's immune response to cancer, to make cancer cells more sensitive to chemotherapy, to place "suicide" genes into cancer cells, or to inhibit angiogenesis. Genes may be delivered to target cells using viruses, liposomes, or other carriers or vectors. This may be done by injecting the gene-carrier composition into the patient directly, or *ex vivo*, with infected cells being introduced back into a patient. Such compositions are suitable for use in the present invention.

Adjuvant therapy

[0144] Adjuvant therapy is a treatment given after the primary treatment to increase the chances of a cure. Adjuvant therapy may include chemotherapy, radiation therapy, hormone therapy, or biological therapy.

[0145] Because the principal purpose of adjuvant therapy is to kill any cancer cells that may have spread, treatment is usually systemic (uses substances that travel through the bloodstream, reaching and affecting cancer cells all over the body). Adjuvant therapy for breast cancer involves chemotherapy or hormone therapy, either alone or in combination.

[0146] Adjuvant chemotherapy is the use of drugs to kill cancer cells. Research has shown that using chemotherapy as adjuvant therapy for early stage breast cancer helps prevent the original

cancer from returning. Adjuvant chemotherapy is usually a combination of anticancer drugs, which has been shown to be more effective than a single anticancer drug.

[0147] Adjuvant hormone therapy deprives cancer cells of the female hormone estrogen, which some breast cancer cells need to grow. Most often, adjuvant hormone therapy is treatment with the drug tamoxifen. Research has shown that when tamoxifen is used as adjuvant therapy for early stage breast cancer, it helps prevent the original cancer from returning and also helps prevent the development of new cancers in the other breast.

[0148] The ovaries are the main source of estrogen prior to menopause. For premenopausal women with breast cancer, adjuvant hormone therapy may involve tamoxifen to deprive the cancer cells of estrogen. Drugs to suppress the production of estrogen by the ovaries are under investigation. Alternatively, surgery may be performed to remove the ovaries.

[0149] Radiation therapy is sometimes used as a local adjuvant treatment. Radiation therapy is considered adjuvant treatment when it is given before or after a mastectomy. Such treatment is intended to destroy breast cancer cells that have spread to nearby parts of the body, such as the chest wall or lymph nodes. Radiation therapy is part of primary therapy, not adjuvant therapy, when it follows breast-sparing surgery.

Neoadjuvant therapy

[0150] Neoadjuvant therapy refers to a treatment given before the primary treatment. Examples of neoadjuvant therapy include chemotherapy, radiation therapy, and hormone therapy. In treating breast cancer, neoadjuvant therapy allows patients with large breast cancer to undergo breast-conserving surgery.

Oncolytic viral therapy

[0151] Viral therapy for cancer utilizes a type of viruses called oncolytic viruses. An oncolytic virus is a virus that is able to infect and lyse cancer cells, while leaving normal cells unharmed, making them potentially useful in cancer therapy. Replication of oncolytic viruses both facilitates tumor cell destruction and also produces dose amplification at the tumor site. They may also act as vectors for anticancer genes, allowing them to be specifically delivered to the tumor site.

[0152] There are two main approaches for generating tumor selectivity: transductional and non-transductional targeting. Transductional targeting involves modifying the specificity of viral coat protein, thus increasing entry into target cells while reducing entry to non-target cells. Non-transductional targeting involves altering the genome of the virus so it can only replicate in cancer cells. This can be done by either transcription targeting, where genes essential for viral replication are placed under the control of a tumor-specific promoter, or by attenuation, which

involves introducing deletions into the viral genome that eliminate functions that are dispensable in cancer cells, but not in normal cells. There are also other, slightly more obscure methods.

[0153] Chen et al (2001) used CV706, a prostate-specific adenovirus, in conjunction with radiotherapy on prostate cancer in mice. The combined treatment results in a synergistic increase in cell death, as well as a significant increase in viral burst size (the number of virus particles released from each cell lysis).

[0154] ONYX-015 has undergone trials in conjunction with chemotherapy. The combined treatment gives a greater response than either treatment alone, but the results have not been entirely conclusive. ONYX-015 has shown promise in conjunction with radiotherapy.

[0155] Viral agents administered intravenously can be particularly effective against metastatic cancers, which are especially difficult to treat conventionally. However, bloodborne viruses can be deactivated by antibodies and cleared from the blood stream quickly *e.g.*, by Kupffer cells (extremely active phagocytic cells in the liver, which are responsible for adenovirus clearance). Avoidance of the immune system until the tumour is destroyed could be the biggest obstacle to the success of oncolytic virus therapy. To date, no technique used to evade the immune system is entirely satisfactory. It is in conjunction with conventional cancer therapies that oncolytic viruses show the most promise, since combined therapies operate synergistically with no apparent negative effects.

[0156] The specificity and flexibility of oncolytic viruses means they have the potential to treat a wide range of cancers including breast cancer with minimal side effects. Oncolytic viruses have the potential to solve the problem of selectively killing cancer cells.

Nanotherapy

[0157] Nanometer-sized particles have novel optical, electronic, and structural properties that are not available from either individual molecules or bulk solids. When linked with tumor-targeting moieties, such as tumor-specific ligands or monoclonal antibodies, these nanoparticles can be used to target cancer-specific receptors, tumor antigens (biomarkers), and tumor vasculatures with high affinity and precision. The formulation and manufacturing process for cancer nanotherapy is disclosed in patent US7179484, and article M. N. Khalid, P. Simard, D. Hoarau, A. Dragomir, J. Leroux, Long Circulating Poly(Ethylene Glycol)Decorated Lipid Nanocapsules Deliver Docetaxel to Solid Tumors, *Pharmaceutical Research,* 23(4), 2006, all of which are herein incorporated by reference in their entireties.

RNA therapy

[0158] RNA including but not limited to siRNA, shRNA, or microRNA may be used to modulate gene expression and treat cancers. Double stranded oligonucleotides are formed by the

assembly of two distinct oligonucleotide sequences where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are generally assembled from two separate oligonucleotides (*e.g.*, siRNA), or from a single molecule that folds on itself to form a double stranded structure (*e.g.*, shRNA or short hairpin RNA). These double stranded oligonucleotides known in the art all have a common feature in that each strand of the duplex has a distinct nucleotide sequence, wherein only one nucleotide sequence region (guide sequence or the antisense sequence) has complementarity to a target nucleic acid sequence and the other strand (sense sequence) comprises a nucleotide sequence that is homologous to the target nucleic acid sequence.

[0159] MicroRNAs (miRNA) are single-stranded RNA molecules of about 21–23 nucleotides in length, which regulate gene expression. miRNAs are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA); instead they are processed from primary transcripts known as pri-miRNA to short stem-loop structures called pre-miRNA and finally to functional miRNA. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression.

[0160] Certain RNA inhibiting agents may be utilized to inhibit the expression or translation of messenger RNA ("mRNA") that is associated with a cancer phenotype. Examples of such agents suitable for use herein include, but are not limited to, short interfering RNA ("siRNA"), ribozymes, and antisense oligonucleotides. Specific examples of RNA inhibiting agents suitable for use herein include, but are not limited to, Cand5, Sirna-027, fomivirsen, and angiozyme.

Small Molecule Enzymatic Inhibitors

[0161] Certain small molecule therapeutic agents are able to target the tyrosine kinase enzymatic activity or downstream signal transduction signals of certain cell receptors such as epidermal growth factor receptor ("EGFR") or vascular endothelial growth factor receptor ("VEGFR"). Such targeting by small molecule therapeutics can result in anti-cancer effects. Examples of such agents suitable for use herein include, but are not limited to, imatinib, gefitinib, erlotinib, lapatinib, canertinib, ZD6474, sorafenib (BAY 43-9006), ERB-569, and their analogues and derivatives.

Anti-Metastatic Agents

[0162] The process whereby cancer cells spread from the site of the original tumor to other locations around the body is termed cancer metastasis. Certain agents have anti-metastatic properties, designed to inhibit the spread of cancer cells. Examples of such agents suitable for use herein include, but are not limited to, marimastat, bevacizumab, trastuzumab, rituximab,

erlotinib, MMI-166, GRN163L, hunter-killer peptides, tissue inhibitors of metalloproteinases (TIMPs), their analogues, derivatives and variants.

Chemopreventative agents

[0163] Certain pharmaceutical agents can be used to prevent initial occurrences of cancer, or to prevent recurrence or metastasis. Such chemopreventative agents in combination with a method provided herein may be used to treat and prevent the recurrence of cancer. Examples of chemopreventative agents suitable for use herein include, but are not limited to, tamoxifen, raloxifene, tibolone, bisphosphonate, ibandronate, estrogen receptor modulators, aromatase inhibitors (letrozole, anastrozole), luteinizing hormone-releasing hormone agonists, goserelin, vitamin A, retinal, retinoic acid, fenretinide, 9-*cis*-retinoid acid, 13-*cis*-retinoid acid, all-*trans*-retinoic acid, isotretinoin, tretinoid, vitamin B6, vitamin B12, vitamin C, vitamin D, vitamin E, cyclooxygenase inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), aspirin, ibuprofen, celecoxib, polyphenols, polyphenol E, green tea extract, folic acid, glucaric acid, interferon-alpha, anethole dithiolethione, zinc, pyridoxine, finasteride, doxazosin, selenium, indole-3-carbinal, alpha-difluoromethylornithine, carotenoids, beta-carotene, lycopene, antioxidants, coenzyme Q10, flavonoids, quercetin, curcumin, catechins, epigallocatechin gallate, N-acetylcysteine, indole-3-carbinol, inositol hexaphosphate, isoflavones, glucanic acid, rosemary, soy, saw palmetto, and calcium. An additional example of chemopreventative agents suitable for use in the present invention is cancer vaccines. These can be created through immunizing a patient with all or part of a cancer cell type that is targeted by the vaccination process.

Methods of treating breast cancer (e.g., locally advanced breast cancer or metastatic breast cancer)

[0164] Provided herein are methods of treating breast cancer (*e.g.*, locally advanced or metastatic breast cancer) in a patient comprising administration of a) 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, and b) irinotecan, or a pharmaceutically acceptable salt or solvate thereof, to said patient. In some embodiments, the breast cancer is locally advanced breast cancer. In some embodiments, the breast cancer is progressing locally advanced breast cancer. In some embodiments, the breast cancer is metastatic breast cancer. In some embodiments, 4-iodo-3-nitrobenzamide or a pharmaceutically acceptable salt thereof (*e.g.*, 4-iodo-3-nitrobenzamide) is administered to the patient. In some embodiments, irinotecan is administered to the patient. In some embodiments, the 4-iodo-3-nitrobenzamide or a pharmaceutically acceptable salt thereof (*e.g.*, 4-iodo-3-nitrobenzamide) is administered at abut 4 mg/kg to about 25 mg/kg (e.g., about 5.6 mg/kg twice weekly, about 8 mg/kg twice weekly, or

11.2 mg/kg once weekly). In some embodiments, the irinotecan is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly.

[0165] In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is hormone receptor-negative ("HR-negative") breast cancer. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is negative for at least one of: estrogen receptor ("ER"), progesterone receptor ("PR") or human epidermal growth factor receptor 2 ("HER2"). In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is negative for at least one of: ER, PR or HER2; and the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is positive for at least one of ER, PR or HER2. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is HR-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is an ER-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is ER-negative and HER2-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is ER-negative and PR-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is ER-negative and both HER2-positive and PR-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is a PR-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is PR-negative and ER-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is PR-negative and HER2-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is PR-negative and both ER-positive and HER2-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is a HER2-negative breast cancer. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is HER2-negative and ER-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is HER2-negative and PR-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is HER2-negative and both ER-positive and PR-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is ER-negative and PR-negative. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is ER-negative, PR-negative and HER-2 positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is ER-negative and HER2-negative. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast

cancer) is ER-negative, HER2-negative and PR-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is PR-negative and HER2-negative. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is PR-negative, HER2-negative and ER-positive. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is ER-negative, PR-negative and HER2-negative.

[0166] In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) does not overexpress HER2. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) overexpresses HER2. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is negative for ER and/or negative for PR. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is positive for ER and/or positive for PR. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is negative for estrogen receptor (ER) expression, negative for progesterone receptor (PR) expression, and does not overexpress HER2. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) expresses estrogen receptor (ER), progesterone receptor (PR), and/or overexpresses HER2.

[0167] In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is deficient in homologous recombination DNA repair. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is BRCA-deficient. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is BRCA1-deficient. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is BRCA2-deficient. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is BRCA1-deficient and BRCA2-deficient. In some embodiments, the patient has breast cancer tissue expressing varying level of BRCA (*e.g.*, BRCA1) compared to normal breast tissue, for example, the patient has reduced level of BRCA (*e.g.*, BRCA1) expression compared to normal breast tissue. In some embodiments, the BRCA (*e.g.*, BRCA1) expression is reduced by at least about 1.5 fold (*e.g.*, at least about any of 2 fold, 3 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, or 10 fold). In some embodiments, the breast cancer comprises at least one mutation in BRCA1 and/or at least one mutation in BRCA2.

[0168] In some embodiments, the breast cancer is metastatic breast cancer. In some embodiments, the breast cancer is locoregional. In some embodiments, the breast cancer is progressing locoregional. In some embodiments, the metastasis is distant metastasis. In some embodiments, the metastasis is systemic metastasis. In some embodiments, the metastasis is brain

metastasis. In some embodiments, the breast cancer is locally advanced breast cancer. In some embodiments, the breast cancer is progressing locally advanced breast cancer.

[0169] In some embodiments, the breast cancer (*e.g.*, locally advanced or metastatic breast cancer) has been previously treated. Prior treatments include, but are not limited to, chemotherapy, radiation, hormonal therapy and/or surgery. For example, prior treatment may include an anthracycline (*e.g.*, daunorubicin (daunomycin), daunorubicin (liposomal), doxorubicin (adriamycin), doxorubicin (liposomal), epirubicin, idarubicin, valrubicin, or mitoxantrone), an anthraquinone (*e.g.*, 9,10-anthraquinone or 9,10-dioxoanthracene, 1,2-, 1,4-, or 2,6-anthraquinone), and/or a taxane (*e.g.*, paclitaxel, docetaxel). For example, in some embodiments, the patient being treated using any one of the methods provided herein has received prior chemotherapy treatment comprising at least one regimen selected from the group consisting of an anthracycline (*e.g.*, daunorubicin (daunomycin), daunorubicin (liposomal), doxorubicin (adriamycin), doxorubicin (liposomal), epirubicin, idarubicin, valrubicin, or mitoxantrone), an anthraquinone (*e.g.*, 9,10-anthraquinone or 9,10-dioxoanthracene, 1,2-, 1,4-, or 2,6-anthraquinone) and a taxane (*e.g.*, paclitaxel, docetaxel). In some embodiments, the breast cancer (*e.g.*, metastatic breast cancer) is refractory to standard treatment or for which no standard therapy is available. In some embodiments, the breast cancer is advanced breast cancer. In some embodiments, the advanced breast cancer is refractory to standard treatment or for which no standard therapy is available. In some embodiments, the patient is refractory to at least one regimen selected from the group consisting of an anthracycline (*e.g.*, daunorubicin (daunomycin), daunorubicin (liposomal), doxorubicin (adriamycin), doxorubicin (liposomal), epirubicin, idarubicin, valrubicin, or mitoxantrone), an anthraquinone (*e.g.*, 9,10-anthraquinone or 9,10-dioxoanthracene, 1,2-, 1,4-, or 2,6-anthraquinone) and a taxane (*e.g.*, paclitaxel, docetaxel). In some embodiments, the patient has received maximum of one adjuvant regimen and two regimens for metastatic disease (whether or not these are based on an anthracycline or a taxane) prior to a treatment described herein. In some embodiments, the breast cancer is metastatic breast cancer. In some embodiments, the breast cancer is locally advanced breast cancer. In some embodiments, the patient has a lesion of at least 2.0 centimeter (e.g., a lesion of bi-dimensionally measuring at least 2.0 centimeter that is, for example, assessed by computed tomography, magnetic resonance imaging, or ultra-sonography).

[0170] Provided herein are methods of treating metastatic triple negative breast cancer (ER-, PR-, HER2-) in a patient, comprising administering to the patient having metastatic triple negative breast cancer an effective amount of 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt thereof, and irinotecan. In some embodiments, the metastasis comprises brain metastases. Also provided herein are methods of treating metastatic triple

negative breast cancer in a patient, where the triple negative breast cancer has metastasized to the brain, comprising administering to the patient having metastatic triple negative breast cancer an effective amount of 4-iodo-3-nitrobenzamide and irinotecan.

[0171] Also provided herein are methods of treating a patient with breast cancer brain metastasis comprising administering to the patient an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof (*e.g.*, 4-iodo-3-nitrobenzamide) and (b) irinotecan or a pharmaceutically acceptable salt thereof (*e.g.*, irinotecan), wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 5.6 mg/kg on days 1, 4, 8, 11 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle. In some embodiments, the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 11.2 mg/kg on days 1 and 8 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle. In some embodiments, the effective amount produces at least one therapeutic effect selected from the group consisting of reduction in size of a breast tumor, reduction in metastasis, complete remission, partial remission, stable disease, increase in overall response rate, or a pathologic complete response. In some embodiments, the brain metastasis is at least about 0.5 centimeter.

[0172] Also provided herein are methods of treating metastatic breast cancer brain metastasis in a patient comprising administering to the patient having the metastatic breast cancer brain metastasis an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan. In some embodiments, the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, the brain metastasis is at least about or larger than about 0.5 centimeter (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter in longest dimension). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new and/or progressive brain metastasis following radiation therapy (e.g., central nervous system ("CNS") radiation therapy or intracranial radiation therapy). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new and/or progressive brain metastasis following radiation therapy (e.g., central nervous system ("CNS") radiation therapy or intracranial radiation therapy) for breast

cancer brain metastases. In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new and/or progressive brain metastasis after prior radiation therapy (e.g., after prior central nervous system radiation therapy or after prior intracranial radiation therapy). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new and/or progressive brain metastasis after prior radiation therapy (e.g., after prior central nervous system radiation therapy or after prior intracranial radiation therapy) for breast cancer brain metastases. In some embodiments, the brain metastasis is new brain metastasis (e.g., new brain metastasis measuring at least about or larger than about 0.5 centimeter) after the radiation therapy. In some embodiments, the brain metastasis is progressive brain metastasis (e.g., progressive brain metastasis measuring at least about or larger than about 0.5 centimeter) after the radiation therapy. In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is asymptomatic and the patient is CNS-radiation therapy naïve patient. In some embodiments, the patient has no prior radiation therapy (e.g., prior intracranial radiation therapy). In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new brain metastasis and the patient has no prior intracranial radiation therapy and/or the intracranial radiation therapy is not emergently indicated for the patient. In some embodiments, the brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) is new brain metastasis (e.g., brain metastasis is found within 2 weeks of initiation of a therapy such as a protocol-based therapy) and the patient is intracranial radiation-naïve patient for whom intracranial radiation therapy is not emergently indicated. In some embodiments, the patient does not have leptomeningeal disease (e.g., the patient does not have diffuse leptomeningeal disease). The effective amount of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and/or irinotecan may be according to any of the dosages described herein. In some embodiments, 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered at about 4 mg/kg to about 25 mg/kg (e.g., about 5.6 mg/kg or about 11.2 mg/kg). In some embodiments, irinotecan is administered at about 50 mg/m^2 to about 200 mg/m^2 (e.g., about 125 mg/m^2). In some embodiments, the treatment comprises at least one treatment cycle of 21-days, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered at about 5.6 mg/kg on days 1, 4, 8, and 11 of the cycle or about 11.2 mg/kg on days 1 and 8 of the treatment cycle, and/or wherein irinotecan is administered at about 125 mg/m^2 on days 1 and 8 of the cycle. In some embodiments, 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable

salt thereof is administered intravenously. In some embodiments, irinotecan is administered intravenously.

[0173] In some embodiments, the breast cancer is carcinoma in situ. In some embodiments, the breast cancer is infiltrating (or invasive) carcinoma. In some embodiments, the breast cancer is lobular carcinoma or ductal carcinoma. In some embodiments, the breast cancer is lobular carcinoma in situ or a ductal carcinoma in situ. In some embodiments, the breast cancer is infiltrating (or invasive) lobular carcinoma or infiltrating (or invasive) ductal carcinoma. In some embodiments, the breast cancer is mammary ductal carcinoma. In some embodiments, the breast cancer is intraductal, invasive, comedo, inflammatory, medullary with lymphocytic infiltrate, mucinous (colloid), papillary, scirrhous, or tubular ductal carcinoma. Other cancers of the breast that can be treated by the methods provided herein are medullary carcinomas, colloid carcinomas, tubular carcinomas, inflammatory breast cancer, nipple carcinoma, and paget disease with intraductal carcinoma or with invasive ductal carcinoma. In some embodiments, the breast cancer described herein is metastatic breast cancer. In some embodiments, the breast cancer described herein is locally advanced breast cancer. In some embodiments, the breast cancer (*e.g.*, locally advanced breast cancer or metastatic breast cancer) is the Luminal B subtype, Luminal A subtype, normal-like subtype, basal-like subtype, claudin-low subtype, or HER2-enriched subtype. In some embodiments, the breast cancer described herein is metastatic breast cancer. In some embodiments, the metastasis comprises brain metastases. In some embodiments, the breast cancer described herein is locally advanced breast cancer.

[0174] In some embodiments, the breast cancer is any of stage 0, stage I, stage II, stage III, or stage IV breast cancer. In some embodiments, the breast cancer is inflammatory breast cancer. In some embodiments, the breast cancer is stage II and/or stage III. In some embodiments, the breast cancer is stage II. In some embodiments, the breast cancer is stage IIIA breast cancer. In some embodiments, the breast cancer is early stage breast cancer, non-metastatic breast cancer, advanced breast cancer, stage IV breast cancer, locally advanced breast cancer, metastatic breast cancer, breast cancer in remission, breast cancer in an adjuvant setting, or breast cancer in a neoadjuvant setting. In some specific embodiments, the breast cancer is in a neoadjuvant setting. In some embodiments, there are provided methods of treating cancer at advanced stage(s). In some embodiments, the patient does not have bilateral breast cancer. In some embodiments, the patient does not have multicentric breast cancer.

[0175] In some embodiments, a method provided herein is used to treat a primary breast tumor. In some embodiments, a method provided herein is used to treat a metastatic breast cancer (that is, cancer that has metastasized from the primary tumor). In some embodiments, the breast cancer is early stage cancer, non-metastatic cancer, primary cancer, advanced cancer, locally advanced

cancer, metastatic cancer, cancer in remission, or recurrent cancer. In some embodiments, the breast cancer has reoccurred after remission. In some embodiments, the breast cancer is progressive cancer. In some embodiments, the breast cancer is localized resectable, localized unresectable, or unresectable. In some embodiments, the breast cancer is locoregional. In some embodiments, the breast cancer is progressing locoregional. In some embodiments, the metastasis is distant metastasis. In some embodiments, the metastasis is systemic metastasis. In some embodiments, the metastasis comprises brain metastasis. In some embodiments, the metastasis is brain metastasis. In some embodiments, the breast cancer is substantially refractory to hormone therapy. In some embodiments, the patient has breast adenocarcinoma (*e.g.*, the breast cancer is breast adenocarcinoma).

[0176] In some embodiments, a method provided herein is used in an adjuvant setting. In some embodiments, a method provided herein is used in a neoadjuvant setting, *i.e.*, the method may be carried out before the primary/definitive therapy such as surgery (*e.g.*, surgery for removing breast cancer tissue from a patient). For example, a method provided herein may be practiced before a surgery for removing breast cancer tissue from the patient. In some embodiments, a method provided herein may be used to treat a patient who has previously been treated. In some embodiments, a method provided herein is used to treat a patient who has not previously been treated. For example, the patient having breast cancer has not received chemotherapy, hormone therapy, surgery, and/or radiation prior to receiving a treatment provided herein. In some embodiments, a method provided herein is used to treat an individual at risk for developing cancer, but has not been diagnosed with cancer. In some embodiments, a method provided herein is used as a first line therapy. In some embodiments, a method provided herein is used as a second line therapy.

[0177] In some embodiments, the patient has not received a prior chemotherapy comprising 4-iodo-3-nitrobenzamide or a metabolite or a pharmaceutically acceptable salt thereof. In some embodiments, the patient has not received a prior chemotherapy comprising a PARP inhibitor (e.g., Olaparib, ABT-888 (Veliparib), AG014699, CEP 9722, MK 4827, KU-0059436 (AZD2281), or LT-673). In some embodiments, the patient has not received a prior chemotherapy comprising gemcitabine. In some embodiments, the patient has not received a prior chemotherapy comprising carboplatin. In some embodiments, the patient has not received a prior chemotherapy comprising cisplatin.

[0178] In some embodiments, a method provided herein is used to treat an individual (*e.g.*, human) who has been diagnosed with or is suspected of having breast cancer. In some embodiments, the individual may be a human who exhibits one or more symptoms associated with breast cancer. In some embodiments, the individual may have advanced disease or a lesser

extent of disease, such as low tumor burden. In some embodiments, the individual is at an early stage of a breast cancer. In some embodiments, the individual is at an advanced stage of breast cancer. In some of the embodiments, the individual may be a human who is genetically or otherwise predisposed (*e.g.*, risk factor) to developing breast cancer who has or has not been diagnosed with breast cancer. In some embodiments, these risk factors include, but are not limited to, age, sex, race, diet, history of previous disease, presence of precursor disease, genetic (*e.g.*, hereditary) considerations, and environmental exposure (*e.g.*, cigarette, pipe, or cigar smoking, exposure to second-hand smoke, radon, arsenic, asbestos, chromates, chloromethyl ethers, nickel, polycyclic aromatic hydrocarbons, radon progeny, other agents, or air pollution).

[0179] In some embodiments of any of the methods described herein, an individual (*e.g.*, human) who has been diagnosed with or is suspected of having breast cancer can be treated. In some embodiments, the individual is human. In some embodiments, the individual is at least about any of 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or 85 years old. In some embodiments, the individual is male. In some embodiments, the individual is a female. In some embodiments, the individual has any of the types of breast cancer described herein. In some embodiments, the individual has a single lesion at presentation. In some embodiments, the individual has multiple lesions at presentation.

[0180] In some embodiments, the effective amount produces at least one therapeutic effect selected from the group consisting of reduction in size of a breast tumor, reduction in metastasis, complete remission, partial remission, stable disease, increase in overall response rate, or a pathologic complete response. In some embodiments, the treatment produces complete response, partial response, or stable disease. The clinical efficacy parameters described herein may be measured according to RECIST version 1.1 criteria, which is described in Eisenhauer EA et al. 2009, Eur J Cancer., 45(2):228-47, the disclosure of which is incorporated by reference in its entirety.

[0181] In some embodiments, there is provided a method of reducing breast tumor size in a patient comprising administering to the patient 4-iodo-3-nitrobenzamide or a metabolite or pharmaceutically acceptable salt thereof, in combination with irinotecan. In some embodiments, the patient has locally advanced breast cancer. In some embodiments, the patient has metastatic breast cancer. The breast cancer may be any of the breast cancers described herein. The dosing regimen may be any of the dosing regimens described herein.

[0182] In some embodiments, there is provided a method of reducing breast cancer metastasis (e.g., breast cancer brain metastasis) in a patient comprising administering to the patient 4-iodo-3-nitrobenzamide or a metabolite or pharmaceutically acceptable salt thereof, in combination with

irinotecan. The breast cancer may be any of the breast cancers described herein. The dosing regimen may be any of the dosing regimens described herein.

[0183] In some embodiments, a treatment described herein reduces breast tumor size by about or at least about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%. In some embodiments, a treatment described herein reduces breast cancer metastasis (e.g., brain metastasis) by about or at least about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%.

[0184] In one aspect, provided herein are methods of treating breast cancer (*e.g.*, metastatic breast cancer) in a patient, comprising administering to the patient having breast cancer (*e.g.*, metastatic breast cancer) an effective amount of 4-iodo-3-nitrobenzamide or a metabolite or pharmaceutically acceptable salt thereof, in combination with irinotecan. In some embodiments, at least one therapeutic effect is obtained, the at least one therapeutic effect being reduction in size of a breast tumor, reduction in metastasis, complete remission, partial remission, pathologic complete response, increase in overall response rate or stable disease. In some embodiments, clinical efficacy of the combination of a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and b) irinotecan may be determined by measuring the clinical benefit rate (CBR). In some emobidiments, the clinical benefit rate is measured by determining the sum of the percentage of patients who are in complete remission (CR), the number of patients who are in partial remission (PR) and the number of patients having stable disease (SD) at a time point at least 6 months out from the end of therapy. The shorthand for this formula is CBR = CR + PR + SD $\geq$ 6 months. The CBR for combination therapy with a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt or solvate thereof, and b) irinotecan may be compared to that of therapy with irinotecan when administered without 4-iodo-3-nitrobenzamide. In some embodiments, the improvement of clinical benefit rate is greater than about any of 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%.

Staging of breast cancer

[0185] Stage 0 may be used to describe non-invasive breast cancers, such as DCIS and LCIS. In stage 0, there is no evidence of cancer cells or non-cancerous abnormal cells breaking out of the part of the breast in which they started, or of getting through to or invading neighboring normal tissue.

[0186] Stage I may describe invasive breast cancer (cancer cells are breaking through to or invading neighboring normal tissue) in which the tumor measures up to 2 centimeters, and no lymph nodes are involved.

[0187] Stage II may be divided into subcategories known as IIA and IIB. Stage IIA may describe invasive breast cancer in which no tumor can be found in the breast, but cancer cells are

found in the axillary lymph nodes (the lymph nodes under the arm), or the tumor measures 2 centimeters or less and has spread to the axillary lymph nodes, or the tumor is larger than 2 centimeters but not larger than 5 centimeters and has not spread to the axillary lymph nodes. Stage IIB may describe invasive breast cancer in which: the tumor is larger than 2 but no larger than 5 centimeters and has spread to the axillary lymph nodes, or the tumor is larger than 5 centimeters but has not spread to the axillary lymph nodes.

[0188] Stage III may be divided into subcategories known as IIIA, IIIB, and IIIC. Stage IIIA may describe invasive breast cancer in which either (1) no tumor is found in the breast; cancer is found in axillary lymph nodes that are clumped together or sticking to other structures, or cancer may have spread to lymph nodes near the breastbone, or (2) the tumor is 5 centimeters or smaller and has spread to axillary lymph nodes that are clumped together or sticking to other structures, or (3) the tumor is larger than 5 centimeters and has spread to axillary lymph nodes that are clumped together or sticking to other structures. Stage IIIB may describe invasive breast cancer in which (1) the tumor may be any size and has spread to the chest wall and/or skin of the breast and (2) may have spread to axillary lymph nodes that are clumped together or sticking to other structures, or cancer may have spread to lymph nodes near the breastbone. Inflammatory breast cancer may be considered at least stage IIIB. Stage IIIC may describe invasive breast cancer in which (1) there may be no sign of cancer in the breast or, if there is a tumor, it may be any size and may have spread to the chest wall and/or the skin of the breast, and (2) the cancer has spread to lymph nodes above or below the collarbone, and (3) the cancer may have spread to axillary lymph nodes or to lymph nodes near the breastbone.

[0189] Stage IV may describe invasive breast cancer that has spread beyond the breast and nearby lymph nodes to other organs of the body, such as the lungs, distant lymph nodes, skin, bones, liver, or brain.

Dosing Regimen, Routes of Administration, and Formulations

[0190] In the methods of treating breast cancer provided herein, any one of the dosage or dosing schedule described herein may be used.

[0191] The dosage of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof may vary depending upon the patient's age, height, weight, overall health, etc. In some embodiments, the dosage of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is in the range of any one of about 0.1 mg/kg to about 50 mg/kg, about 1 mg/kg to about 50 mg/kg, about 1 mg/kg to about 100 mg/kg, about 1 mg/kg to about 25 mg/kg, about 1 mg/kg to about 15 mg/kg, about 1 mg/kg to about 6 mg/kg, about 2 to about 70 mg/kg, about 2 mg/kg to about 50 mg/kg, about 2 mg/kg to about 40 mg/kg, about 3

mg/kg to about 30 mg/kg, about 4 mg/kg to about 20 mg/kg, about 4 to about 15 mg/kg, about 4 to about 100 mg, about 4 to about 25 mg/kg, about 5 to about 15 mg/kg, about 5 to about 10 mg/kg, about 50 to about 100 mg/kg or about 25 to about 75 mg/kg. In some embodiments, the dosage of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is greater than or at least about any of 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 8.5 mg/kg, 9 mg/kg, 9.5 mg/kg, 10 mg/kg, 12 mg/kg, or 15 mg/kg. In some embodiments, the dosage of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is about any of 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 5.5 mg/kg, 5.6 mg/kg, 6 mg/kg, 6.5 mg/kg, 7 mg/kg, 7.5 mg/kg, 8 mg/kg, 8.5 mg/kg, 9 mg/kg, 9.5 mg/kg, 10 mg/kg, 10.5 mg/kg, 11 mg/kg, 11.2 mg/kg, 11.5 mg/kg, 12 mg/kg, 15 mg/kg, 20 mg/kg, 30 mg/kg, 50 mg/kg, 75 mg/kg, or 100 mg/kg. 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof may be administered intravenously, *e.g.,* by IV infusion over about 10 to about 300 minutes, about 30 to about 180 minutes, about 45 to about 120 minutes or about 60 minutes (*i.e.* about 1 hour). In some embodiments, 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof may alternatively be administered orally. In this context, the term "about" has its normal meaning of approximately. In some embodiments, about means ±10% or ±5%.

[0192] In some embodiments, 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered at about any of 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the MTD ("maximum tolerated dose"). In some embodiments, the MTD of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is in the range of about 1 mg/kg to about 100 mg/kg, about 2 mg/kg to about 50 mg/kg, about 1 to about 25 mg/kg, about 2 to about 70 mg/kg, about 4 to about 100 mg, about 4 to about 25 mg/kg, about 4 to about 20 mg/kg, about 5 to about 15 mg/kg, about 5 to about 10 mg/kg, about 50 to about 100 mg/kg or about 25 to about 75 mg/kg. In some embodiments, the MTD of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is greater than or at least about any of 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 8.5 mg/kg, 9 mg/kg, 9.5 mg/kg, 10 mg/kg, 12 mg/kg, or 15 mg/kg. In some embodiments, the MTD of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is about any of 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 5.5 mg/kg, 5.6 mg/kg, 6 mg/kg, 6.5 mg/kg, 7 mg/kg, 7.5 mg/kg, 8 mg/kg, 8.5 mg/kg, 9 mg/kg, 9.5 mg/kg, 10 mg/kg, 10.5 mg/kg, 11 mg/kg, 11.2 mg/kg, 11.5 mg/kg, 12 mg/kg, 15 mg/kg, 20 mg/kg, 30 mg/kg, 50 mg/kg, 75 mg/kg, or 100 mg/kg. MTD for 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof may determined by methods known to one skilled in the art.

[0193] The dosage of irinotecan may vary depending upon the patient's age, height, weight, overall health, etc. In some embodiments, the dosage of irinotecan is in the range of about 10 mg/m^2 to about 1000 mg/m^2, about 25 mg/m^2 to about 500 mg/m^2, about 50 mg/m^2 to about 200 mg/m^2, about 75 mg/m^2 to about 200 mg/m^2, about 75 mg/m^2 to about 150 mg/m^2, or about 80 mg/m^2 to about 125 mg/m^2. In some embodiments, the dosage of irinotecan is greater than or at least about any of 25 mg/m^2, 50 mg/m^2, 75 mg/m^2, 80 mg/m^2, 100 mg/m^2, 125 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 250 mg/m^2, or 300 mg/m^2. In some embodiments, the dosage of irinotecan is about any of 50 mg/m^2, 75 mg/m^2, 80 mg/m^2, 100 mg/m^2, 125 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 250 mg/m^2, or 300 mg/m^2. Irinotecan may be administered intravenously, *e.g.* by IV infusion over about 10 to about 500 minutes, about 10 to about 300 minutes, about 30 to about 180 minutes, about 45 to about 120 minutes, about 60 minutes (*i.e.* about 1 hour), or about 90 minutes. In some embodiments, irinotecan may alternatively be administered orally.

[0194] In some embodiments, the MTD of irinotecan is in the range of about 10 mg/m^2 to about 1000 mg/m^2, about 25mg/m^2 to about 500 mg/m^2, about 50 mg/m^2 to about 200 mg/m^2, about 75 mg/m^2 to about 200 mg/m^2. In some embodiments, the MTD of irinotecan is greater than about any of 25 mg/m^2, 50 mg/m^2, 75 mg/m^2, 100 mg/m^2, 125 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 250 mg/m^2, or 300 mg/m^2. In some embodiments, the MTD of irinotecan is about any of 50 mg/m^2, 75 mg/m^2, 80 mg/m^2, 100 mg/m^2, 125 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 250 mg/m^2, or 300 mg/m^2. MTD for irinotecan or a pharmaceutically acceptable salt thereof may determined by methods known to one skilled in the art.

[0195] In some embodiments, the treatment includes 1 cycle, 2 cycles, 3 cycles, 4 cycles, 5 cycles, 6 cycles, 7 cycles, 8 cycles, 9 cycles, 10 cycles, 11 cycles, 12 cycles, 13 cycles, 14 cycles, or 15 cycles. Cycle means treatment cycle here. In some embodiments, the treatment includes at most any of 2 cycles, 3 cycles, 4 cycles, 5 cycles, 6 cycles, 7 cycles, 8 cycles, 9 cycles, 10 cycles, 11 cycles, 12 cycles, 13 cycles, 14 cycles, or 15 cycles. In some embodiments, the treatment includes at least any of 2 cycles, 3 cycles, 4 cycles, 5 cycles, 6 cycles, 7 cycles, 8 cycles, 9 cycles, or 10 cycles. In some embodiments, the treatment comprises a treatment cycle of at least about any of 1 week, 10 days, 11 days, 2 weeks, 3 weeks, 4 weeks, 30 days, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 12 weeks, or 15 weeks. A treatment cycle may be a period of about any of 1 week, 10 days, 11 days, 2 weeks, 3 weeks, 4 weeks, 30 days, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, or 10 weeks. In some embodiments, a treatment cycle is about 11 to about 30 days in length. In some embodiments, the treatment schedule comprises a resting period, wherein neither 4-iodo-3-nitrobenzamide nor irinotecan is administered to the patient. In some

embodiments, the resting period is 3 days, 4 days, 5 days, 6 days, 1 week, 10 days, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 12 weeks, or 15 weeks.

[0196] Administration of (i) 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt thereof; (ii) irinotecan may be on different days of a treatment cycle, such as the treatment cycles described herein. The interval between administration of 4-iodo-3-nitrobenzamide and irinotecan vary within a treatment cycle (*e.g.,* administration is not always spaced apart by 7 day, but may be at intervals of 1 day followed by an interval of 9 days, etc.). Similarly, at certain times during the treatment cycle, 4-iodo-3-nitrobenzamide and irinotecan may be administered at the same time, and at other points during the treatment administered at different times.

[0197] 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt thereof may be administered every day of the treatment cycle, or administered on certain days but not on every day of the treatment cycle. In some embodiments, 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt thereof is administered daily, once a week, twice a week, three times a week, four times a week, five times a week, six times a week, once 10 days, once two weeks, twice every three weeks, four times every three weeks, once three weeks, once four weeks, once six weeks, or once eight weeks. 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt thereof may be administered on the selected days of each treatment cycle, for example, 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt thereof is administered daily for the period of 2 (or 3, 4, 5, 6, 7, 8, 9, 10, or 11) days of the treatment cycle, and 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt thereof is not administered on other days of the treatment cycle. In some embodiments, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered to said patient. In some embodiments, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 1 mg/kg to about 25 mg/kg. In some embodiments, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 4 mg/kg to about 20 mg/kg. 4-iodo-3-nitrobenzamide (or a metabolite thereof, or a pharmaceutically acceptable salt thereof) may be administered (e.g., at about 5.6 mg/kg) on 4 days of a treatment cycle, e.g., on days 1, 4, 8, 11 of a 21-day treatment cycle. 4-iodo-3-nitrobenzamide (or a metabolite thereof, or a pharmaceutically acceptable salt thereof) may be administered (e.g., at about 11.2 mg/kg) on 2 days of a treatment cycle, e.g., on days 1 and 8 of a 21-day treatment cycle. In some embodiments, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about any of 5.6 mg/kg, 8 mg/kg, and 11.2 mg/kg.

[0198] In some embodiments, the treatment comprises a treatment cycle of at least 11 days, wherein on days 1, 4, 8 and 11 of the cycle, the patient receives about 10 to about 100 mg/kg of 4-iodo-3-nitrobenzamide or a molar equivalent of a metabolite or pharmaceutically acceptable salt thereof. In some embodiments, the treatment comprises a treatment cycle of at least 11 days, wherein on days 4, 8 and 11 of the cycle, the patient receives about 1 to about 50 mg/kg of 4-iodo-3-nitrobenzamide or a molar equivalent of a metabolite or pharmaceutically acceptable salt thereof. In some embodiments, the treatment comprises a treatment cycle of at least 11 days, wherein on days 1, 4, 8 and 11 of the cycle, the patient receives about 1, 2, 3, 4, 5, 5.6, 6, 7, 8, 9, 10, 11, 11.2, 12, 13, 14, 15, 16, 18, or 20 mg/kg of 4-iodo-3-nitrobenzamide, a metabolite or pharmaceutically acceptable salt thereof.

[0199] Irinotecan may be administered daily, *e.g.,* every day of the treatment cycle, or administered on certain days but not on every day of the treatment cycle. In some embodiments, irinotecan is administered daily, once a week, twice a week, twice every 3 weeks, three times a week, four times a week, five times a week, six times a week, once every 10 days, once every two weeks, once every three weeks, once every four weeks, once every six weeks, or once every eight weeks. Irinotecan may be administered on the selected days of each treatment cycle, for example, irinotecan is administered daily on 2 (or 3, 4, 5, 6, 7, 8, 9, 10) days of the treatment cycle, and irinotecan is not administered on other days of the treatment cycle. Irinotecan may be administered (e.g., at about 1000 mg/m^2) on 2 days of a treatment cycle, e.g., on days 1 and 8 of a 21-day treatment cycle.

[0200] In some embodiments, there is provided a method of treating breast cancer (e.g., metastatic triple negative breast cancer) in a patient, comprising administering to the patient an effective amount of: 4-iodo-3-nitrobenzamide or a metabolite or pharmaceutically acceptable salt thereof and irinotecan or a pharmaceutically acceptable salt thereof. In some embodiments, 4-iodo-3-nitrobenzamide or a metabolite or pharmaceutically acceptable salt thereof is administered at a dose of 1.0-6.0 mg/kg twice weekly (e.g., 5.6 mg/kg on days 1, 4, 8, and 11 of a 21-day treatment cycle). In some embodiments, the effective amount of 4-iodo-3-nitrobenzamide or a metabolite or pharmaceutically acceptable salt thereof is administered at a dose of 1.0-15.0 mg/kg once weekly (e.g., 11.2 mg/kg on days 1 and 8 of a 21-day treatment cycle). In some embodiments, irinotecan is administered at a dose of 50-200 mg/m^2 once weekly (e.g., 125 mg/m^2 on days 1 and 8 of a 21-day treatment cycle). In some embodiments, the method comprises at least one cycle, wherein the cycle includes at least 11 days, wherein on days 1 and 8, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 11.2 mg/kg, and on days 1 and 8, irinotecan is administered at about 80 mg/m^2 to about 125 mg/m^2. In some embodiments, the method comprises a 21-day treatment cycle, wherein 4-iodo-3-

nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 5.6 mg/kg on days 1, 4, 8, 11 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

[0201] Provided herein are methods of treating locally advanced or metastatic breast cancer in a patient, comprising administering to the patient having locally advanced or metastatic breast cancer an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof (*e.g.*, 4-iodo-3-nitrobenzamide), and (b) irinotecan or a pharmaceutically acceptable salt thereof (*e.g.*, irinotecan), wherein the method comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 (*e.g.*, about 125 mg/m^2) once weekly for two weeks of the cycle.

[0202] In some embodiments, the method comprises at least one cycle, wherein each cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan is administered at about 80 mg/m^2 to about 125 mg/m^2 twice the cycle (*e.g.*, once weekly for two weeks of the cycle). In some embodiments, the method comprises at least one cycle, wherein each cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle (*e.g.*, on days 1, 4, 8 and 11), and wherein irinotecan or a pharmaceutically acceptable salt thereof (*e.g.*, irinotecan) is administered at about 80 mg/m^2 to about 125 mg/m^2 weekly for two weeks of the cycle (*e.g.*, on days 1 and 8). In some embodiments, the method comprises at least one cycle, wherein each cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for the first two weeks of the cycle (*e.g.*, on days 1, 4, 8 and 11), wherein irinotecan is administered at about 80 mg/m^2 to about 125 mg/m^2 weekly for the first two weeks of the cycle (*e.g.*, on days 1 and 8), wherein neither 4-iodo-3-nitrobenzamide (or the pharmaceutically acceptable salt thereof) nor irinotecan is administered during the third week of the cycle. For example, in some embodiments, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg on days 1, 4, 8, and 11 of the 21-day cycle, and irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the 21-day cycle.

[0203] In some embodiments, the method comprises at least one cycle, wherein the cycle includes at least 11 days, wherein on days 1 and 8, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 11.2 mg/kg, and on days 1 and 8, irinotecan is administered at about 80 mg/m^2 to about 125 mg/m^2. In some embodiments, the method comprises at least one cycle, wherein the cycle includes at least 11 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 11.2 mg/kg once weekly for two weeks of the cycle, and wherein irinotecan is administered at about 125 mg/m^2 once weekly for two weeks of the cycle. In some embodiments, the method comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 11.2 mg/kg once weekly for two weeks of the cycle, and wherein irinotecan is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle. In some embodiments, the method comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 11.2 mg/kg once weekly for two weeks of the cycle, and wherein irinotecan is administered at about 125 mg/m^2 once weekly for two weeks of the cycle. In some embodiments, the method comprises at least one cycle, wherein the cycle is a period of 21 days, wherein on days 1 and 8, 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 11.2 mg/kg and irinotecan is administered at about 125 mg/m^2.

[0204] 4-iodo-3-nitrobenzamide (or a metabolite thereof) and irinotecan may be co-administered to a patient. Co-administration is meant to include simultaneous or sequential administration of the compounds individually or in combination (more than one compound), such as described herein.

[0205] Simultaneous administration in this context means that a first compound and second compound are administered with a time separation of no more than about 15 minutes, such as no more than about any of 10, 5, or 1 minutes. When the first compound and second compound are administered simultaneously, the first compound and second compound may be contained in the same composition (*e.g.*, a composition comprising both first compound and second compound) or in separate compositions (*e.g.*, a first compound in one composition and a second compound is contained in another composition). The first compound described herein may be 4-iodo-3-nitrobenzamide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and the second compound may be irinotecan. Or, the first compound may be irinotecan and the second compound may be 4-iodo-3-nitrobenzamide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof).

[0206] Sequential administration described herein means that a first compound and second compound are administered with a time separation of more than about 15 minutes, such as more than about any of 20, 30, 40, 50, 60, or more minutes. Either the first compound or the second compound may be administered first. The first compound and second compound are contained in separate compositions, which may be contained in the same or different packages or kits. The first compound described herein may be 4-iodo-3-nitrobenzamide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and the second compound may be irinotecan. Or, the first compound may be irinotecan and the second compound may be 4-iodo-3-nitrobenzamide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof).

[0207] 4-iodo-3-nitrobenzamide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and/or irinotecan may be continuously or not continuously given to a patient. "Not continuously" means that the compound or composition provided herein is not administered to the patient over a period of time, *e.g.,* there is a resting period when the patient does not receive the compound or composition. It may be that one compound is administered continuously administered to a patient while the second compound is not administered continuously administered to the patient.

[0208] In some cases, a beneficial effect is achieved when the administration of irinotecan is temporally removed from the administration of the 4-iodo-3-nitrobenazmide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) by a significant period of time (*e.g.,* about 12 hours, about 24 hours, about 36 hours, about 48 hours, etc.). Or, for example, administration is spaced apart by at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, etc.). For example, administration on different days of a treatment cycle, such as the treatment cycles described herein. The interval between administration of the 4-iodo-3-nitrobenzamide and irinotecan may vary within a treatment cycle (*e.g.,* administration is not always spaced apart by 1 day, but may be intervals of 1 day followed by an interval of 3 days, etc.). Similarly, at certain times during the treatment cycle, the 4-iodo-3-nitrobenzamide and irinotecan may be administered at the same time, and at other points during the treatment administered at different times.

[0209] 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) may be formulated in separate formulations or in the same formulation. 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof) may be administered through different administration route or using same administration routes. In some embodiments, there are provided formulations (*e.g.,* pharmaceutical formulations) comprising 4-iodo-3-nitrobenzamide (or a metabolite thereof, or a

pharmaceutically acceptable salt thereof) and irinotecan (or a pharmaceutically acceptable salt thereof), and a carrier, such as a pharmaceutically acceptable carrier. The formulations may include optical isomers, diastereomers, carriers of the compounds disclosed herein. In some embodiments, the carrier is a cyclodextrin, or a derivative thereof, *e.g.,* hydroxypropyl-ß-cyclodextrin (HPBCD). In some embodiments the formulations are formulated for intravenous administration.

[0210] A formulation may comprise both the 4-iodo-3-nitrobenzamide compound and acid forms in particular proportions, depending on the relative potencies of each and the intended indication. The two forms may be formulated together or in different formulations. They may be in the same dosage unit e.g. in one cream, suppository, tablet, capsule, or packet of powder to be dissolved in a beverage; or each form may be formulated in a separate unit, *e.g.,* two creams, two suppositories, two tablets, two capsules, a tablet and a liquid for dissolving the tablet, a packet of powder and a liquid for dissolving the powder, etc.

[0211] The pharmaceutical compositions of the present invention may be provided as a prodrug and/or may be allowed to interconvert to 4-iodo-3-nitrobenzamide form *in vivo* after administration. That is, either 4-iodo-3-nitrobenzamide or metabolites thereof or pharmaceutically acceptable salts may be used in developing a formulation for use in the present invention.

[0212] Also provided herein are synergistic compositions used for treating breast cancer (*e.g.*, metastatic breast cancer) in a patient comprising a) 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, b) irinotecan (or a pharmaceutically acceptable salt thereof).

[0213] The pharmaceutical compositions of the 4-iodo-3-nitrobenzamide (or pharmaceutically acceptable salt or solvate thereof, or metabolite thereof) and irinotecan can be combined with other active ingredients, such as other chemotherapeutic agents as described herein. The two compounds and/or forms of a compound may be formulated together, in the same dosage unit *e.g.,* in one cream, suppository, tablet, capsule, or packet of powder to be dissolved in a beverage; or each form may be formulated in separate units, *e.g.,* two creams, two suppositories, two tablets, two capsules, a tablet and a liquid for dissolving the tablet, a packet of powder and a liquid for dissolving the powder, etc.

[0214] In some embodiments, the composition is administered in unit dosage form. In some embodiments, the unit dosage form is adapted for oral or parenteral administration. In some embodiments, upon administration of the composition, at least one therapeutic effect is obtained, said at least one therapeutic effect being reduction in size of a tumor, reduction in metastasis, complete remission, partial remission, pathologic complete response, or stable disease. In some

embodiments, upon administration of the composition, an improvement of clinical benefit rate ("CBR") is obtained as compared to treatment with irinotecan but without 4-iodo-3-nitrobenzamide or the metabolite thereof or the pharmaceutically acceptable salt thereof. In some embodiments, the improvement of clinical benefit rate is at least about any of 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%. In some embodiments, CBR = CR + PR + SD $\geq$ 6 months. In some embodiments, CBR = CR + PR + SD $\geq$ 6 cycles.

[0215] In some embodiments, 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered intravenously. In some embodiments, irinotecan or a pharmaceutically acceptable salt thereof is administered intravenously.

[0216] For injection, the 4-iodo-3-nitrobenzamide or pharmaceutically acceptable salt thereof may be formulated for administration in aqueous solutions, preferably in physiologically compatible buffers such as phosphate buffers, Hank's solution, or Ringer's solution. Such compositions may also include one or more excipients, for example, preservatives, solubilizers, fillers, lubricants, stabilizers, albumin, and the like. Formulations of 4-iodo-3-nitrobenzamide are described in US Pat. Publ. No. 2008/0176946 A1, which is incorporated by reference in its entirety, particularly with reference to intravenous (*e.g.,* hydroxypropyl-β-cyclodextrin, etc.) and oral (*e.g.,* sodium lauryl sulfate, etc.) formulations. In some embodiments, 4-iodo-3-nitrobenzamide is formulated in 25% (w/v) hydroxypropyl-β-cyclodextrin and 10 mM phosphate buffer for intravenous administration as described in U.S. Patent Application Publication No. 2010/0160442, which is incorporated herein by reference.

[0217] Additional methods of formulation, such as for irinotecan (or a pharmaceutically acceptable salt thereof), are known in the art, for example, as disclosed in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA. Compositions described herein may also be formulated for transmucosal administration, buccal administration, for administration by inhalation, for parental administration, for transdermal administration, and rectal administration.

[0218] Compositions described herein may also be formulated for transmucosal administration, buccal administration, for administration by inhalation, for parental administration, for transdermal administration, and rectal administration.

[0219] Typical salts for compositions, formulations, and methods provided herein may be those of the inorganic ions, such as, for example, sodium, potassium, calcium and magnesium ions. Such salts include salts with inorganic or organic acids, such as hydrochloric acid, hydrobromic acid, phosphoric acid, nitric acid, sulfuric acid, methanesulfonic acid, p-toluenesulfonic acid, acetic acid, fumaric acid, succinic acid, lactic acid, mandelic acid, malic acid, citric acid, tartaric acid or maleic acid. In addition, where compounds contain a carboxy

group or other acidic group, it may be converted into a pharmaceutically acceptable addition salt with inorganic or organic bases. Examples of suitable bases include sodium hydroxide, potassium hydroxide, ammonia, cyclohexylamine, dicyclohexyl-amine, ethanolamine, diethanolamine and triethanolamine. In some embodiments, 4-iodo-3-nitrobenzamide is formulated in 25% (w/v) hydroxypropyl-β-cyclodextrin and 10 mM phosphate buffer for intravenous administration as described in U.S. Patent Application Publication No. 2010/0160442, which is incorporated herein by reference.

[0220] Pharmaceutical compositions suitable for use as described herein include compositions wherein the active ingredients are present in an effective amount, *i.e.*, in an amount effective to achieve therapeutic and/or prophylactic benefit in a breast cancer (*e.g.*, metastatic breast cancer) described herein. The actual amount effective for a particular administration will depend on the breast cancer (*e.g.*, metastatic breast cancer) being treated, the condition of the individual, the formulation, and the route of administration, as well as other factors known to those of skill in the art in view of the specific teaching provided herein. In light of the disclosure herein, optimization of an effective amount of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and irinotecan or a pharmaceutically acceptable salt thereof, within the ranges specified, may be determined.

[0221] The compositions described herein may be administered to a patient through appropriate route, such as, but are not limited to intravenous, intra-arterial, intraperitoneal, intrapulmonary, inhalation, intravesicular, intramuscular, intra-tracheal, subcutaneous, intraocular, intrathecal, transmucosal, transdermal, intranasal, epidural, and oral routes. In some embodiments, the composition or compound(s) provided herein is administered by the parenteral route, *e.g.*, intravenously, intraperitoneally, subcutaneously, intradermally, or intramuscularly. In some embodiments, sustained continuous release of the formulations or compositions described herein are administered.

[0222] Compositions provided herein may also be administered by a convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.,* oral mucosa, rectal and intestinal mucosa, etc.) and may be administered in combination with other biologically active agents, *e.g.,* such as described herein. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Kits, uses, articles of manufacture

[0223] Provided herein are kits for administration of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and irinotecan. In certain embodiments the kits may include a dosage amount of at least one composition as disclosed herein. Kits may further comprise suitable packaging and/or instructions for use of the formulation. Kits may also comprise a means for the delivery of the formulation thereof.

[0224] In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan or a pharmaceutically acceptable salt thereof. In some embodiments, the kit further comprises instructions (e.g., instructions on package insert, product insert or label) for using (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan to treat locally advanced or metastatic breast cancer in a patient according to a method provided herein. In some embodiments, there is provide a kit comprising (i) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (ii) instructions (e.g., instructions on package insert, product insert or label) for using (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan to treat locally advanced or metastatic breast cancer in a patient according to a method provided herein. A kit described herein may comprise packaging.

[0225] In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan or a pharmaceutically acceptable salt thereof. In some embodiments, the kit further comprises instructions (e.g., instructions on package insert, product insert, or label) for using (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan or a pharmaceutically acceptable salt thereof to treat metastatic breast cancer brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) according to a method described herein. Also provided herein are kits comprising (i) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (ii) instructions (e.g., instructions on package insert, product insert, or label) for using (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan or a pharmaceutically acceptable salt thereof to treat metastatic breast cancer brain metastasis (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter) in a patient according to a method described herein. In some embodiments, the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, the brain metastasis is at least about or larger than about 0.5 centimeter (e.g., brain metastasis measuring at least about or larger than about 0.5 centimeter in longest dimension). The dosage or treatment regimen for (a)

4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and/or (b) irinotecan may be included in the instructions of any of the kits described herein and may be according to any of the dosages or treatment regimens described herein.

[0226] The kits may optionally include appropriate instructions for preparation and administration of the composition, side effects of the composition, and any other relevant information. The instructions may be in any suitable format, including, but not limited to, printed matter, videotape, computer readable disk, optical disc or directions to internet-based instructions.

[0227] For example, in some embodiments, there is provided a kit for treating a patient with breast cancer brain metastasis comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, the kit further comprises instructions for using effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof to treat the patient with breast cancer brain metastasis. In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) instructions for using an effective amount of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, in combination with irinotecan or a pharmaceutically acceptable salt thereof to treat a patient with breast cancer brain metastasis, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing. In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, (b) irinotecan or a pharmaceutically acceptable salt thereof, and (c) instructions for using 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and irinotecan or a pharmaceutically acceptable salt thereof to treat locally advanced or metastatic breast cancer in a patient, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle. In some embodiments, there is provided a kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) instructions for using 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, in combination with irinotecan or a pharmaceutically acceptable salt thereof to treat locally advanced or metastatic breast cancer in a patient, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt

thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle.

[0228] The kits may include other pharmaceutical agents (such as the side-effect limiting agents, chemotherapy agents, gene therapy agents, DNA therapy agents, RNA therapy agents, viral therapy agents, nanotherapy agents, small molecule enzymatic inhibitors, anti-metastatic agents, etc.), for use in conjunction with 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and irinotecan or a pharmaceutically acceptable salt thereof. These agents may be provided in a separate form, or mixed with 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and irinotecan, provided such mixing does not reduce the effectiveness of 4-iodo-3-nitrobenzamide (or a metabolite thereof or a pharmaceutically acceptable salt thereof) or irinotecan or a pharmaceutically acceptable salt thereof, and is compatible with the route of administration. Similarly the kits may include additional agents for adjunctive therapy or other agents known to the skilled artisan as effective in the treatment or prevention of breast cancer (*e.g.*, metastatic breast cancer) described herein.

[0229] In another aspect, provided are kits for treating a patient who suffers from or is susceptible to the breast cancer (*e.g.*, locally advanced metastatic breast cancer) described herein, comprising a first container comprising a dosage amount of a formulation as disclosed herein, and instructions for use. The container may be any of those known in the art and appropriate for storage and delivery of intravenous formulation. In certain embodiments, the kit further comprises a second container comprising a pharmaceutically acceptable carrier, diluent, adjuvant, etc. for preparation of the composition to be administered to the patient.

[0230] Kits may also be provided that contain sufficient dosages of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and/or irinotecan or a pharmaceutically acceptable salt thereof (including formulation thereof) as disclosed herein to provide effective treatment for a patient for an extended period, such as 1-3 days, 1-5 days, a week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months or more. Kits may also include multiple doses of the compounds and instructions for use and packaged in quantities sufficient for storage and use in pharmacies, for example, hospital pharmacies and compounding pharmacies.

[0231] The kits may include the compounds as described herein packaged in either a unit dosage form or in a multi-use form. The kits may also include multiple units of the unit dose form. In certain embodiments, provided are the compound described herein in a unit dose form. In other embodiments the compositions may be provided in a multi-dose form (*e.g.*, a blister pack, etc.).

[0232] Also provided are medicines for treating breast cancer (*e.g.*, locally advanced metastatic breast cancer). In some embodiments, the medicine comprises a) a composition comprising 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt thereof, and irinotecan. 4-iodo-3-nitrobenzamide (or a metabolite thereof, or a pharmaceutically acceptable salt thereof) and irinotecan can be present in separate containers or in a single container. It is understood that the medicine may comprise one distinct composition or two or more compositions wherein one composition comprises 4-iodo-3-nitrobenzamide (or a metabolite thereof, or a pharmaceutically acceptable salt thereof) and one composition comprises irinotecan.

[0233] Also provided herein are uses of 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, in combination with irinotecan, a pharmaceutically acceptable salt or solvate thereof for the manufacture of a medicament for the treatment or prevention of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) described herein. Also provided herein are uses of 4-iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof for the manufacture of a medicament for the treatment or prevention of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) in combination with irinotecan, a pharmaceutically acceptable salt or solvate thereof described herein. In certain embodiments, the medicament is provided for the treatment of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis). Also provided herein are uses of -iodo-3-nitrobenzamide, a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, in combination with irinotecan, a pharmaceutically acceptable salt or solvate thereof, for treatment of breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) in a patient. Also provided herein are synergistic compositions used for treating breast cancer (*e.g.*, locally advanced breast cancer, metastatic breast cancer, breast cancer brain metastasis, or ER-negative, PR-negative, and HER2-nonoverexpressing breast cancer brain metastasis) in a patient comprising a) 4-iodo-3-nitrobenzamide, or a metabolite thereof, or a pharmaceutically acceptable salt or solvate thereof, and b) irinotecan, or pharmaceutically acceptable salt or solvate thereof, to said patient. The uses described herein may in accordance with any of the methods provided herein.

[0234] Also provided are articles of manufacture comprising the compositions described herein in suitable packaging. Suitable packaging for compositions described herein are known in the art, and include, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed. Also provided are unit dosage forms comprising the compositions described herein. These unit dosage forms can be stored in a suitable packaging in single or multiple unit dosages and may also be further sterilized and sealed.

EXAMPLES

[0235] The examples below are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way. The following examples and detailed description are offered by way of illustration and not by way of limitation.

Example 1: A phase 1/1b dose escalation study evaluating 4-iodo-3-nitrobenzamide (BA) as a single agent and in combination with irinotecan in subjects with advanced solid tumors

[0236] This study includes treatment of advanced solid tumors including locally advanced or metastatic breast cancer. The study has two phases: phase 1 and phase 1b.

Objectives

[0237] The primary objectives of phase 1 are to assess the safety, pharmacokinetics and to determine the maximum tolerated dose (MTD) of 4-iodo-3-nitrobenzamide as a single agent in patients with advanced solid tumors that are refractory to standard therapy. The primary objectives of phase 1b are to determine safety and maximum tolerated dose of 4-iodo-3-nitrobenzamide in combination with irinotecan in patients with locally advanced or metastatic breast cancer and to investigate the effect of this maximum tolerated dose of 4-iodo-3-nitrobenzamide in combination with irinotecan in patients with locally advanced or metastatic breast cancer. The secondary objectives are to assess safety profiles such as significant laboratory changes and adverse events (AEs) not defined as a dose limiting toxicity (DLT). The exploratory objectives include: to study whether BRCA activity is down-regulated in metastatic breast cancer; to quantitatively measure total cellular levels of topoisomerase I as well as levels within the nucleus and cytoplasm in order to determine a nuclear to cytoplasmic ratio, which may be predictive of response to inhibitors of topoisomerase I.

Study design

[0238] Phase 1: 4-iodo-3-nitrobenzamide is administered intravenously twice weekly (days 1 and 4 of each week) for 3 weeks, followed by a one week 4-iodo-3-nitrobenzamide treatment-free

period per one 28-day cycle. Cycle one (day 1 through day 28) is defined as the Safety Phase of the study during which DLTs is assessed and the MTD is determined. The remainder of the study is termed the Maintenance Phase. A subject may participate in this study until he/she experiences a drug intolerance or disease progression. The first assessment of tumor response is performed during week 8 of this portion of the study, and approximately every 8 weeks thereafter. The modified Response Evaluation Criteria in Solid Tumors (RECIST) is used to establish disease progression. For non-measurable disease, best medical practices are used to determine disease progression.

[0239] In cohort A (starting dose), a single subject receives 4-iodo-3-nitrobenzamide twice weekly at a dose level of 0.5 mg/kg based on weight measured at screening. If this subject experiences a grade 2 toxicity or higher, then 3 additional subjects would be enrolled in this cohort (only 2 additional subjects would be enrolled in this cohort if the initial subject receives at least 80% of the planned cycle 1 doses). If no additional subjects dosed in this cohort experience a DLT, then dose escalation would occur as below. If no DLT occurs in the initial subject, dose escalation would occur as below. The safety review group has the ability to recommend dose escalation (opening of a new cohort), without expansion with additional subjects, for cases of grade 2 toxicity seen in a single subject that are deemed not to be clinically relevant and/or study treatment-related. Dose Escalation Prior to Grade 2 Toxicity (Potential Cohorts B-J): Until a subject experiences a grade 2 toxicity or higher, one subject would be initially enrolled in all subsequent cohorts at planned 100% dose level increases, with possible cohort expansion as described for cohort A. Safety data are reviewed after 6 doses of 4-iodo-3-nitrobenzamide, and a decision to escalate to the next cohort would be made if no subject experiences a grade 2 toxicity or higher. If 1 subject in this cohort experiences a grade 2 toxicity or higher, then 3 additional subjects would be enrolled in this cohort (only 2 additional subjects would be enrolled in this cohort if the initial subject receives at least 80% of the planned cycle 1 doses). If none of these three additional subjects dosed in this cohort experience a DLT, then further dose escalation would occur. If 1 of 3 subjects experience a DLT, then 3 additional subjects would be enrolled in the same cohort with the same dose. If 0 of these 3 subjects experience a DLT then escalation would occur. If one or more of the additional subjects in a cohort experience a DLT, then the previous lower dose level would be defined as the MTD. Additional subjects may be accrued at the MTD if needed to ensure at least 18 subjects receive 4-iodo-3-nitrobenzamide in the Phase 1 portion of the study. Dose Escalation After Grade 2 Toxicity Level (Potential Cohorts B-J): After the dose associated with the initial grade 2 toxicity is expanded and cleared for dose escalation to the next level, then three subjects would be initially enrolled in all future cohorts (Cohorts B, C, D, E, F, G, H, I, or J). If 0 of the 3 initial subjects experience a DLT, then dose escalation to the

next cohort would proceed. If 1 of 3 subjects experience a DLT, then 3 additional subjects would be enrolled in the same cohort with the same dose. If 0 of these 3 subjects experience a DLT then escalation would occur. If one or more of the additional subjects in a cohort experience a DLT, then the previous lower dose level would be defined as the MTD. Additional subjects may be accrued at the MTD if needed to ensure that at least 18 subjects receive 4-iodo-3-nitrobenzamide in the Phase 1 portion of the study. Overall Dose Escalation Limitations: When a grade 2 toxicity has been observed and that dose level subsequently cleared, individual dose escalations between cohorts would be more conservative, and would be limited to approximately a maximum 40% increase from the previous dose level until a grade 3 toxicity is seen, with subsequent escalations limited to approximately 25% dose increases. See Table 1. Absolute dose escalation is decided by the safety review group after review of all available data.

TABLE 1

Toxicity at a given dose level	Dosage increment for next cohort
Grade 0-1	100%
Grade 2	40%
Grade >=3 toxicity	25%

[0240] Maximum Tolerated Dose (MTD) is defined as the highest dose level with an observed incidence of a DLT in < 33% of the subjects enrolled in the cohort. Dose Limiting Toxicity (DLT) is defined as a grade 3, or 4, severe hematological or non-hematological toxicity thought to be possibly due to study drug, during the initial 28 days of study except fatigue, nausea, diarrhea, vomiting, neutropenia, febrile neutropenia, thrombocytopenia, anemia, AST, and ALT, which would be defined as: Grade 3 fatigue, persistent for more than 7 days; Grade 3 nausea, diarrhea, and/or vomiting despite maximum supportive care, or any Grade 4 nausea, diarrhea, and/or vomiting; Grade 4 neutropenia (ANC < 0.5 x 10^9/L) for more than 5 days; Grade 4 thrombocytopenia (platelet count < 25.0 x 10^9/L); Grade 4 anemia; Grade 2 neurotoxicity; AST or ALT > 5 x ULN. Subjects that require GCF and/or platelet support should be considered as having a DLT.

[0241] Phase 1b: Phase 1b enrollment proceeds in cohorts of 3 patients with locally advanced or metastatic breast cancer in accordance with standard Phase I dose escalation rules (3+3). No intra-subject dose escalation is allowed in this portion of the study. Irinotecan is administered intravenously over 90 minutes on day 1 and then on day 8 , beginning at a dose of 80 mg/m^2, and escalating up to 125 mg/m^2. 4-iodo-3-nitrobenzamide is administered intravenously over 60 minutes on day 1 beginning immediately after completion of the irinotecan infusion, and again on day 4, at a dose of 8.0 mg/kg. This twice-weekly dosing is repeated over a total of 2 weeks. Week 3 is a rest period in which neither irinotecan nor 4-iodo-3-nitrobenzamide is dosed. The dose of 4-

iodo-3-nitrobenzamide selected for the phase 1b portion of the study is partially based on clearance of the 8.0 mg/kg dose level in the phase 1 portion of the study. This overall three week period is defined as one study cycle and is repeated every three weeks. Also, the initial three week cycle (Cycle 1) is the basis for decisions regarding tolerability and dose escalation to the next cohort. Dose escalation (or reduction) is done following guidance in Table 2 starting at dose level 1b-1.

TABLE 2

Phase 1b cohort/Level	Irinotecan Dose	4-iodo-3-nitrobenzamide Dose
1b-0	60 mg/m^2	8.0 mg/kg
1b-1 (initial dose level)	80 mg/m^2	8.0 mg/kg
1b-2	100 mg/m^2	8.0 mg/kg
1b-3	125 mg/m^2	8.0 mg/kg

[0242] Once the highest well tolerated combination is identified, that dose would be studied in an additional 18 subjects with metastatic breast cancer. During accrual in this latter group, subjects continue to be monitored for toxicity. If >6 of 18 subjects experience grade 3 or 4 drug related DLT (as defined in the protocol), the dosing of study drugs would be reassessed and the protocol would either be terminated or amended to investigate a less toxic dose and/or schedule.

[0243] The first assessment of tumor response is performed after week 6 (completion of 2 cycles of therapy), and then after every 6 weeks in addition to the initial staging done at baseline. The modified Response Evaluation Criteria in Solid Tumors (RECIST) is used to establish disease response or progression. Subjects may participate in this study until a subject experiences a drug intolerance or disease progression. Subjects with complete regression (CR) receive 4 additional treatment cycles, and subjects with partial regression (PR) or stable disease (SD) may continue therapy indefinitely, at the investigator's discretion.

[0244] Maximum Tolerated Dose (MTD) is defined as the highest dose level with an observed incidence of a DLT in < 33% of the subjects enrolled in the cohort. Dose Limiting Toxicity (DLT) is defined as any grade 3 or 4, severe hematological or non-hematological toxicity, thought possibly due to irinotecan or potentiated by 4-iodo-3-nitrobenzamide, during the initial 21 days of study except fatigue, nausea, diarrhea, vomiting, neutropenia, febrile neutropenia, thrombocytopenia, anemia, hypertension, AST, and ALT, which would be defined as: Grade 3 fatigue, persistent for more than 7 days; Grade 3 or 4 nausea, diarrhea, and/or vomiting despite maximum supportive care; Grade 3 or 4 neutropenia with fever > 38.50C; Grade 4 neutropenia (ANC < 0.5 x 10^9/L) for more than 7 days; Grade 4 thrombocytopenia (platelet count < 25.0 x

10^9/L); Grade 4 hypertension despite anti-hypertensives; AST or ALT > 2 grade change from baseline.

[0245] The overall study is planned for as many as 6 cycles of 4-iodo-3-nitrobenzamide treatment (with or without irinotecan) per subject at the MTD level and as many as 12 cycles of total 4-iodo-3-nitrobenzamide treatment, dependent on the date of subject study enrollment. In the event that the study subject(s) demonstrate persistent clinically favorable response that is accompanied by documented radiographic response (complete response, partial response, and stable disease without any tumor growth), the sponsor would, if possible, maintain such subject(s) on 4-iodo-3-nitrobenzamide treatment. It is anticipated that each subject would be on the study for 2 weeks of screening, for as long as 48 weeks of treatment, and 30 days of follow-up.

Endpoints

[0246] Phase 1 - Primary endpoints: safety/tolerability to characterize DLT; PK profiles: 4-iodo-3-nitrobenzamide half life (t½), maximum observed concentration (Cmax), area under the plasma concentration-time curve (AUC), and clearance (CL). Secondary endpoints: Tumor response per RECIST criteria; safety profiles: significant laboratory changes and other AEs (not defined as a DLT). Exploratory endpoints include reduction in circulating tumor cell (CTC) levels.

[0247] Phase 1b – Primary endpoints: Determination of the safety and tolerability of the combination of irinotecan plus 4-iodo-3-nitrobenzamide; Determination of the effect of this combination in subjects with locally advanced or metastatic breast cancer by Objective Response Rate (ORR; PR + CR). Secondary endpoints: Determination of the effect of this combination in subjects with metastatic breast cancer by Clinical Benefit Rate (CBR; ORR + stable disease). Exploratory points include: To study the formation of double strand DNA breaks and homologous recombination in pre- and post-treatment tumor tissues by staining r-H2AX foci and RAD51 foci; To study the status of BRCA on response in subjects with metastatic breast cancer; To study the expression of hypoxia markers in metastatic breast cancer; To quantitatively measure total cellular levels of topoisomerase I as well as levels within the nucleus and cytoplasm in order to determine a nuclear to cytoplasmic ratio, which may be predictive of response to inhibitors of topoisomerase I.

Subject eligibility criteria

[0248] Inclusion criteria include the following: ≥ 18 years old with a pathologically documented, advanced solid tumor that is refractory to standard treatment or for which no standard therapy is available (phase 1 only); ≥ 18 years old with a histologically documented, adenocarcinoma of the breast with progressing locally advanced or metastatic disease and at least

one bi-dimensionally measurable indicator lesion of at least 2.0 cm as assessed by computed tomography, magnetic resonance imaging, or ultra-sonography (phase 1b only); Prior treatment with at least one regimen containing an anthracycline, an anthraquinone, a taxane, or doxorubicin is required (phase 1b only); Maximum of one adjuvant regimen and two regimens for metastatic disease, whether or not all of these were based on anthracycline or taxane (phase 1b only); Eastern Oncology Cooperative Group (ECOG) performance status of ≤ 2; Completion of prior chemotherapy at least 3 weeks prior to trial entry and recovery from toxicity of prior chemotherapy; Radiation therapy must be completed at least 3 weeks prior to trial entry, and radiated lesions may not serve as target lesions; Subjects may have CNS metastases if individual does not require steroids, whole brain XRT, or gamma/cyber knife, and brain metastases are clinically stable without symptomatic progression; Adequate organ function defined as: ANC $\geq 1{,}500/mm^3$, platelets $\geq 100{,}000/mm^3$, creatinine clearance > 50mL/min, ALT and AST < 2.5 x upper limit of normal (ULN) (or < 5 x ULN in case of liver metastases); total bilirubin < 1.5 mg/dL; Tissue block (primary or metastatic) available for pharmacogenomic studies is recommended, although its absence would not exclude subjects from participating; Women of child-bearing potential must have documented negative pregnancy test within two weeks of trial entry and agree to use acceptable birth control during the duration of the trial therapy; Signed, IRB-approved written informed consent.

[0249] Exclusion criteria include the following: Lesions identifiable only by PET; Major medical conditions that might affect trial participation (uncontrolled pulmonary, renal, or hepatic dysfunction, uncontrolled infection); Significant history of uncontrolled cardiac disease; i.e., uncontrolled hypertension, unstable angina, recent myocardial infarction (within prior 6 months), uncontrolled congestive heart failure, and cardiomyopathy that is either symptomatic or asymptomatic but with decreased ejection fraction < 45%; Other significant co-morbid condition which the investigator feels might compromise effective and safe participation in the trial; Subject enrolled in another investigational device or drug trial, or is receiving other investigational agents; Concurrent or prior (within 7 days of trial day 1) anticoagulation therapy (low dose for port maintenance allowed); Concurrent radiation therapy is not permitted throughout the course of the trial; Inability to comply with the requirements of the trial; Pregnant or lactating women are excluded; Leptomeningeal disease or brain metastases requiring steroids or other therapeutic intervention.

[0250] An adverse event (AE) is an undesirable medical occurrence (*e.g.*, sign, symptom, or diagnosis) or worsening of a pre-existing medical condition that occurs after the initial dose of 4-iodo-3-nitrobenzamide or irinotecan and as much as 30 days after the last dose of 4-iodo-3-nitrobenzamide or irinotecan or until another tumor treatment is initiated (whichever is first)

whether or not it is considered to be related to the investigational product. A worsening of an existing medical condition occurs when a condition present at the time the informed consent form is signed (*e.g.*, cancer, diabetes, migraine headaches, gout) becomes more severe, more frequent, or increased in duration during investigational product treatment. Hospitalizations for pre-treatment conditions (*e.g.*, elective cosmetic procedures) or surgeries that were planned before entry into the study are not considered adverse events. Abnormal laboratory values should not be reported as an AE; however, any clinical consequences of the abnormality should be reported as an AE.

[0251] A serious adverse event (SAE) is defined by regulatory agencies as one that suggests a significant hazard or adverse event, regardless of the investigator's or sponsor's opinion on the relationship to investigational product. This includes, but may not be limited to, any event at any dose that: is fatal; is life threatening (places the subject at immediate risk of death); requires in-patient hospitalization or prolongation of existing hospitalization; results in persistent or significant disability/incapacity; is a congenital anomaly/birth defect; is any other significant medical hazard. A hospitalization is considered to meet the regulatory requirement for "serious" when it entails any inpatient hospital admission that requires an overnight stay. Elective hospitalizations for the administration of chemotherapy, or therapeutic or prophylactic transfusions are not considered SAEs. However, prolonged hospitalization or readmission, after the subject had already been discharged post-treatment, is considered an SAE. Any event that does not exactly meet this definition, but in the investigator's opinion represents a significant hazard (*e.g.*, emergency room visit or outpatient surgery), can be assigned the "other significant hazard" regulatory reporting serious criteria. Additionally, important medical events that may not be immediately life threatening or result in death or hospitalization but that may jeopardize the subject or require intervention to prevent one of the outcomes listed above, or result in urgent investigation, may be considered serious. Examples include allergic bronchospasm, convulsions, and blood dyscrasias.

[0252] Efficacy Analyses: The overall response rate (ORR) is estimated by the proportion of patients who achieve an overall response (CR+PR).

[0253] Biomarker Analyses: Analyses to assess associations between differences in any pharmacogenomic results (*e.g.*, BRCA) from samples taken before, during, and after 4-iodo-3-nitrobenzamide treatment are considered and offered by the subject on a voluntary basis.

[0254] Clinically applicable serum tumor markers are analyzed. Levels for carcinoembryonic antigen (CEA), CA-125, CA-19-9, or other as appropriate are measured.

[0255] Detailed PK analysis is done on samples from the phase 1 portion of the study. Blood level data obtained from the phase 1b portion of the study are listed.

[0256] Correlative analyses of markers of hypoxia (*e.g.* HIF-1, CA9, PHD3, PGK1, and PNIP3) with clinical outcome are exploratory and descriptive in nature. Voluntary tumor biopsy samples at pre- and post-4-iodo-3-nitrobenzamide infusion are analyzed for the expression of hypoxia markers by using immunohistochemistry. Formalin-fixed paraffin tumor block from the prior surgery or biopsy is collected, and analyzed for the expression of hypoxia markers. The extent of DNA damage induced by irinotecan and presence of homologous DNA repair is analyzed by immunohistochemical staining of voluntarily obtained tumor biopsy samples at pre- and post-4-iodo-3-nitrobenzamide infusion. Immunohistochemical staining of tumor samples analyzes the formation of γ -H2AX foci and Rad51 foci. γ-H2AX foci are formed at the site of DNA double strand breaks, and the number of γ -H2AX per cell reflects the extent of DNA damage. Rad51 foci are formed at the DNA double strand break sites with presence of intact homologous DNA repair pathway. Defects in homologous DNA repair such as BRCA-deficiency result in absence of Rad51 foci formation in the presence of γ -H2AX foci.

Modified RECIST criteria

[0257] Definitions: Measurable disease - the presence of at least one measurable lesion. If the measurable disease is restricted to a solitary lesion, its neoplastic nature should be confirmed by cytology/histology. Measurable lesions - lesions that can be accurately measured in at least one dimension with longest diameter ≥20 mm using conventional techniques or ≥10 mm with spiral CT scan. Non-measurable lesions - all other lesions, including small lesions (longest diameter <20 mm with conventional techniques or <10 mm with spiral CT scan), i.e., bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusion, inflammatory breast disease, lymphangitis cutis/pulmonis, cystic lesions, and also abdominal masses that are not confirmed and followed by imaging techniques.

[0258] All measurements should be taken and recorded in metric notation, using a ruler or calipers. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 14 days before study day 1. The same method of assessment and the same technique should be used to characterize each identified and reported lesion throughout the trial. Clinical lesions are only considered measurable when they are superficial (*e.g.*, skin nodules and palpable lymph nodes). For the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

[0259] Methods of Measurement: CT and MRI are the best currently available and reproducible methods to measure target lesions selected for response assessment.

[0260] Evaluation of Target Lesions - Complete Response (CR): Disappearance of all target lesions; Partial Response (PR): At least a 30% decrease in the sum of the LD of target lesions, taking as reference the baseline sum LD; Progressive Disease (PD): At least a 20% increase in the

sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions; Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started.

[0261] Evaluation of Non-Target Lesions - Complete Response (CR): Disappearance of all non-target lesions and normalization of tumor marker level; Incomplete Response/ Stable Disease (SD): Persistence of one or more non-target lesion(s) or/and maintenance of tumor marker level above the normal limits; Progressive Disease (PD): Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions.

[0262] Evaluation of Best Overall Response: The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for PD the smallest measurements recorded since the treatment started). In general, the subject's best response assignment depends on the achievement of both measurement and confirmation criteria.

TABLE 3

Target Lesions	Non-Target Lesions	New Lesions	Overall Response
CR	CR	No	CR
CR	Incomplete response/SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

[0263] Subjects with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be classified as having "symptomatic deterioration".

[0264] Duration of Overall Response: The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever status is recorded first) until the first date that recurrence or PD is objectively documented, taking as reference for PD the smallest measurements recorded since the treatment started.

[0265] Duration of Stable Disease: SD is measured from the start of the treatment until the criteria for disease progression are met, taking as reference the smallest measurements recorded since the treatment started.

Example 2: Safety assessment of administration of 4-iodo-3-nitrobenzamide (BA) at various dosages

[0266] 24 subjects (advanced solid tumors) were treated with 4-iodo-3-nitrobenzamide monotherapy at doses of 0.5, 1.0, 1.4, 2.8, 4.0, 5.6, and 8.0 mg/kg. Safety data indicated that 4-iodo-3-nitrobenzamide was well tolerated at all dose levels tested to date; no dose limiting toxicities (DLTs) were observed at any dose level. A total of 13 serious adverse events were reported for 5 trial participants. Any serious adverse events (SAEs) reported in the study were deemed not related to study drug by the study investigators. Best response measured to date was stable disease present through at least cycle 2 of the study for six subjects, with 1 subject not having reached end of cycle 2 for a disease assessment to be performed. One subject had completed 9 cycles of treatment with a staging of continued stable disease.

[0267] 42 subjects (advanced solid tumors) were treated with 4-iodo-3-nitrobenzamide in combination with cytotoxic chemotherapeutics agents (topotecan, temozolomide, gemcitabine, or carboplatin/taxol) at 4-iodo-3-nitrobenzamide doses of 1.1, 2.0, 2.8, 4.0 and 5.6 mg/kg. Preliminary safety data indicated that 4-iodo-3-nitrobenzamide was well tolerated at all dose levels tested. Any serious adverse events reported in the study were deemed not related to study drug by the study investigators. Best response measured was stable disease present through at least cycle 2 of the study for eight subjects, stable disease through cycle 4 for three subjects, three subjects with partial response through at least cycle 2 and still on study, and one subject demonstrating a complete response thru cycle 6 (reached end of treatment). Several subjects enrolled did not yet reach end of cycle 2 for a disease assessment to be performed.

[0268] There were no SAEs attributed to 4-iodo-3-nitrobenzamide in either study. Safety data indicated that 4-iodo-3-nitrobenzamide would not cause any additional toxicities when combined with standard cytotoxic chemotherapy. Additionally, there was no evidence that 4-iodo-3-nitrobenzamide would potentiate any known toxicities associated with standard chemotherapeutic agents.

Example 3: A phase 1b study to assess the tolerability of the 4-iodo-3-nitrobenzamide (BA) in combination with irinotecan for the treatment of patients with metastatic breast cancer (MBC)

[0269] This study was conducted to determine the maximum-tolerated dose (MTD) of irinotecan that can be used in combination with fixed dose 4-iodo-3-nitrobenzamide in patients with locally advanced or MBC.

[0270] Patients were treated with a 3+3 study design to determine safety of 4-iodo-3-nitrobenzamide (8 mg/kg IV twice-weekly on Days 1, 4, 8, and 11 every 21 days) in combination with escalating doses of irinotecan (80 – 125 mg/m^2 IV on Days 1 and 8 every 21 days).

Response rates were measured per modified RECIST criteria. Serial pharmacokinetic (PK) assessments were performed during Cycle 1, and archived paraffin-embedded tumor samples were collected to correlate markers of hypoxia (CAIX) with response.

[0271] The median age of 34 patients who received therapy was 50 years (range, 32-84), and the median number of prior therapies was 2 (range, 0-6). Preliminary analysis showed that tumors from 22 (65%) patients were ER-/PR-/HER2- (triple-negative), 10 (29%) were ER+/HER2-, and 1 (3%) was HER2+. One DLT of Grade 3 diarrhea with lower GI bleed was observed, thus establishing the optimal regimen from this study at 8 mg/kg 4-iodo-3-nitrobenzamide and 125 mg/m^2 irinotecan. Other toxicities included neutropenia, anemia and diarrhea. Of 26 patients evaluable for response, 5 (19%) had partial response (PR), 10 (38%) had stable disease (SD) for >4 cycles of therapy, and 9 (35%) developed progressive disease (PD) as best response. Of 10 patients who had reached 6 cycles at time of analysis, 5 (50%) had SD. Median follow-up time was 10 weeks.

[0272] Thus, an MTD of 8 mg/kg 4-iodo-3-nitrobenzamide and 125 mg/m^2 irinotecan was identified in patients with MBC. 4-iodo-3-nitrobenzamide in combination with irinotecan was well tolerated and showed efficacy, with evidence of clinical benefit (RR + SD ≥6 cycles) in 50% in patients with treatment-refractory MBC.

Example 4: A phase 1b study to assess the safety and tolerability of 4-iodo-3-nitrobenzamide (iniparib) in combination with irinotecan for the treatment of patients with metastatic breast cancer (MBC)

[0273] The primary endpoints for this study were (1) safety and tolerability and (2) objective response rate (ORR). The secondary endpoint for this study was clinical benefit rate (CBR defined as the sum of ORR and stable disease (SD) >6 cycles).

[0274] Methods: Patients were treated with a 3+3 study design to determine safety of 4-iodo-3-nitrobenzamide (8 mg/kg IV twice-weekly on Days 1, 4, 8, and 11 every 21 days) in combination with escalating doses of irinotecan (80 – 125 mg/m^2 IV on Days 1 and 8 every 21 days). The treatment scheme is shown in Figure 1. ORR was measured per modified RECIST criteria. Serial pharmacokinetic (PK) assessments were performed during Cycle 1, and archived paraffin-embedded tumor samples were collected to correlate markers of hypoxia (CAIX) with response.

[0275] The key eligibility criteria for this study were (1) ≥18 years of age; (2) Histologically documented adenocarcinoma of the breast with progressing locoregional or metastatic disease and at least one bi-dimensionally measurable indicator lesion of at least 2.0 cm, as assessed by CT, MRI, or ultrasonography; (3) Prior treatment with at least one regimen containing an anthracycline, an anthraquinone, or a taxane; (4) Maximum of one adjuvant regimen and two regimens for metastatic disease, whether or not all of these were based on an anthracycline or

taxane; (5) ECOG PS 0–2; (6) No symptomatic or untreated brain metastases requiring concurrent treatment; (7) Written, informed consent.

[0276] Patient characteristics are shown in Table 4.

TABLE 4 Patient Characteristics

	4-iodo-3-nitrobenzamide 8 mg/kg + Irinotecan dose		
	80 mg/m^2	100 mg/m^2	125 mg/m^2
N	3	6	25
Age, median (range)	58 (48 – 78)	52 (41 – 68)	50 (32 – 84)
Race, n (%)			
White	3 (100)	5 (83.3)	20 (80.0)
Black/African-American	0	1 (16.7)	5 (20.0)
ECOG PS, n (%)			
0	2 (66.7)	3 (50.0)	11 (44.0)
1	1 (33.3)	3 (50.0)	13 (52.0)
Missing/Unknown	0	0	1 (4.0)
Receptor status, n (%)			
TN: ER-/PR-/HER2-	3 (100)	2 (33.3)	17 (68.0)
HR+/HER2-	0	3 (50.0)	7 (28.0)
HR-/HER2+	0	0	1 (4.0)
Missing/Unknown	0	1 (16.7)	0
Prior therapy, n (%)			
Chemotherapy	3 (100)	6 (100)	25 (100)
Radiation therapy	3 (100)	6 (100)	19 (76.0)
Hormonal	0	4 (66.7)	6 (24.0)
Surgery	3 (100)	6 (100)	20 (80.0)
Other	1 (33.3)	0	8 (32.0)

ECOG PS, Eastern Cooperative Oncology Group Performance Status; TN, triple negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human growth hormone receptor-2; HR, hormone receptor

[0277] Summary of Results: The median age of 34 patients who received therapy was 50 years (range, 32–84), and the median number of prior therapies was 2 (range, 0–6). Tumors from 22 (64.7%) patients were ER-/PR-/HER2- (triple-negative), 10 (29.4%) were ER+/HER2-, and 1 (2.9%) was HER2+. One DLT of Grade 3 diarrhea with lower GI bleed was observed, thus establishing the optimal regimen from this study at 8 mg/kg 4-iodo-3-nitrobenzamide and 125 mg/m^2 irinotecan. Other toxicities included neutropenia, anemia and diarrhea. Of 22 evaluable patients at the highest irinotecan dose, 7 (31.8%) had an objective response, 6 (22.7%) had stable

disease (SD) >4 cycles, 3 (13.6%) had SD>6 cycles, and 5 (22.7%) had progressive disease (PD) as best response. The clinical benefit rate (CBR, defined as ORR+SD>6 cycles) was 45.5%.

[0278] The efficacy data is shown in Table 5.

TABLE 5 Efficacy Summary

	4-iodo-3-nitrobenzamide 8 mg/kg + Irinotecan dose		
	80 mg/m^2 (N=3)	100 mg/m^2 (N=6)	125 mg/m^2 (N=22)
	Number of patients (%)		
Objective response rate (ORR)	0	1 (16.7)	7 (31.8)
Complete response	0	0	1 (4.5)
Partial response	0	1 (16.7)	6 (27.3)
Stable disease	1 (33.3)	2 (33.3)	10 (45.5)
Stable Disease >4 cycles	1 (33.3)	0	6 (22.7)
Stable Disease >6 cycles	1 (33.3)	0	3 (13.6)
Progressive disease	2 (66.7)	3 (50.0)	5 (22.7)
Missing	0	0	3 (13.6)
Clinical Benefit Rate (ORR+SD>6 cycles)	1 (33.3)	1 (16.7)	10 (45.5)

[0279] The effect of the treatment with 4-iodo-3-nitrobenzamide in combination with irinotecan on tumor size in triple negative breast cancer ("TNBC") and non-TNBC is shown in Figure 2.

[0280] Tumoral BRCA1, ER, PR and HER2 were evaluated in 11 out of 30 patients. BRCA1 protein levels were measured using M110 (Ab-1; 1:1000; Calbiochem) with AQUA™ technology. Clinical benefit from 4-iodo-3-nitrobenzamide and irinotecan was observed in breast cancers that were ER+/PR+ and with varying levels of tumoral BRCA1 staining by AQUA™. See Figure 3 and Table 6.

TABLE 6. BRCA1 Staining

Patient	**Best overall response**	**Max tumor change**	**Breast cancer type**	**Avg BRCA1 staining**	**Max BRCA1 staining**
# 1	CR	100% decr	TN	10.604	23.929
# 2	PR	75% decr	ER+/PR+	15.158	23.79
# 3	PR	50% decr	TN	31.599	65.81
# 4	PR	40% decr	ER+/PR+	17.732	39.804
# 5	SD	5% decr	ER+/PR+	10.548	17.988
# 6	SD	5% decr	TN	12.228	51.855
# 7	SD	slight decr	TN	7.194	17.799
# 8	SD	5% incr	TN	21.313	63.854
# 9	PD	20% incr	TN	13.36	42.625
# 10	PD	60% incr	TN	10.993	21.182

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; TN, triple-negative; decr: decrease; incr: increase.

[0281] The safety data are summarized in Table 7.

TABLE 7. Treatment-emergent adverse events (TEAE) of interest

	4-iodo-3-nitrobenzamide 8 mg/kg + Irinotecan Dose							
	80 mg/m2 (n=3)		100 mg/m2 (n=6)		125 mg/m2 (n=25)		All Patients (N=34)	
	Gr 3/4	All grades	Gr 3/4	All grades	Gr 3/4	All grades	Gr 3/4	All grades
	Number of patients (%)							
HEMATOLOGIC								
Neutropenia	0	1 (33.3)	1 (16.7)	1 (16.7)	8 (32.0)	11 (44.0)	9 (26.5)	13 (38.2)
Leukopenia	0	0	1 (16.7)	1 (16.7)	5 (20.0)	9 (36.0)	6 (17.6)	10 (29.4)
Anemia	0	1 (33.3)	0	3 (50)	2 (8.0)	12 (48.0)	2 (5.9)	16 (47.1)
Hematocrit decreased	0	1 (33.3)	0	2 (33.3)	0	2 (8%)	0	5 (14.7)
NON-HEMATOLOGIC								
Diarrhea	0	1 (33.3)	0	3 (50.0)	3 (12.0)	17 (68.0)	3 (8.8)	21 (61.8)
Nausea	0	1 (33.3)	0	4 (66.7)	1 (4.0)	16 (64.0)	1 (2.9)	21 (61.8)
Fatigue	0	2 (66.7)	0	4 (66.7)	3 (12.0)	14 (56.0)	3 (8.8)	20 (58.8)
Constipation	0	2 (66.7)	0	1 (16.7)	1 (4.0)	9 (36.0)	1 (2.9)	12 (35.3)
Any TEAE	1 (33.3)	2 (66.7)	2 (33.3)	4 (66.7)	14 (56.0)	20 (80.0)	17 (50.0)	26 (76.5)

[0282] Conclusion: The maximum protocol-defined dose combination was attained and tested in patients with metastatic breast cancer ("mBC"). No true MTD was reached. 4-iodo-3-nitrobenzamide in combination with irinotecan was well tolerated and was associated with a manageable rate of Grade 3/4 adverse events. Rates of Grade 3/4 neutropenia and diarrhea were lower than expected compared with reported data from single-agent irinotecan (Perez EA et al., J Clin Oncol. 2004, 22(14):2849-55). The highest dose evaluated (8 mg/kg 4-iodo-3-nitrobenzamide and 125 mg/m^2 irinotecan) was well tolerated and showed promising efficacy, with evidence of clinical benefit in 45.5% of patients with treatment-refractory MBC. Preliminary results from this study supported the promising safety and efficacy profile of 4-iodo-3-nitrobenzamide in combination with DNA-damaging chemotherapy.

Example 5: Phase 1/1b Dose Escalation Study Evaluating 4-iodo-3-nitrobenzamide (BA) as a Single Agent and in Combination With Irinotecan in Subjects With Advanced Solid Tumors

[0283] The purpose of this study is to assess the safety, establish the maximum tolerated dose (MTD) and generate pharmacokinetic profiles of 4-iodo-3-nitrobenzamide after IV administration

in adult subjects with histologically documented advanced solid tumors that are refractory to standard therapy or for which no standard therapy is available. Additionally, the safety and tolerability and clinical response of 4-iodo-3-nitrobenzamide and irinotecan are investigated in patients with metastatic breast cancer in the phase 1b portion of the study. The primary outcome measure is maximum tolerated dose and the secondary outcome measure is clinical Response.

[0284] There are two arms for this study. Arm 1 includes 4-iodo-3-nitrobenzamide, i.v., twice weekly. Arm 2 includes 4-iodo-3-nitrobenzamide, i.v., twice weekly and irinotecan, i.v., weekly.

[0285] Inclusion criteria include the following: Pathologically documented, advanced solid tumor that is refractory to standard therapy or for which no standard therapy is available; ECOG performance status of 0, 1, or 2; Adequate hematological status; Any prior toxicity from prior chemotherapeutic treatment recovered to grade 1 or grade 0; 18 years of age or older; For phase 1b portion only: metastatic breast cancer.

[0286] Exclusion criteria include the following: Hematologic malignancies; Symptomatic or untreated brain metastases requiring concurrent treatment, inclusive of but not limited to surgery, radiation, and corticosteroids; Myocardial infarction within 6 months of study day 1, unstable angina, congestive heart failure with NYHA > class II, uncontrolled hypertension; Known positive test for HIV or hepatitis C virus, or chronic active hepatitis; Major surgery within 1 month of study day 1; History of second neoplasm, except for curatively treated non-melanoma skin cancer, carcinoma in situ of the cervix and other primary cancer with no known active disease present and no curative treatment administered for the last 3 years; History of seizure disorder or currently on anti-seizure medication; Systemic chemotherapy or radiation therapy within 28 days of study day 1; Antibody therapy for treatment of underlying malignancy within 1 month of study day 1; Evidence of liver disease shown by elevated enzymes; Evidence of renal disease shown by serum creatinine > 1.5 x upper limit of normal; Currently receiving platelet of GCF support for any medical condition; Concurrent use of herbal medications taken with the intent to treat cancer; Enrolled in or not yet completed at least 30 days since ending other investigational device or drug study.

Example 6: Treating triple negative breast cancer brain metastasis with 4-iodo-3-nitrobenzamide in combination with irinotecan

[0287] The purpose of the study is to investigate the response rate for triple negative breast cancer patients with brain metastasis when 4-iodo-3-nitrobenzamide is used in combination with irinotecan.

[0288] Primary outcome measure is efficacy as measured by intra or extra cranial time to progression (TTP). Secondary outcome measure is response rate as measured by RECIST.

[0289] Patients are treated with 4-iodo-3-nitrobenzamide at 5.6 mg/kg intravenously on days 1, 4, 8, and 11 and irinotecan at 125 mg/m^2 intravenously on days 1 and 8 of a 21-day treatment cycle.

[0290] The ages eligible for study are 21 years and older. The genders eligible for study are both male and female.

[0291] Inclusion criteria are: (1) Histologically-confirmed, ER negative, PR negative and Her2 non-overexpressing adenocarcinoma of the breast with brain lesion on radiographic imaging; (2) ECOG Performance Status of 0-2; (3) Life expectancy of >12 weeks; (4) No limit to prior therapies with last anti-cancer treatment ≥ 2 weeks from initiation of protocol-based therapy provided all toxicities (other than alopecia) have resolved to ≤Grade 1 or baseline; (5) No active serious infection or other comorbid illness which would impair ability to participate in the trial; (6) Stable or decreasing dose of steroids for ≥ 7 days; (7) Interval ≥ 4 weeks between open brain biopsy and initiation of protocol-based therapy; and (8) Patients must have adequate organ function.

[0292] Exclusion criteria are: (1) Pregnant or breast-feeding; (2) Prior allergic reaction to 4-iodo-3-nitrobenzamide; (3) Prior allergic reaction to irinotecan; (4) Evidence of hemorrhage or impending herniation on baseline brain imaging; (5) Evidence of diffuse leptomeningeal disease on brain MRI or by previously documented CSF cytology-NOTE: discrete dural metastases are permitted; (6) Clinically significant cardiac, renal, hepatic, infectious or pulmonary disease which might affect trial participation; (7) Concurrent or planned radiation, hormonal, chemotherapeutic, experimental or targeted biologic therapy; (8) Contraindication to gadolinium-enhanced MRI imaging; and (9) Inability to comply with study and/or follow-up procedures.

Example 7: Phase II study of 4-iodo-3-nitrobenzamide plus irinotecan to treat triple negative breast cancer ("TNBC") brain metastases ("BM")

[0293] This study is conducted to evaluate the effect of 4-iodo-3-nitrobenzamide in combination with irinotecan in patients with TNBC brain metastases. Patients with TNBC BM measuring >0.5cm are eligible. There are no limits to prior therapies, including 4-iodo-3-nitrobenzamide; stable or decreasing steroids ≥ 7 days prior to study entry. Patients with leptomeningeal disease are excluded. Patients receive irinotecan (125 mg/m^2 IV Days 1, 8) prior to 4-iodo-3-nitrobenzamide (5.6mg/kg IV Days 1, 4, 8, and 11) of a 21-day cycle.

[0294] Detailed inclusion criteria for this study are: 1) Histologically-confirmed, ER negative, PR negative and Her2 negative (0-1+ or FISH non-amplified; by clinical assay on either primary or metastatic tumor) adenocarcinoma of the breast with brain lesion on radiographic imaging; 2) Cohort 1: Patients with unequivocal evidence of new and/or progressive brain metastases (>5.0

mm in longest dimension) on radiographic imaging after prior intracranial radiation therapy (i.e. WBRT, SRS, GK or local equivalent), or Cohort 2: Intracranial radiation-naïve patients for whom intracranial radiation therapy ("ICR") is not emergently indicated (at treating physician's discretion) with a >5.0 mm (in longest dimension) brain lesion on radiographic imaging within 2 weeks of initiation of protocol-based therapy; 3) ECOG Performance Status of 0-2; 4) Life expectancy of >12 weeks; 5) No limit to prior therapies with last anti-cancer treatment ≥ 2 weeks from initiation of protocol-based therapy provided all toxicities (other than alopecia) have resolved to ≤Grade 1 or baseline; 6) No active serious infection or other comorbid illness which would impair ability to participate in the study; 7) Stable or decreasing dose of steroids for ≥ 7 days; 8) Interval ≥ 4 weeks between open brain biopsy and initiation of protocol-based therapy; 9) Patients must have adequate organ function as evidenced by: Absolute neutrophil count ≥ 1.5/μL; Platelet count ≥ 100,000/μL; Bilirubin ≤ 1.5 X's upper limit of normal (ULN); AST or ALT ≤ 2.5 X's ULN (≤ 5 X's ULN if liver metastases are present); Creatinine clearance ≥ 30 mL/min; 10) Not pregnant or nursing and able to use appropriate contraception; 11) At least 21 years of age; 12) Archived, paraffin-embedded tissue block (primary or metastatic) available for genomic studies required; 13) Signed, Institutional Review Board ("IRB") approved written informed consent.

[0295] Detailed exclusion criteria for this study are: 1) Pregnant or breast-feeding; 2) Prior allergic reaction to 4-iodo-3-nitrobenzamide; 3) Prior allergic reaction to irinotecan; 4) Evidence of hemorrhage or impending herniation on baseline brain imaging; 5) Evidence of diffuse leptomeningeal disease on brain MRI or by previously documented CSF cytology (discrete dural metastases are permitted); 6) Clinically significant cardiac, renal, hepatic, infectious or pulmonary disease which might affect trial participation; 7) Concurrent or planned radiation, hormonal, chemotherapeutic, experimental or targeted biologic therapy; 8) Contraindication to gadolinium-enhanced MRI imaging; 9) Inability to comply with study and/or follow -up procedures; 10) Patients unable or unwilling to discontinue (and substitute if necessary) use of CYP3A4 inducing drugs, including the anti-convulsants (e.g. phenytoin, phenobarbital or carbamazepine) and strong CYP3A4 inhibiting drugs (e.g., ketoconazole) as these can significantly change plasma concentrations of irinotecan and its active metabolites (patients must not have received any of the prohibited drugs for at least 2 weeks prior to Day 1 of study drug administration).

[0296] Intra- and extracranial disease is assessed every 9 weeks (wks) by gadolinium-enhanced brain MRI and CT chest/abdomen/pelvis, respectively. The co-primary endpoint is intracranial (modified RECIST) and extracranial (RECIST 1.1) time to progression ("TTP"). Secondary objectives include CNS and non-CNS response rates, progression free survival, overall survival,

quality of life, and correlative science endpoints. Time to progression is defined as the time from treatment initiation to documented disease progression as defined below. Progression free survival (PFS) is defined as the time from the start of treatment until documented disease progression as defined below. Overall survival (OS) is defined as the time from the start of treatment until death due to any cause. Intracranial response is also assessed by 3-dimensional (3-D) estimates of tumor volume, and by CNS composite response criteria.

Assessment of intracranial disease

[0297] Tumor Measurement: Intracranial tumor lesions are evaluated via gadolinium-enhanced brain MRI. Measurable disease is defined as the presence of at least one measurable brain lesion that can be accurately measured in at least one dimension (longest diameter to be recorded) as > 5.0mm via gadolinium-enhanced brain MRI. The same method of assessment and the same techniques are used to characterize each identified and reported lesion at baseline and during follow-up. A maximum of 5 target lesions are identified and followed during the course of study.

[0298] Modified RECIST Criteria for Evaluation of Intracranial Disease: Complete Response (CR)- Disappearance of all target lesions; Partial Response (PR)- at least a 30% decrease in the sum of the longest diameter (LD) of target lesions, taking as reference the baseline sum longest diameter and an absolute decrease of at least 5mm in at least one target lesion; Stable Disease (SD)- neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum longest diameter since the treatment started; Progressive Disease (PD) – at least a 20% increase in the sum LD of target lesions, taking as reference the smallest sum longest diameter recorded since the treatment started and an absolute increase in size of at last 5 mm in at least one target lesion or the appearance of one or more new lesions of at least 6 mm in size.

[0299] CNS Composite Response Criteria: CNS Response- ≥50% volumetric reduction of target CNS lesion(s) as measured via volumetric MRI in the absence of: new lesions, progression in any non-target CNS lesions, the need for increased dose of steroids, progressive neurological signs/symptoms, or progressive extracranial disease; CNS Disease Progression- Either a ≥40% volumetric increase from nadir in target CNS lesion(s) as measured via volumetric MRI, an increase in steroid requirements, or progression of neurological signs/symptoms.

Assessment of Extracranial Disease-Measurement Based on RECIST 1.1

[0300] Extracranial tumor lesions are evaluated via CT scan of the chest, abdomen and pelvis (and bone scan if clinically indicated). Measurable disease is defined as the presence of at least one measurable lesion that can be accurately measured in at least one dimension with the longest diameter a minimum size of: ≥10mm by CT scan (CT scan slice thickness no greater than 5 mm); 10mm caliper measurement by clinical exam (lesions which cannot be accurately measured with

calipers should be recorded as non-measurable); 20 mm by chest x-ray. For malignant lymph nodes to be considered pathologically enlarged and measurable, a lymph node must be ≥15mm in short axis when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5mm). At baseline and in follow-up, only the short axis is measured and followed. All other lesions, including small lesions (longest diameter <10mm or pathological lymph nodes with ≥10 to <15 mm short axis) as well as truly non-measurable lesions, are considered non-measurable. Lesions considered truly non-measurable include: leptomeningeal disease; ascites; pleural/pericardial effusion; inflammatory breast disease; lymphangitic involvement of skin or lung, abdominal masses/abdominal organomegaly identified by physical exam that is not measurable by reproducible imaging techniques.

Baseline Documentation of Extracranial Target and Non-Target Lesions

[0301] All measurable lesions up to a maximum of 5 lesions total (and a maximum of two lesions per organ) representative of all involved organs are identified as target lesions and recorded and measured at baseline. Target lesions are selected on the basis of their size (lesions with the longer diameter), are representative of all involved organs, and in addition are those that lend themselves to reproducible repeated measurements. A sum of the diameters (longest for non-nodal lesions, short axis for nodal lesions) for all target lesions is calculated and reported as the baseline sum diameters. If lymph nodes are to be included in the sum, only the short axis is added into the sum. The baseline sum diameters are used as reference to further characterize the objective tumor response of the measurable dimension of the disease. All other lesions (or sites of disease) including pathological lymph nodes are identified as non-target lesions and recorded at baseline.

RECIST 1.1 Criteria for Evaluation of Extracranial Disease

[0302] Evaluation of Target Lesions: Complete response (CR)–Disappearance of all target lesions (any pathological lymph node (LN) target or no must have decreased in short axis to <10mm); Partial response (PR)–At least a 30% decrease in the sum of the LD of the target lesions taking as reference the baseline sum LD; Progressive Disease (PD)–At least a 20% increase in the sum of the LD of the target lesions taking as reference the smallest sum LD recorded since the treatment started including baseline if that is the smallest on study (in addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5mm) (the appearance of one or more new lesions also constitutes PD); Stable disease (SD)–Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD taking as references the smallest sum LD since the treatment started.

[0303] Evaluation of Non-Target Lesions: Complete response (CR)–Disappearance of all non-target lesions and normalization of tumor marker levels (all LN must be non-pathological in size (<10mm short axis)); Non-complete response (non-CR)/non-progression (non-PD)–Persistence of one or more non-target lesion(s) or/and maintenance of tumor marker level above the normal limits; Progressive disease (PD)–Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions.

[0304] Evaluation of Best Overall Response: The best overall response is the best response recorded from the start of the study treatment until the end of treatment provided the confirmation criteria are met. To be assigned a status of PR or CR, changes in tumor measurements must be confirmed by repeat studies that should be performed > 4 weeks after the criteria for response are first met. If a CR/PR cannot be confirmed, the original "response" should be considered stable disease. The best overall response is defined according to Table 8:

TABLE 8

Overall Response First Time Point	Overall Response Subsequent Time Point	BEST Overall Response
CR	CR	CR
CR	PR	SD, PD, or PR[1]
CR	SD	SD provided minimum criteria for SD duration met, otherwise, PD
CR	PD	SD provided minimum criteria for SD duration met, otherwise, PD
CR	NE2	SD provided minimum criteria for SD duration met, otherwise, NE2
PR	CR	PR
PR	PR	PR
PR	SD	SD
PR	PD	SD provided minimum criteria for SD duration met, otherwise, PD
PR	NE2	SD provided minimum criteria for SD duration met, otherwise, NE2
NE	NE2	NE[2]

[1.] If a CR is truly met at first time point, then any disease seen at a subsequent time point, even disease meeting PR criteria relative to baseline, makes the disease PD at that point (since disease must have reappeared after CR). Best response would depend on whether minimum duration for SD was met. However, sometimes 'CR' may be claimed when subsequent scans suggest small lesions were likely still present and in fact the patient had PR, not CR at the first time point. Under these circumstances, the original CR should be changed to PR and the best response is PR.
[2.] NE=inevaluable.

[0305] Statistical considerations: A sample size of 32 evaluable patients has 80% power to detect a difference between the null (2.0 months TTP) and the alternative hypothesis (3.15 months, 57.5% improvement) at a 0.05 significance level. Assuming a 20% drop-out rate, 40 patients are enrolled.

[0306] Correlative studies: Brain magnetic resonance angiography pre- and 9 wks post-therapy to determine if intracranial vasculature dynamics predict response. Archival primary and/or metastatic tissues are required from all patients to evaluate intrinsic breast cancer subtype and alterations in DNA repair genes. Among patients who consent for testing, BRCA1/2 status is correlated with response. Optional pre- and post-therapy (non-CNS) biopsies are expected from 25% of patients to assess *in vivo* effects on proliferation, apoptosis, and gene expression.

[0307] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention.

Abbreviations

[0308] "(%)TGD:" (percent) tumor growth delay; "AE:" Adverse event; "AUC:" Area under the plasma concentration-time curve; "BA:" 4-iodo-3-nitrobenzamide; "biwk to end:" twice weekly (dosing) for the duration of the study; "BUN:" Blood urea nitrogen; "BW:" body weight; "CBC:" complete blood count; "CHF:" Congestive heart failure; "CL:" Clearance; "Clr:" Renal clearance; "Cmax:" Maximum observed concentration; "CNS:" Central nervous system; "CO2:" Carbon dioxide; "CR:" complete regression or complete response; "CrCl24hr:" 24 hour creatinine clearance; "CRF:" Case report form; "CT:" Computed tomography; "CTC:" Circulating tumor cells; "CTCAE:" Common terminology criteria for adverse events; "Cut:" Concentration in urine for time; "D:" Day (of the study); "dC/dtmax:" Maximum rate of concentration change; "DCE-MRI:" Dynamic contrast enhanced magnetic resonance imaging; "DLT:" Dose limiting toxicity; "DNA:" Deoxyribonucleic acid; "ECHO:" Echocardiogram; "ECOG:" Eastern Cooperative Oncology Group; "EKG:" Electrocardiogram; "End of Study (EOS):" Last dose of 4-iodo-3-nitrobenzamide + 30 days; "Enrollment:" Study day 1 when 4-iodo-3-nitrobenzamide first administered; "FDA:" Food and Drug Administration; "FIH:" First in human; "GBM:" glioblastoma multiforme; "GCP:" Good clinical practice; "GLU:" Glucose; "HCT:" Hematocrit; "HED:" Human equivalent dose; "Hgb:" Hemoglobin; "HPBCD:" 25% hydroxypropyl-ß-cyclodextrin; "i.p.:" intraperitoneal(ly); "IC50:" 50% inhibitory concentration; "IC90:" 90% inhibitory concentration; "ICH:" International Conference on Harmonization; "In vitro:" In an artificial environment; "In vivo:" Within the living body; "iniparib:" 4-iodo-3-nitrobenzamide (BA); "IRB:" Institutional review board; "IV:" Intravenous; "K_2EDTA:" potassium ethylenediaminetetraacetic acid; "LD:" Longest diameter; "MedRA:" Medical Dictionary for Regulatory Submissions; "Mins:" Minutes; "MRI:" Magnetic resonance imaging; "MTD:" maximum tolerated dose; "MTV (n):" median tumor volume in mm3 of the number of animals, n,

remaining on the last day of the study; "MTV:" median tumor volume; "MUGA:" Multiple gated acquisition; "n:" number of mice in a group per protocol; number of evaluable mice in a group for analyses; "ne:" not evaluated; "NOAEL:" No observable adverse effect level; "ns:" not significant; "NTR:" non-treatment-related (death); "NTRm:" non-treatment-related (death) due to metastasis and/or tumor invasion; "NYHA:" New York Heart Association; "OMP:" miniature osmotic infusion pump; "p.o.:" by mouth (per os); "PARP:" poly (ADP-ribose) polymerase; "PD:" Pharmacodynamic or Progressive Disease; "PK:" Pharmacokinetic; "PLT:" Platelets; "PR:" partial regression or partial response; PT:" Prothrombin time; "PTT:" Partial thromboplastin time; "Q2W:" Twice weekly dosing; "qd x 5:" once daily (dosing) for five days; "QD:" Once daily dosing; "QT:" Part of electrocardiographic wave representing ventricular repolarization; "QTc:" Corrected QT; "Rad:" Randomization; "RBC:" Red blood cell; "RECIST:" Response evaluation criteria in solid tumor; "RNA:" Ribonucleic acid; "s.c.:" subcutaneous(ly); "SAE:" Serious adverse event; "Screening:" Point where subject signs the informed consent form; "SD:" Stable disease; "SMC:" Safety Monitoring Committee; "STD:" Severe toxic dose; "STD10:" One-tenth of the severe toxic dose; "Study Day 1:" Day 4-iodo-3-nitrobenzamide is first administered; "T1/2:" Terminal elimination half-life; "TBILI:" Total bilirubin (direct and indirect); "TFS:" tumor-free survivor(s); "TGI:" tumor growth inhibition; "Tmax:" Time to reach maximum plasma concentration; "TP:" Total protein; "TR:" treatment-related (death); "TTE:" time to endpoint; "ULN:" Upper limit of normal; "US:" Ultrasound; "WBC:" White blood cell.

CLAIMS

WE CLAIM:

1. A method of treating a patient with breast cancer brain metastasis comprising administering to the patient an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof and (b) irinotecan or a pharmaceutically acceptable salt thereof, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing.

2. The method of claim 1, wherein the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 5.6 mg/kg on days 1, 4, 8, 11 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

3. The method of claim 1, wherein the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 11.2 mg/kg on days 1 and 8 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

4. A method of treating locally advanced or metastatic breast cancer in a patient, comprising administering to the patient having locally advanced or metastatic breast cancer an effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof, wherein the method comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle.

5. The method of claim 4, wherein the patient has a breast cancer that is negative for human epidermal growth factor 2 receptor ("HER2-neu").

6. The method of claim 4, wherein the patient has a breast cancer that is positive for HER2-neu.

7. The method of any one of claims 4-6, wherein the patient has a breast cancer that is negative for estrogen receptor ("ER").

8. The method of any one of claims 4-6, wherein the patient has a breast cancer that is positive for ER.

9. The method of any one of claims 4-8, wherein the patient has a breast cancer that is negative for progresterone receptor ("PR").

10. The method of any one of claims 4-8, wherein the patient has a breast cancer that is positive for PR.

11. The method of any one of claims 4-10, wherein the breast cancer is locally advanced breast cancer.

12. The method of any one of claims 4-10, wherein the breast cancer is metastatic breast cancer.

13. The method of claim 12, wherein the metastasis comprises brain metastases.

14. The method of any one of claims 1-3 and 13, wherein the brain metastasis is at least about 0.5 centimeter.

15. The method of claim 14, wherein the brain metastasis is new brain metastasis after radiation therapy.

16. The method of claim 14, wherein the brain metastasis is progressive brain metastasis after radiation therapy.

17. The method of any one of claims 1 and 4-16, wherein irinotecan is administered at about 125 mg/m^2.

18. The method of claim 17, wherein 4-iodo-3-nitrobenzamide is administered at about 8 mg/kg on days 1, 4, 8, and 11 of the 21-day cycle, and wherein irinotecan is administered at about 125 mg/m^2 on days 1 and 8 of the 21-day cycle.

19. The method of any one of claims 1-18, wherein the patient has breast adenocarcinoma.

20. The method of any one of claims 4-10, wherein the breast cancer is locoregional.

21. The method of any one of claims 4-10, wherein the patient has distant metastasis.

22. The method of any one of claims 1-10, 12-19, and 21, wherein the patient has systemic metastasis.

23. The method of any one of claims 1-22, wherein the patient has received prior chemotherapy treatment comprising at least one regimen selected from the group consisting of an anthracycline, an anthraquinone, and a taxane.

24. The method of claim 23, wherein the patient is refractory to at least one regimen selected from the group consisting of an anthracycline, an anthraquinone, and a taxane.

25. The method of any one of claims 1-24, wherein 4-iodo-3-nitrobenzamide or the metabolite thereof or the pharmaceutically acceptable salt thereof is administered intravenously.

26. The method of any one of claims 1-25, wherein irinotecan or the pharmaceutically acceptable salt thereof is administered intravenously.

27. The method of any one of claims 1-26, wherein the method further comprises surgery, radiation therapy, chemotherapy, gene therapy, DNA therapy, viral therapy, RNA therapy, adjuvant therapy, neoadjuvant therapy, immunotherapy, nanotherapy or a combination thereof.

28. The method of claim 27, wherein the radiation therapy comprises administering to the patient gamma irradiation.

29. The method of any one of claims 1-28, wherein the effective amount produces at least one therapeutic effect selected from the group consisting of reduction in size of a breast tumor, reduction in metastasis, complete remission, partial remission, stable disease, and a pathologic complete response.

30. A kit for treating a patient with breast cancer brain metastasis comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing.

31. The kit of claim 30 further comprising a package insert or label containing instructions for using the effective amount of (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) irinotecan or a pharmaceutically acceptable salt thereof is administered to the patient to treat the patient with breast cancer brain metastasis.

32. The kit of claim 31, wherein the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 5.6 mg/kg on days 1, 4, 8, 11 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

33. The kit of claim 31, wherein the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 11.2 mg/kg on days 1 and 8 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

34. A kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) a package insert or label containing instructions for using an effective amount of 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, in combination with irinotecan or a pharmaceutically acceptable salt thereof to treat a patient with breast cancer brain metastasis, wherein the breast cancer is ER-negative, PR-negative, and HER2-nonoverexpressing.

35. The kit of claim 34, wherein the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 5.6 mg/kg on days 1, 4, 8, 11 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

36. The kit of claim 34, wherein the effective amount is administered over a 21-day treatment cycle, wherein 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof is administered to the patient at about 11.2 mg/kg on days 1 and 8 of the treatment cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 125 mg/m^2 on days 1 and 8 of the cycle.

37. A kit for treating locally advanced or metastatic breast cancer in a patient comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, (b) irinotecan or a pharmaceutically acceptable salt thereof, and (c) a package insert or label containing

instructions for using 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and irinotecan or a pharmaceutically acceptable salt thereof to treat locally advanced or metastatic breast cancer in the patient, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle.

38. A kit comprising (a) 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, and (b) a package insert or label containing instructions for using 4-iodo-3-nitrobenzamide or a metabolite thereof or a pharmaceutically acceptable salt thereof, in combination with irinotecan or a pharmaceutically acceptable salt thereof to treat locally advanced or metastatic breast cancer in a patient, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 21 days, wherein 4-iodo-3-nitrobenzamide or the pharmaceutically acceptable salt thereof is administered at about 8 mg/kg twice weekly for two weeks of the cycle, and wherein irinotecan or a pharmaceutically acceptable salt thereof is administered at about 80 mg/m^2 to about 125 mg/m^2 once weekly for two weeks of the cycle.

Figure 1

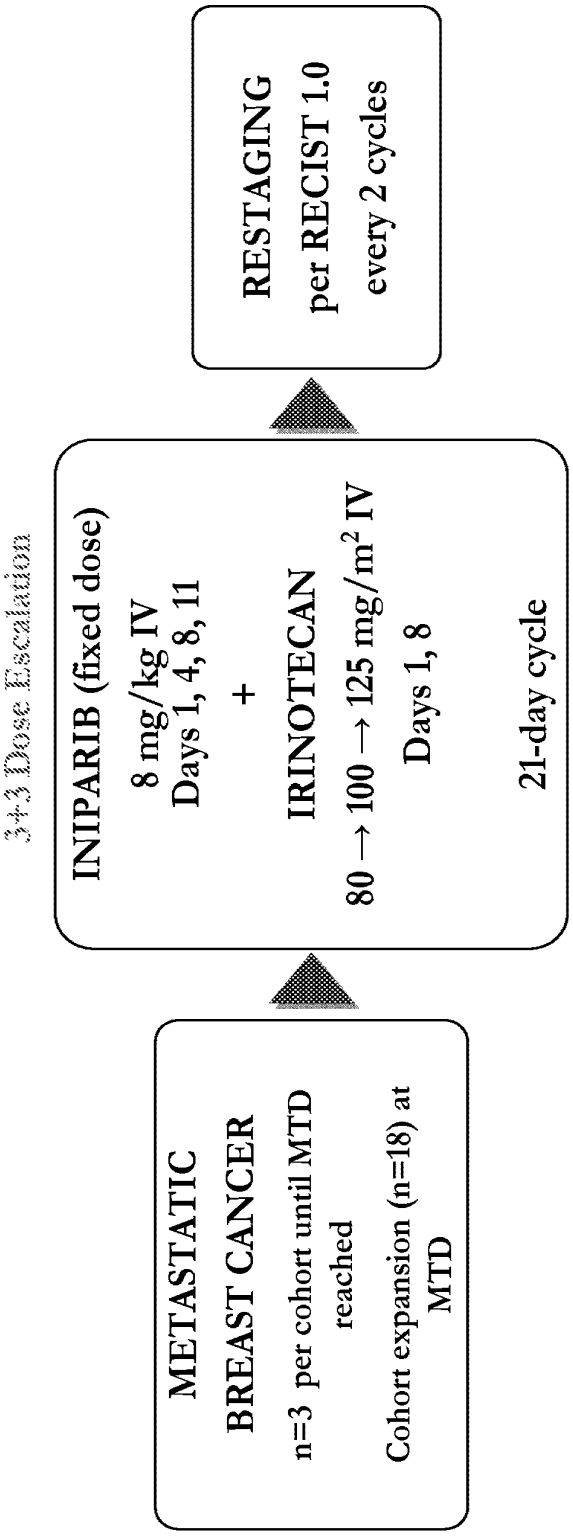
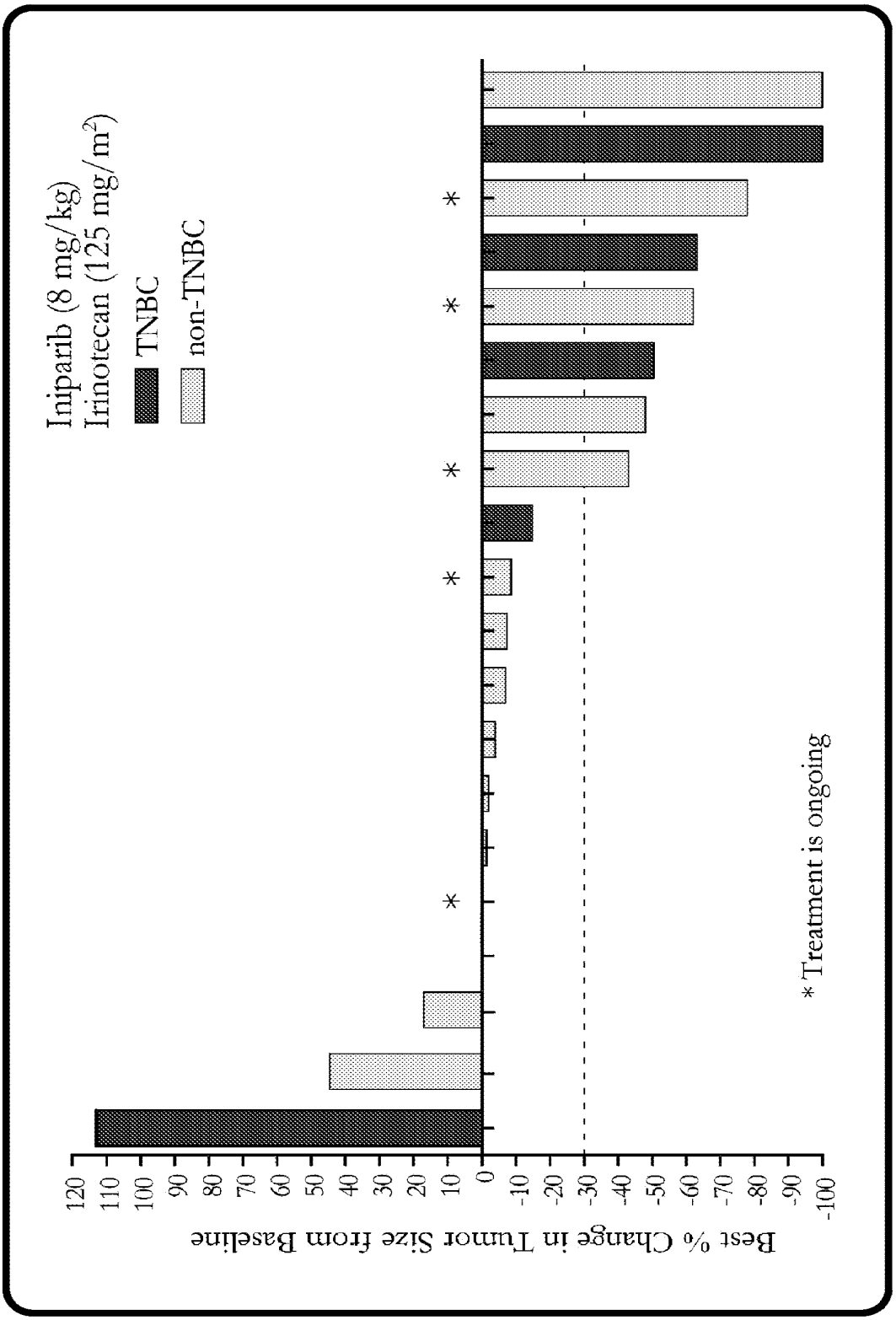
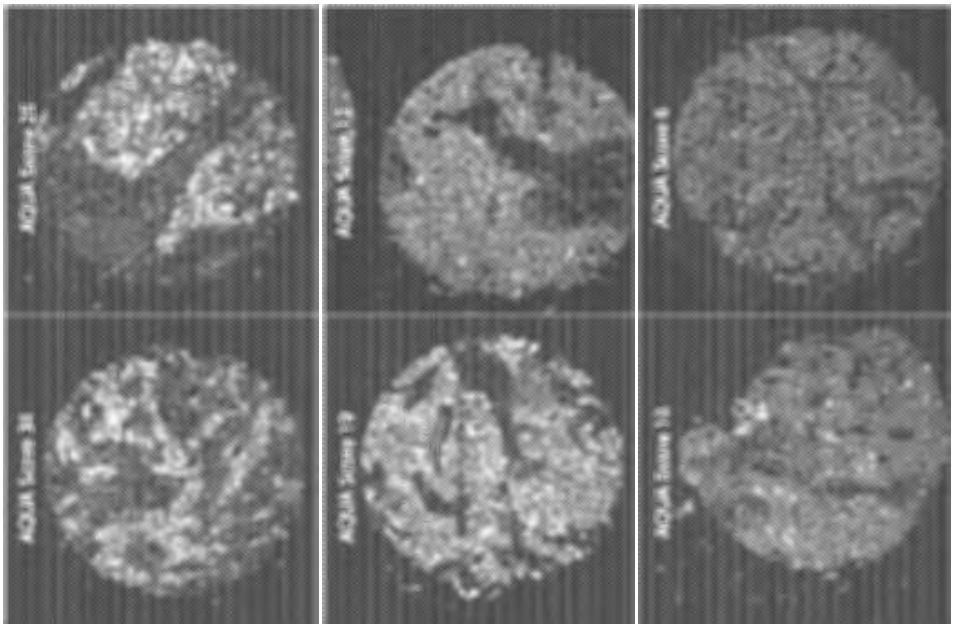

METASTATIC
BREAST CANCER
n=3 per cohort until MTD reached
Cohort expansion (n=18) at MTD
3+3 Dose Escalation
INIPARIB (fixed dose)
8 mg/kg IV
Days 1, 4, 8, 11
+
IRINOTECAN
80 → 100 → 125 mg/m² IV
Days 1, 8
21-day cycle
RESTAGING
per RECIST 1.0
every 2 cycles

Figure 2



Bars represent best single measurement of target lesion from each patient

Figure 3



IHC Staining for BRCA1 on Tumor Samples

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/044569

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61K 31/166 (2006.01) *A61P 35/00* (2006.01)
A61K 31/4745 (2006.01) *A61P 35/04* (2006.01)

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)

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)
EPOQUE (WPI, Medline, EPODOC): Iniparib, BSI201, 4-iodo-3-nitrobenzamide, nitrobenzamide, nitrosobenzamide, hydroxyaminobenzamide, benzamide, aminobenzoic acid, PARP inhibitor, irinotecan, Camptosar, Campto, Irinotecanum, CPT-11, topoisomerase 1 inhibitor, cancer
STN (HCAPLUS): Iniparib, BSI201, 4-iodo-3-nitrobenzamide, 160003-66-7/RN,PARP inhibitor, irinotecan, Camptosar, Campto, 97682-44-5/RN, CPT-11,topoisomerase 1 inhibitor

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 2009/0131529 A1 (SHERMAN, B.M. ET AL) 21 May 2009 paragraphs 0011, 0087, 0123, 0252-0256, 0477; example 3	30-38 1,19,22,25,29
Y	VREDENBURGH, J.J. *et al*, "Experience with irinotecan for the treatment of malignant glioma", *Neuro-Oncology*, 2009, Vol. 11, No. 1, Pages 80-91 Abstract	1,19,22,25,29

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

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 October 2011	06/10/2011
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **MS CORRINA PARKER** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6222 3661

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US NATIONAL INSTITUTES OF HEALTH, "A Study Evaluating INIPARINB in Combination With Chemotherapy to Treat Triple Negative Breast Cancer Brain Metastasis", 28 July 2010 [online], [retrieved on 15 August 2011]. Retrieved from the Internet <URL: http://www.clinicaltrials.gov/ct2/show/record/NCT01173497> Whole Document	1
X,P	UNC LINEBERGER COMPREHENSIVE CANCER CENTER, "Breast Cancer Brian Metastases – Overview of current and future therapeutic approaches", April 2011 [online], [retrieved on 23 August 2011]. Retrieved from Internet <URL: www.lbbc.org/content/download/3124/28007/.../**Brain**%20Mets_Anders.pdf > Slide 35	1,2

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	2009131529	AR	069291	AU	2008321128	AU	2008321382
		AU	2008333786	CA	2705417	CA	2705537
		CA	2708157	CN	101888777	CN	101917982
		CN	101918003	CO	6290649	CO	6290650
		DO	P2010000141	DO	P2010000142	EC	SP10010235
		EC	SP10010240	EP	2217227	EP	2217244
		EP	2224804	JP	2011503071	JP	2011503111
		JP	2011506343	KR	20100102607	KR	20100102609
		KR	20100102637	MX	2010005221	MX	2010005222
		MX	2010006154	US	7732491	US	2009123419
		US	2009149397	US	2010003192	US	2010009930
		WO	2009064444	WO	2009064738	WO	2009073869

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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A61K 9/127 (2006.01)

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6 December 2011 (06.12.2011)

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61/420,688	7 December 2010 (07.12.2010)	US
61/449,602	4 March 2011 (04.03.2011)	US

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(74) Agents: **RUSSETT, Mark, D.** et al.; Edwards Wildman Palmer LLP, P.O. Box 55874, Boston, MA 02205 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: DOSAGE AND ADMINISTRATION FOR PREVENTING CARDIOTOXICITY IN TREATMENT WITH ERBB2-TARGETED IMMUNOLIPOSOMES COMPRISING ANTHRACYCLIN CHEMOTHERAPEUTIC AGENTS

(57) Abstract: Methods for determining dosage of HER2-targeted anthracycline -containing immunoliposomes are disclosed, as are methods of treating cancer patients with HER2- positive tumors using dosages so determined. Upon administration, the dosages share the low cardiotoxicity profile of standard dosages of non-immunoliposomal (untargeted), anthracycline -containing liposomes.

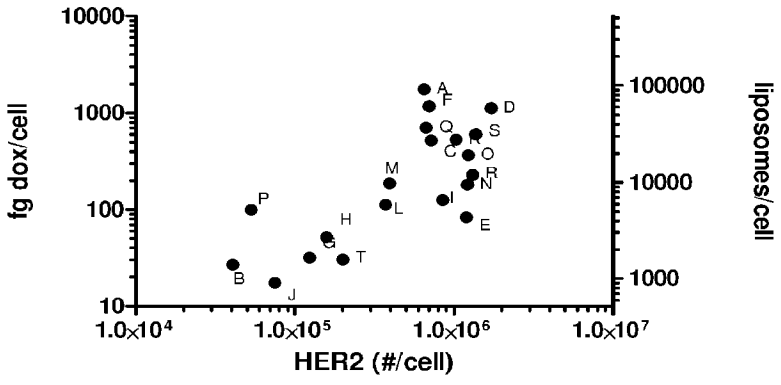


Figure 1A

WO 2012/078695 A2

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

DOSAGE AND ADMINISTRATION FOR PREVENTING CARDIOTOXICITY IN TREATMENT WITH ERBB2-TARGETED IMMUNOLIPOSOMES COMPRISING ANTHRACYCLINE CHEMOTHERAPEUTIC AGENTS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Patent Application Nos. 61/420,225, filed December 6, 2010; 61/420,688, filed December 7, 2010; and 61/449,602, filed March 4, 2011. The contents of each of the foregoing applications are incorporated herein by reference in their entitrety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK

NOT APPLICABLE

BACKGROUND OF THE INVENTION

Anthracyclines have been an effective backbone of cancer therapies for decades. Despite consistent clinical benefit observed with anthracycline-based regimens in breast cancer, significant toxicities such as acute and/or chronic cardiac dysfunction associated with such treatment have limited more expansive therapeutic use. While liposomal doxorubicin formulations have succeeded in reducing cardiotoxicity to some extent, they have failed to demonstrate clear-cut efficacy advantages and can involve other toxicities such as palmar-plantar erythrodysesthesia (hand foot syndrome). In an effort to improve upon efficacy of currently available anthracyclines, a new immunoliposomal formulation, MM-302, has been prepared that targets doxorubicin to HER2 (ErbB2)-overexpressing tumor cells. Antibody fragments that bind to HER2 without blocking HER2-mediated signaling are coupled to the outer surface of pegylated liposomal doxorubicin.

Doxorubicin (dox) is an anthracyline chemotherapeutic agent used to treat a variety of cancers. The use of doxorubicin is dose-limited by the cardiotoxicity of the drug. In order to address this problem, doxorubicin has been formulated as a pegylated liposomal preparation. Liposomal encapsulation of drugs enables delivery of potent cytotoxic drugs with an improved therapeutic index or therapeutic window. doxorubicin HCl liposome injection (DOXIL®) is a pegylated liposome-encapsulated (liposomal) form of doxorubicin. DOXIL is a commercial form of pegylated liposomal doxorubicin (PLD). DOXIL alters the tissue distribution and pharmacokinetic profile of doxorubicin. Use of DOXIL results in a

significantly lower rate of left ventricular cardiac dysfunction and symptomatic congestive heart failure as compared to therapy with free doxorubicin, both alone and in combination with trastuzumab in anthracycline-naïve and previously treated patients. DOXIL® is approved for use to treat Kaposi's sarcoma, ovarian cancer, and multiple mycloma. Doxorubicin HCl liposome injection is also sold as CAELYX®.

Immunoliposomes are antibody (typically antibody fragment) targeted liposomes that provide advantages over non-immunoliposomal preparations because they are selectively internalized by cells bearing cell surface antigens targeted by the antibody. Such antibodies and immunoliposomes are described, for example, in the following US patents and patent applications: US 2010-0068255, 6,214,388, 7,135,177, and 7,507,407 ("Immunoliposomes that optimize internalization into target cells"); 6,210,707 ("Methods of forming protein-linked lipidic microparticles and compositions thereof"); 7,022,336 ("Methods for attaching protein to lipidic microparticles with high efficiency") and US 2008-0108135 and 7,244,826 ("Internalizing ErbB2 antibodies."). The following US and international patents and patent applications describe assays, cell lines, and related technologies that are relevant to this disclosure: US 7,846,440 ("Antibodies against ErbB3 and uses thereof") and US 12/757,801, PCT/US2009/040259, and PCT/US2009/60721 ("Human Serum Albumin Linkers and Conjugates Thereof").

Immunoliposomes targeting ErbB2 (HER2) can be prepared in accordance with the foregoing patent disclosures. Such HER2 targeted immunoliposomes include MM-302, which comprises the F5 anti-HER2 antibody fragment and contains doxorubicin. MM-302 contains 45 copies of mammalian-derived F5-scFv (anti-HER2) per liposome. The F5-scFv was selected for its ability to internalize while not affecting HER2 signaling. Characterization of the F5-scFv indicates that it does not cross react with mouse, rat or rabbit HER2 and does not interfere with HER2 signaling in the free scFv form. Because cardiomyocytes are known to express HER2, concerns have been expressed regarding the potential cardiotoxicity of MM-302 and related HER2-targeted immunoliposomes.

Dosage and administration of commercially available liposomal doxorubicin:

DOXIL® (doxorubicin HCl liposome injection) is an exemplary liposomal anthracycline chemotherapeutic drug. DOXIL is typically administered intravenously at a dose indicated in mg/m^2 and characterized as doxorubicin HCl equivalent (dox equiv., meaning the total mass of doxorubicin in each dose). Each dose is typically administered at an interval measured in weeks, to yield a dosage of x mg/m^2 (dox equiv.) every y weeks. The first liposomal doxorubicin dose is typically administered at an initial rate of 1 mg/min to minimize the risk of infusion-related reactions. If no infusion-related adverse reactions are observed, the infusion rate is typically increased to complete the administration of the drug over one hour.

Patients With Ovarian Cancer:

DOXIL is typically administered to ovarian cancer patients intravenously at a dose of 50 mg/m^2 dox equiv. The patient is typically dosed once every 4 weeks, for as long as the patient does not progress, shows no evidence of cardiotoxicity and continues to tolerate treatment. A minimum of 4 courses is recommended because median time to response in clinical trials was 4 months. To manage adverse reactions such as hand-foot syndrome (HFS), stomatitis, or hematologic toxicity the doses may be delayed or reduced. Pretreatment with or concomitant use of antiemetics should be considered.

Patients With AIDS-Related Kaposi's Sarcoma (KS):

DOXIL is typically administered to KS patients intravenously at a dose of 20 mg/m^2 (dox equiv.). In KS patients the dose is typically repeated once every three weeks, for as long as patients respond satisfactorily and tolerate treatment.

Patients With Multiple Myeloma:

To treat patients with multiple myeloma, DOXIL is administered with VELCADE® (bortezomib). Bortezomib is administered at a dose of 1.3 mg/m^2 as intravenous bolus on days 1, 4, 8 and 11, every three weeks. DOXIL is typically administered to these patients at a dose of 30 mg/m^2 as a 1-hr intravenous infusion following each day 4 bortezomib administration. Patients are typically treated for up to 8 cycles until disease progression or the occurrence of unacceptable toxicity.

HERCEPTIN® (trastuzumab) is a therapeutic anti-HER2 antibody that is very widely used to treat HER2 overexpressing tumors. A key dosage-limiting effect of trastuzumab is cardiotoxicity. Cardiomyocytes are known to express HER2, and trastuzumab-mediated cardiotoxicity is generally accepted as being caused by damage to HER2-expressing cardiomyocytes resulting from trastuzumab binding to the cardiomyocyte-expressed HER2 -- see, e.g., Hysing J and Wist E, "Cardiotoxic Effects of Trastuzumab," . Tidsskr Nor Laegeforen, 2011 Nov 15;131(22):2239-2241. Anthracycline drugs such as doxorubicin are known to exert dose-limiting cardiotoxic effects, which are considered a major limitation in their use – see, e.g., Sawyer et al., "Mechanisms of Anthracycline Cardiac Injury: Can we identify strategies for cardio-protection?" Prog Cardiovasc Dis., 2010 Sep-Oct;53(2):105-13.

Doxorubicin-induced cardiac damage is irreversible, resulting in acute injury and also damage that can manifest itself years after treatment. Exposure to cumulative concentrations of doxorubicin above 550 mg/m^2 increases the potential for cardiomyopathy and heart failure. The development of HER2-directed therapy for the treatment of HER2-positive breast cancer has led to the investigation of the clinical benefit of the combination of doxorubicin and trastuzumab. The clinical efficacy of doxorubicin plus trastuzumab was superior to that of paclitaxel plus trastuzumab; however, there was an increased incidence of cardiac toxicity observed on the doxorubicin plus trastuzumab arm of the study, and the combination was not

approved for marketing. The clinical benefit of anthracycline-based therapy, specifically in HER2-positive breast cancer, remains controversial.

Liposomal encapsulation of drugs has enabled delivery of potent cytotoxic drugs with an improved therapeutic index. Pegylated liposomal doxorubicin (PLD) alters the tissue distribution and pharmacokinetic profile of doxorubicin. PLD has demonstrated a significantly lower rate of left ventricular cardiac dysfunction and symptomatic congestive heart failure as compared to therapy with conventional doxorubicin, alone and in combination with trastuzumab in anthracycline-naïve and previously treated patients. A proposed mechanism for the reduced cardiotoxicity of PLD is that its greater size relative to conventional doxorubicin prevents it from crossing the endothelial barrier in the heart, thereby minimizing doxorubicin exposure to heart tissue.

MM-302 is a HER2-targeted, pegylated liposome designed to deliver doxorubicin directly to HER2-overexpressing cancers. HER2-targeted PLD deposits in tumors through the enhanced permeability and retention effect similar to PLD. In the tumor microenvironment, targeting HER2-overexpressing cells with HER2-targeted PLD results in superior efficacy relative to PLD in preclinical models. During the development of MM-302, concern was expressed by regulatory authorities that due to its HER2-targeting, MM-302 would deliver cardiotoxic doxorubicin directly to cardiomyocytes, resulting in increased cardiotoxicity compared to doxorubicin HCl liposome injection, and reduced dosages of MM-302 were suggested to avoid such life-threatening toxicities.

BRIEF SUMMARY OF THE INVENTION

The present invention provides methods to determine safe doses and to safely use anti-HER2 immunoliposomal anthracyclins to treat HER2-expressing cancers, e.g., without increased risk of cardiotoxicity as compared to doxorubicin HCl liposome injection (DOXIL), and provides other advantages.

It has now been discovered that anti-ErbB2 targeted, anthracycline-containing immunoliposomes, e.g., MM-302, are not any more cardiotoxic than doxorubicin HCl liposome injection (DOXIL®), and can be dosed using exactly the same dosages,(i.e., dose and administration amounts and schedules) as used for doxorubicin HCl liposome injection without any increase in cardiotoxicity risk or decrease in efficacy. Furthermore, it has now been demonstrated that MM-302 can be effectively targeted to cells expressing 200,000 or more ErbB2 (HER2) receptors per cell *in vitro* and *in vivo*, indicating that it can be used to treat patients with HER2- overexpressing tumors that are either HER2 "3+" (e.g., by HERCEPTEST®), HER2 FISH+ (fluorescence in situ hybridization for HER2 gene amplification) or HER2 "2+" (e.g., by HERCEPTEST).

Therefore, disclosed herein are methods for determining a safe and effective dosage for use in treating a human cancer patient by administration of anthracycline-comprising anti-HER2 immunoliposomes, the patient being diagnosed with a cancer characterized by expression of HER2 receptor,

the methods comprising determining a first dosage, such a dosage indicating a dose magnitude and frequency of dosing, for a patient diagnosed with a cancer characterized by expression of HER2 receptor, the first dosage being for a liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome, which dosage is determined to provide to the patient a safe and effective amount of the liposomal anthracycline chemotherapeutic agent; and determining a dosage for the administration of the anthracycline-comprising anti-HER2 immunoliposomes, a plurality of which immunoliposomes is each bearing a plurality of anti-HER2 antibody molecules on its surface and each containing the anthracycline chemotherapeutic agent, where the safe and effective dosage for the administration of the anthracycline-comprising anti-HER2 immunoliposomes is the first dosage.

Also disclosed are methods of treating a human cancer patient by administration of anthracycline-comprising anti-HER2 immunoliposomes, the methods comprising determining a first dosage, such a dosage indicating a dose magnitude and frequency of dosing, for a patient diagnosed with a cancer characterized by expression of HER2 receptor, the first dosage being for a liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome, which dosage is determined to provide to the patient a safe and effective amount of the liposomal formulation, and administering anthracycline-comprising anti-HER2 immunoliposomes, a plurality of which immunoliposomes is each bearing a plurality of anti-HER2 antibody molecules on its surface and each containing the anthracycline chemotherapeutic agent, where the anthracycline-comprising anti-HER2 immunoliposomes are administered to the patient at the first dosage.

In certain aspects the anthracycline is doxorubicin.In other aspects the liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome is doxorubicin HCl liposome injection and the HER2-targeted immunoliposomes are MM-302. In others, the cancer is breast cancer, Kaposi's sarcoma, ovarian cancer, or multiple myeloma. In yet other aspects, the first dosage is 50 mg/m^2, 40 mg/m^2, 30 mg/m^2, 20 mg/m^2, or 10 mg/m^2 every two weeks or every three weeks or every four weeks. In other aspects the cancer characterized by expression of HER2 receptor is further characterized as being HER2^{2+}, HER2^{3+}, or HER2 FISH positive. In others the cancer characterized by expression of ErbB2 receptor is further characterized as expressing an average of at least 200,000 cell surface ErbB2 receptors per cell. In yet others, the administration of the immunoliposomes at the first dosage is effective to treat the cancer and in others the administration of the

immunoliposomes at the first dosage does not result in increased cardiotoxicity as compared to administration at the first dosage of the liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome. In other aspects, the administration of the immunoliposomes to the patient at the first dosage results in a peak concentration of the immunoliposomes in the patient's bloodstream, and treating human cardiomyocytes in vitro by culturing in medium comprising the immunoliposomes at about the peak concentration does not reduce, or reduces by no more than 5%, heregulin-stimulated increase of pERK or pAKT in the cultured cardiomyocytes as compared to in control human cardiomyocytes cultured in medium free of the immunoliposomes. In other aspects the immunoliposome concentration in the patient's bloodstream is measured as a serum immunoliposome concentration. In yet other aspects each of the HER2 immunoliposomes bears on its surface, on average, 45 anti-HER2 antibody molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Effect of Her2 Levels on Uptake: Multiple cell lines expressing various levels of HER2 were treated with 15 μg/ml of MM-302 (A) and untargeted pegylated liposomal dox (UT-PLD) (B) for 2h and total cellular doxorubicin was quantified by HPLC. The y-axes represent femtograms dox per cell (left) and liposomes per cell (right)and the x-axes represent the number of HER2 receptors per cell (log scale). Mouse tumor 4T1 cells (C) and endogenously low HER2 expressing HeLa cells (D) were transfected with human HER2 to generate stable clones with varying levels of expression. Individual clones (represented by triangles, circles, or squares) were treated with F5- targeted liposomes containing a fluorescent marker (DiI5-F5-PL), and total binding/uptake was determined by FACS.

Figure 2. (A) HER2-overexpressing BT474-M3 cells were treated with 15 μg/ml of MM-302 (circle), PLD (square), and free doxorubicin (triangle) for the indicated times (x-axis, in min.). Total cellular doxorubicin was quantified by HPLC (y-axis, femtograms/cell). (B) Nuclear doxorubicin delivery was quantified by high content microscopy (y-axis, signal per cell above background) 24 h following the indicated incubation times (x-axis, in min.). (C) The anti-tumor activity of MM-302 and PLD were compared in a BT474-M3 orthotopic breast cancer model. Both MM-302 (square) and PLD (triangle) significantly inhibited tumor growth compared to control (circle) (t-test at day 55; $p<0.0001$). MM-302 resulted in a stronger inhibition of tumor growth relative to PLD (t-test at day 55; $p=0.0310$). The y-axis indicates tumor volume in mm^3 and the x-axis indicates days after inoculation. Figure 2D shows pharmacokinetics of MM-302 and UT-PLD in BT474-M3 xenografts administered with 3

mg/kg or 6 mg/kg (dox equiv) at q7d. The y-axis is µg/ml dox in plasma and the x-axis is time in hours.

Figure 3. Role of HER2 Levels *in vivo*: Mice bearing BT474-M3 xenograft tumors in the mammary fat pad were injected with DiI5-labeled MM-302 (DiI5-F5-PLD) or UT-PLD (DiI5-UT-PL). A tumor single cell suspension is prepared and stained with FITC-HER2 antibodies. DiI5-positive-HER2-positive cells were determined by FACS. (A) A graph showing HER2 level (receptors per cell, x-axis) as a function of liposome binding to tumor cells (percent cells positive, y-axis). (B) A graph demonstrating the heterogeneity of HER2 expression on a single cell basis as measured in tumor tissue sections.

Figure 4. Uptake of MM-302 (circle), PLD (square) and doxorubicin (triangle) was measured in human cardiomyocytes. Embryonic stem cell-derived (ESCd) (A) and induced pluripotent stem cell-derived (iPSd) (B) cardiomyocytes were treated with 15 µg/ml of MM-302, PLD and free doxorubicin for the indicated times. Total cellular doxorubicin was quantified by HPLC. The y-axes represents uptake in femtograms/cell and the x-axes represent incubation time in min. (C) Cell viability: ESCd cardiomyocytes were treated for 3 h with drug at the indicated concentrations and incubated for an additional 24 h with fresh media and cell viability was assessed. The y-axis represents cell viability as % compared to control and the x-axis represents concentration in µg/ml. (D) Cell viability: iPSd cardiomyocytes were treated with the indicated concentration of free doxorubicin (circle), PLD (square) or MM-302 (triangle) for 24 hours. The supernatant was collected and a PrestoBlue® cell viability assay was performed on the remaining cells. All values were normalized to the untreated population. (E) A human Troponin I ELISA was performed on the supernatant collected in (D). The untreated line (dotted line) represents the value of soluble Troponin I detectable in untreated wells. All values are normalized against dilutions of a supplied standard.

Figure 5. ESCd cardiomyocytes were treated for 3 h with MM-302 (circle), PLD (square), and free doxorubicin (triangle) at the indicated concentrations and then incubated for an additional 24 h with fresh media. Cells were stained and imaged using high-content microscopy. Single cell intensity for each stain was quantified and represented as the mean relative intensity of individual cells. Cells were stained for the DNA damage marker gamma-H2AX (A). In addition, cells were stained for the cell stress proteins phospho-p53 (B) and phospho-HSP27 (C), and the cleaved form of the apoptosis protein PARP (cPARP) (D). The y-axes represent relative intensity and the x-axes represent concentration in µg/ml.

Figure 6. (A) F5 scFv alone, or F5 scFv bound to empty (i.e. without encapsulated dox) liposomes ("F5 lipo" - liposomes equivalent to MM-302 but not containing doxorubicin) minimally decrease basal pERK levels and do not decrease heregulin stimulated pERK levels in iPS-derived human cardiomyocytes, while Herceptin® (trastuzumab) decreases basal levels to a greater extent and both trastuzumab and lapatinib decrease heregulin stimulated levels to a greater extent than do F5 scFv or F5 lipo. (B) None of the tested agents changed basal pAKT levels in these cells and that trastuzumab and lapatinib both decrease heregulin stimulated levels to a significantly greater extent than do F5 scFv or F5 lipo.

Figure 7. (A) The biodistribution of MM-302 (square), PLD (triangle) and free doxorubicin (circle) was studied. NCI-N87 tumor bearing mice (n=4/time point/group) were given a single dose (3 mg/kg) of either doxorubicin, MM-302 or PLD. Mice were sacrificed at 0.5, 4 and 24 h post injection and the doxorubicin accumulation in heart tissue (A), tumor tissue (B) and paw (C) was quantified by HPLC. (D) MM-302 induces lower nuclear doxorubicin accumulation in heart tissue compared to free doxorubicin, and comparable to PLD. Nu/nu mice were injected intravenously with MM-302-DiI5, PLD-DiI5, and free doxorubicin at 3 mg/kg (dox equiv.). At the designated time points, hearts were collected for the preparation of cryosections to analyze the microdistribution of liposomes and doxorubicin. FITC-lectin was injected to visualize functional/perfused blood vessels. Heart sections were counterstained with Hoechst and imaged by confocal fluorescence microscopy (40X magnification). Doxorubicin positive nuclei are shown. Doxorubicin positive nuclei are visible in the free doxorubicin treated samples at 0.5 h and 4 h and not in MM-302 treated samples (see "merge panels"). (E) A higher magnification (2x) of the overlay of the nuclei and doxorubicin signal of the above fields for the 0.5 h time points for doxorubicin and MM-302 is shown. (F) The percent of doxorubicin positive nuclei was quantified using Definiens® Developer XD™ (F).

Figure 8: Modeling: A computational model was developed and calibrated on literature and in-house data for free and liposomal doxorubicin. It is applied to understand the competing kinetic processes that determine drug concentration and exposure for liposomal versus free doxorubicin in various tissues. (A) PK and Biodistribution: In contrast with free doxorubicin, liposomal delivery results in a much longer circulation time. (B) Tissue Deposition: The model is able to capture typical liposome deposition data in mouse. (C) Microdistribution: Kinetic modeling is able to provide a framework for understanding the role of HER2 expression in MM-302 uptake and doxorubicin cellular trafficking.

DETAILED DESCRIPTION OF THE INVENTION

An MM-302 liposome is a unilamellar lipid bilayer vesicle of approximately 75-110 nm in diameter that encapsulates an aqueous space which contains doxorubicin in a gelated or precipitated state. The lipid membrane is composed of phosphatidylcholine, cholesterol, and a polyethyleneglycol-derivatized phosphatidylethanolamine in the amount of approximately one PEG molecule for 200 phospholipid molecules, of which approximately one PEG chain for each 1780 phospholipid molecules bears at its end an F5 single-chain Fv antibody fragment that binds to HER2. MM-302 liposomes are prepared from HSPC (Hydrogenated soy phosphatidylcholine):Cholesterol (plant-derived):PEG-DSPE (polyethylene glycol-disteroylphosphoethanolamine) at a molar ratio of 3:2:0.3. The total HSPC lipid concentration of MM-302 is about 40 mmol/L. MM-302 contains about 10 mmol/L of lipid, and about 2 mg/mL of doxorubicin. MM-302 comprises 1.8-2.2 mg/mL of doxorubicin in liposomes that contain 0.16-0.30 mg/mL DSPE-PEG-F5 (prepared as described in US 6,210,707). F5 is an anti-ErbB2 (HER2) scFv antibody fragment (encoded by ATCC plasmid deposit designation PTA-7843). MM-302 liposomes comprise 130-170 g doxorubicin / mol phospholipid and 12-22g F5-PEG-DSPE / mol phospholipid. MM-302 is formulated in sterile 10 mM/L histidine-HCl as a buffer (pH 6.5), and 10% sucrose to maintain isotonicity. MM-302 liposomes are loaded using pre-loaded ammonium sulfate

MM-302 dosing

	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5
Every week	10 mg/m^2	20 mg/m^2	30 mg/m^2	40 mg/m^2	50 mg/m^2
Every two weeks	10 mg/m^2	20 mg/m^2	30 mg/m^2	40 mg/m^2	50 mg/m^2
Every three weeks	10 mg/m^2	20 mg/m^2	30 mg/m^2	40 mg/m^2	50 mg/m^2
Every four weeks	10 mg/m^2	20 mg/m^2	30 mg/m^2	40 mg/m^2	50 mg/m^2
Every five weeks	10 mg/m^2	20 mg/m^2	30 mg/m^2	40 mg/m^2	50 mg/m^2

"mg/m^2" indicates mg of doxorubicin (formulated as MM-302) per square meter of body surface area of the patient. For breast cancer, dose 3, 4, or 5 is preferred. For Kaposi's sarcoma dose 1, 2, or 3 is preferred, for ovarian cancer, dose 3, 4, or 5 is preferred and for multiple myeloma dose 2, 3, 4, or 5 is preferred. Dosing regimens may vary in patients with solid tumors that are "early" (pre-metastatic, e.g., adjuvant breast cancer) as compared to "advanced" (metastatic tumors). Preferred tumors are those in which the tumor cells overexpress HER2. A tumor that overexpresses HER2 is one that is identified as being HER2 "3+" or HER2 "2+" by HercepTest™, or HER2 FISH+ by fluorescence in situ hybridization.

Alternatively, a preferred tumor that overexpresses HER2 is one that expresses an average of 200,000 or more receptors per cell, as quantified by the methods described in the Examples.

MM-302 therapy of advanced breast cancer

MM-302 is administered once every 4 weeks by intravenous (IV) infusion over 60 minutes at 8, 16, 30, 40, or 50 mg/m^2 to patients with locally advanced/unresectable or metastatic advanced breast cancer that overexpresses HER2 as determined by FISH or by IHC or by determination of the average number of HER2 receptors per cell. Patients should have adequate bone marrow reserves as evidenced by: 1) absolute neutrophil count (ANC) ≥ 1,500/μL; 2) platelet count ≥ 100,000/μL and 3) hemoglobin ≥ 9 g/dL (Transfusions allowed). Patients should have adequate hepatic function as evidenced by:1) serum total bilirubin ≤1.5x ULN and 2) Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline Phosphatase (ALP) normal or up to 2.5 x upper limit of normal (ULN; 5 x ULN is acceptable for ALP if liver metastases and/or bone metastases are present). Patients should have adequate renal function as evidenced by a serum creatinine ≤ 1.5 x ULN. Patients should be recovered from any clinically relevant toxic effects of any prior surgery, radiotherapy or other therapy intended for the treatment of breast cancer. Women of childbearing potential as well as fertile men and their partners must be warned to abstain from sexual intercourse or to use an effective form of contraception during treatment and for 90 days following the last dose of MM-302. Patients should have adequate cardiac function as evidenced by a measured left ventricular ejection fraction of ≥ 50% by ECHO or MUGA within approximately 30 days of treatment. Patients who are pregnant or lactating and those with NYHA Class III or IV congestive heart failure or left ventricular ejection fraction (LVEF) < 50%, or a prolonged QTc interval (≥ 460 ms), are preferably not be treated with MM-302.

The following Examples are merely illustrative and should not be construed as limiting the scope of this disclosure in any way as many variations and equivalents will become apparent to those skilled in the art upon reading the present disclosure.

EXAMPLES

Materials and Methods Used in these Examples:

Materials: Doxorubicin is from SIGMA-ALDRICH, Inc. (St. Louis, MO). FITC-conjugated lectin (lycopersicon esculentum (tomato) lectin, Cat # FL-1171) is purchased from Vector Laboratories, Inc. (Burlingame, CA). Acetic acid, Methanol, and Acetonitrile are from EMD Chemicals Inc. (Gibbstown, NJ). Water and Trifluoroacetic Acid (TFA) are from J. T. Baker (Phillipsburg, NJ). HOECHST 33342 trihydrochloride trihydrate, ProLong Gold®, and

DiIC18(5)-DS (DiI5) are from Invitrogen (Carlsbad, CA). Cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (PEG-DSPE) are from Avanti Polar Lipids Inc. Hydrogenated soy phosphatidylcholine (HSPC) is from Lipoid (Newark, NJ). RPMI is from Lonza (Walkersville, MD), Fetal Bovine Serum (FBS) is from Tissue Culture Biologicals and penicillin G/streptomycin sulphate mixture is from GIBCO (Invitrogen).

Preparation of immunoliposomes: Liposomes are prepared and loaded with doxorubicin using an ammonium sulfate gradient as previously described (Kirpotin et. al., Cancer Res. 2006;66:6732-40; Park et al., Clin Cancer Res. 2002;8:1172-81). The lipid components are HSPC, cholesterol, and PEG-DSPE (3:2:0.3, mol:mol:mol). The anti-ErbB2 (F5)-PEG-DSPE conjugate is prepared and inserted into the liposome to form immunoliposomes as reported by Nellis et al., (Biotechnol Prog. 2005;21:205-20; Biotechnol Prog. 2005;21:221-32). The DiI-5-labelled liposomes, MM-302-DiI5 and PLD-DiI5, are prepared as above with the difference that the DiIC18(5)-DS (DiI5) dye is solubilized with the lipid components at a concentration of 0.3 mol % of total phospholipid. In all cases unloaded free doxorubicin is removed using a Sephadex® G-75 size exclusion column eluted with Hepes buffered saline (pH 6.5). F5-lipo-DiI5 is prepared in a similar fashion as above but without doxorubicin, and incorporating an aqueous solution of HEPES buffered saline (pH 6.5).

Tumor cell culture: BT474-M3 cells (see Noble, Cancer Chemother. Pharmacol. 2009 64:741-51), are HER2-overexpressing human breast cancer cells. BT474-M3 cells are grown in RPMI medium containing 10% FBS and 1% penicillin G/streptomycin sulphate. Embryonic stem cell-derived (ESCd) cardiomyocytes are obtained from P.W. Zandstra, Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada; (Bauwens et al., Tissue Eng Part A. 2011 Apr 25, PMID 21417693;). These cells have been shown to express appropriate cellular markers of cardiomyocytes such as LIM domain homeobox gene Isl-1, Troponin T, and Myosin Light Chain 2c. The percentage of Troponin T positive cells is determined following differentiation. Batches containing less than 70% positive for Troponin T are discarded. Induced pluripotent stem cell-derived (iPSd) cells are obtained from Cell Dynamics International and are handled per the manufacturer's protocol.

Xenograft Studies: 5-7-week-old female nude mice are purchased from Charles River Laboratories or Taconic Farms, Inc (NCr nude mice). Unless otherwise indicated, mice are inoculated with BT474-M3 breast cancer cells or NCI-N87 gastric cancer cells (NCI-DTP, 10×10^6 cells in 100 µl RPMI) into the right dorsal flank of the mice (subcutaneous injection,

s.c.). When the tumors reach an average volume of ~200 mm^3, studies are performed as described below.

Testing of tumor HER2 levels: Homozygous NCr nude mice are are inoculated with 15 x 10^6 BT474-M3 cells in the mammary fat pad 2nd from the top right hand side.. BT474-M3 tumors are injected with a single dose of 4 mg/ml fluorescently-labeled (with DiI5 as described below) HER2-targeted or untargeted liposomes without doxorubicin. Twenty-four hours later, the tumors are excised and dissociated by mechanical and enzymatic means. After surface staining with anti-HER2 cells are analyzed by flow cytometry on a FACSCalibur™ instrument (BD Biosciences). The flow cytometry dataset is analyzed for the relationship between HER2 surface expression levels and uptake of liposomes above a given threshold by plotting the overall percentage of cells with elevated liposome content in narrowly gated cell subsets defined by increasing HER2 signals.

Single cell distribution of cell surface HER2: BT474-M3 tumor xenograft tissue is stained with an anti-HER2 antibody and counterstained with DAPI. The slide is imaged with an Aperio® Scanscope® at 20X magnification and the image is analyzed. The intensity of the HER2 membrane staining is quantified on a single-cell basis as the (mean of the inner border of the HER2 layer) + (mean of the outer border of the HER2 layer).

Efficacy study: Mice are randomized into three treatment groups (n=7/group) based on an average tumor volume from mice that receive PBS (control), MM-302 or PLD, dosed at 3 mg/kg (q7d, n=3 total doses). Tumors are measured twice/week with a caliper. Tumor volumes are calculated using the formula: $width^2 \times length \times 0.52$. Mice are weighed twice/week to monitor weight loss.

Uptake of liposomes in HER2-expressing cell lines: Multiple cell lines expressing various levels of HER2 are treated with 15 µg/ml of MM-302 or PLD for 2h and total cellular doxorubicin is quantified by HPLC. Murine 4T1 breast cancer cells and human HeLa cervical cancer cells are obtained from the ATCC and propagated according to ATCC recommendations. Cells are characterized for human HER2 expression by flow cytometry using a commercial anti-HER2 antibody (BD Biosciences #340552). This antibody does not cross-react with murine HER2 but detects human HER2. A neomycin-selectable expression vector encoding human HER2 is obtained from GeneCopeia (Z2866). 4T1 and HeLa cells are transfected with this construct using the non-lipid polymer transfection reagent MegaTran® 1.0 (Origene) according to the manufacturer's instructions. Transfected cells are selected with 400-500µg/ml Geneticin/Neomycin (Invitrogen). Surviving cells are allowed to expand under

reduced Geneticin/Neomycin concentrations (100 µg/ml) and are sorted on a BD Biosciences FACSAria™ instrument to obtain enriched cell populations with human HER2 expression exceeding those observed in parental HeLa cells. The sort-enriched cells are then sub-cloned by limited dilutions, and colonies are ranked by HER2 surface levels to obtain representative populations of 4T1 and HeLa cells that express different ranges of HER2. Fluorescent intensity of HER2 surface staining measured by flow cytometry is compared to staining with the same antibody bound to Quantum™ Simply Cellular® anti-mouse IgG microspheres (Bangs Laboratories #815) according to the manufacturer's instructions to calculate the number of HER2 surface receptors of the cells.

Uptake of liposomes in cardiomyocytes: iPSd cardiomyocytes are plated per the manufacturer's instructions (Cell Dynamics International Cat# CMC-100-110-001) in a 24-well tissue culture plate at 250,000 cells/well. Two days later, the 0.5ml of media in the wells is removed and replaced with 0.5ml of 15.0 µg/ml (dox equiv.) of MM-302, PLD or free doxorubicin. The plates are swirled in a "figure 8 fashion" 20 times to maximize exposure of the cells to the liposomes. Cells are incubated with MM-302, PLD or free doxorubicin for the indicated time period after which the media are removed and the cells are washed once with 0.5ml of PBS. The PBS is removed and 0.5ml of 0.05% trypsin is added to each well. The cells are monitored, and once detachment begins, 0.5ml of medium containing FBS is added to each well to inactivate the trypsin. The cells are collected and placed in microcentrifuge tubes. The cells are spun at 1,500 rpm for 5 minutes at 4°C. The cell pellet is resuspended by vortexing and pipetting in 0.5ml of 1.0% acetic acid in methanol and placed at -80°C for 1 hour to extract the doxorubicin. The microcentrifuge tube containing the resuspended cell pellet is spun at 10,000rpm for 10 minutes in the cold room, and 450µl of the supernatant is transferred to a HPLC tube. Samples are run on the HPLC machine and concentration per sample is determined by relating values to a doxorubicin standard curve.

Biodistribution of liposomes: Mice are randomized into 4 groups that received a single intravenous (i.v.) dose of either PBS, doxorubicin, MM-302-DiI5 or PLD-DiI5 (all at 3 mg/kg), respectively. Mice (n=4/time point/group) are sacrificed at 0.5, 4, 24 and 96 h (doxorubicin) or 168 h (MM-302-DiI5 and PLD-DiI5) after the single dose. Five minutes before sacrificing, mice are injected i.v. with 100 µl of FITC-lectin, to label the vasculature.

HPLC quantification of doxorubicin: Heart tissues are weighed and disaggregated with 1 mL H_2O using a TissueLyser™ (Qiagen) for 3 min. 100 µl of the homogenate is then transferred into a new tube and 900 µl of 1% acetic acid/methanol is added. For the cultured cells, cells are treated with drug, as described above, trypsinized and lysed using 1.0% acetic

acid in methanol. Lysates are vortexed for 10 sec. and placed at -80°C for 1 h. Samples are spun at room temperature (RT) for 10 min at 10,000 RPM. Supernatants and doxorubicin standards are analyzed by HPLC (Dionex) using a C18 reverse phase column (Synergi™ POLAR-RP® 80Å, 250x4.60mm 4µm column). Doxorubicin is eluted running a gradient from 30% acetonitrile; 70% 0.1% trifluoroacetic acid (TFA)/H_2O to 55% acetonitrile; 45% 0.1% TFA/H2O during a 7 min span at a flow rate of 1.0 ml/min. The doxorubicin peak is detected at 6.5 min using an in-line fluorescence detector excited at 485 nm, and emitting at 590 nm. The extraction efficiency of doxorubicin from the heart tissue was 83% as determined by a control heart spiked with a known amount of doxorubicin.

Confocal microscopy and image analysis of heart snap-frozen sections: 10 µm-thick heart sections are air-dried for 30 min at RT, counterstained with Hoechst® 33342 diluted 1:10,000 in mounting media (ProLong® Gold, Invitrogen) and mounted. Slides are imaged on a LSM 510 Zeiss® confocal microscope equipped with Enterprise (351, 364 nm), Argon (458, 477, 488, 514 nm), HeNe1 (543 nm) and HeNe2 (594 nm) lasers with a Plan-Neofluar® 40x/1.3 oil DIC objective. Image analysis and quantification of nuclear doxorubicin is carried out using Definiens® Developer XD™ (Definiens, Parsippany, NJ). Nuclei are segmented in the Hoechst channel. Doxorubicin positive nuclei are segmented in the doxorubicin channel. The percentage of doxorubicin positive nuclei is quantified as a ratio of the number of objects in the doxorubicin channel divided by the total nuclei objects in the Hoechst channel.

Receptor quantification: Stem cell-derived cardiomyocytes are trypsinized, washed, and HER2 levels are determined as described above under "**Uptake of liposomes in HER2-expressing cell lines.**"

Viability: ESCd cardiomyocytes are treated for 3 h at the indicated concentrations of MM-302, PLD and doxorubicin. Cells are washed twice with PBS, fresh medium is added and the cells are incubated for an additional 24 h. Cell viability is assessed using CellTiter-Glo® from Promega (Madison, WI) and the percent of viable cells is determined relative to the untreated population.

Troponin I ELISA: 15,000 iPSd (iCELL®) cardiomyocytes (Cellular Dynamics International, Madison WI) cells are plated per the manufacturer's protocol. Cells are treated for 24 h with the indicated concentrations of free doxorubicin, PLD or MM-302. The supernatant is collected and analyzed using a human Troponin I ELISA (Catalog #: GWB-83A61F, Genway Biotech, San Diego CA) per the manufacturer's protocol. A PrestoBlue®

Cell Viability Assay (Catalog #: A-13261, Invitrogen, Grand Island, NY) is performed on the remaining cells in 100µl per the manufacturer's protocol.

High-Content Analysis: Cardiomyocytes are treated as described above. Cells are fixed using 3.7% formaldehyde, washed twice with PBS containing 0.1% Tween-20 (PBS-T), and permeabilized with methanol. Cells are blocked using a 1:1 mixture of LI-COR® Odyssey® Blocking Buffer (Lincoln, NE) and PBS-T for 1 h at room temperature (RT). Cells are stained with a 1:400 dilution of the indicated primary antibody from Cell Signaling Technology (Beverly, MA) and incubated shaking at 4°C overnight. Cells are washed and incubated with a 1:2,000 dilution of the fluorescently labeled secondary antibody for 1 h at RT. Cells are stained with a 1:10,000 dilution of Hoechst 33342 and 1:1,000 dilution of Whole Cell Stain from Pierce Protein Research Products (Rockford, IL) for 30 min at RT to allow visualization of DNA and the whole cell, respectively. Plates are scanned using the Applied Precision Instruments ArrayWorx® High Content Scanner (Issaquah, WA) with a 10x objective for Hoechst 33342/Whole Cell Stain (460 nm), doxorubicin (595 nm), and APC/DiI5 (657 nm). Images are analyzed using the software ImageRail (as described in Millard et al., Nat Methods. 2011;8:487-92). An intensity threshold is established for nuclear and whole cell signals. This threshold is then applied to all images and used to segment individual cells. Data is presented as the mean pixel intensity for all cells in a given well for the indicated channel.

Signaling in cardiomyocytes: iPS-derived cardiomyocytes (iCell™ iPS-derived human cardiomyocytes - Cellular Dynamics International (CDI), Madison, Wisconsin - CDI # CMC-100-010-001, Lot 1258680) are cultured using iCell™ Plating Medium (CDI # CMM-100-110-005, Lot 1013740 and iCell™ Maintenance Medium (CDI # CMM-100-120-005, Lot 1000305) and are exposed to components of MM-302. Levels of phospho-AKT (pAKT) and phospho-ERK (pERK) in the cardiomyocytes are then measured using immunostaining and high content microscopy. Cells are pretreated for 24 hours with either trastuzumab, lapatinib, or the MM-302 antibody (F5-scFv) and an MM-302 molecule (liposome) not containing doxorubicin (F5-lipo) at an equivalent concentration to 5.0ug/ml of MM-302. Cells are stained and imaged using high content microscopy as described above for (A) pAKT and (B) pERK following a10 minute stimulation with 10nM and 5nM of heregulin, respectively. The following primary antibodies are used at a 1:400 dilution in blocking buffer: pERK antibody - Cell Signaling Technology (CST - Danvers Massachusetts) - Catalog 9106L (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse mAb #9106); pAKT antibody - CST - Catalog 4060L (Phospho-Akt (Ser473) (D9E) XP™ Rabbit mAb #4060). Secondary antibodies are Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) – CST – Catalog 4410; Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647

Conjugate) – CST – Catalog 4414. Whole cell stain and DNA stain (Hoechst 33342) are used as described above. Images are analyzed and individual cells are segmented on the basis of Hoechst 33342 nuclear staining. Single cell signal intensity for each stain is quantified and represented as the mean relative intensity of individual cells.

The results in the following Examples were obtained using the above methods or minor variations thereof. Cellular uptake studies in tumor cell lines expressing various levels of HER2 demonstrate that MM-302 delivers significantly higher doxorubicin levels to HER2 over-expressing tumor cells compared to PLD as well as similar or higher levels than highly permeable free doxorubicin. However, in human cardiomyocytes, while free doxorubicin was again taken up at high levels, doxorubicin uptake was dramatically lower with both MM-302 and PLD. Pharmacokinetic studies in mice demonstrate that MM-302 has a similar half-life, clearance, and organ distribution compared to PLD. In HER2-overexpressing BT474 breast and NCI-N87 gastric tumor xenografts, MM-302 exhibits superior anti-tumor activity to both free anthracyclines and PLD. Tumor microdistribution studies further suggest that differences in the localization of doxorubicin in the tumor may be responsible for the enhanced activity of MM-302 compared to free doxorubicin and PLD.

Example 1: Correlation between HER2 expression and MM-302 uptake *in vitro*: The level of cell surface HER2 expression on multiple cell lines was determined as described above. These same cell lines were then treated with 15 μg/ml of MM-302 (Figure 1A, Table 1) or PLD (Figure 1B, Table 2) for 180 minutes, after which the cells were collected and the amount of cell associated doxorubicin was quantified by HPLC. By plotting the number of HER2 receptors per cell for each cell line vs. the quantity of doxorubicin present per cell in that same cell line following treatment, a relationship between increasing HER2 levels and increasing doxorubicin becomes evident. Through this representation, there appears to be an inflection point at approximately 200,000 HER2 receptors per cell where cells expressing greater than this number appear to have consistently higher levels of cell associated doxorubicin. Taken together, these results support the specificity of MM-302, with high uptake by cells expressing levels of HER2 above the inflection point (such as HER2 overexpressing cancers) and no-to-minimal uptake in cells expressing levels of HER2 below the inflection point (such as cells in normal tissues, e.g., cardiomyocytes).

Table 1: HER2 levels vs MM-302 uptake

Fig 1A	Cell line	Source	HER2 (#/cell)	fg dox/cell
A	4T1-clone-12W7	ATCC # CRL-2539™	650,000	1,758.63
B	ADRr	ATCC # HTB-22™ (MCF7 derivative)	40,792	26.47
C	AdRr-Her2	ATCC # HTB-22™, stably transfected with HER2	722,000	519.04
D	BT474-M3	Noble, Cancer Chemother. Pharmacol. 2009 64:741-51	1,706,601	1,123.51
E	Calu-3	ATCC # HTB-55™	1,196,976	84.06
F	HCC1954	ATCC # CRL-2338™	700,000	1,174.60
G	HeLa	ATCC # CCL-2™	123,713	31.61
H	IGROV1	NCI 60-cell panel from NCI-DTP, DCTD TUMOR REPOSITORY, Operated by Charles River Laboratories, Inc. (NCI-DTP)	158,418	51.54
I	JIMT-1	DSMZ # ACC-589	850,000	126.85
J	MCF7	ATCC # HTB-22	74,745	17.52
K	MCF7-c18	Gift from Dr. Christopher Bentz, Director, Cancer and Developmental Therapeutics Program, Buck Institute for Age Research, UCSF	1,031,247	531.31
L	MDA-MB-361	ATCC # HBT-27™	371,731	112.79
M	MDA-MB-453	ATCC # HBT-131™	393,441	185.51
N	MKN-7	Health Science Research Resource Bank of the Japanese Health Sciences Foundation #JCRB1025	1,217,989	181.73
O	NCI-N87	ATCC # CRL-5822™	1,233,479	366.10
P	OVCAR8	NCI 60-cell panel from NCI-DTP	53,272	99.79
Q	OVCAR8-Her2	NCI 60-cell panel from NCI-DTP, stably transfected with HER2	673,300	711.87
R	SkBr3	ATCC # HBT-30™	1,315,512	228.68
S	SKOV3	ATCC # HTB-77™	1,377,661	600.81
T	ZR75-1	ATCC # CRL-1500™	199,132	30.32

(Results for MKN-45 cells were below detection level)

Table 2 HER2 levels vs PLD uptake

Fig 1B	Cell Line	Source	HER2 (#/cell)	fg dox/cell
A	4T1-clone-12W7	ATCC # CRL-2539™	650,000	13.54
B	ADRr	ADR-RES (NCI-DTP)	40,792	23.24
D	BT474-M3	Noble, Cancer Chemother. Pharmacol. 2009 64:741-51	1,706,601	106.60
E	Calu-3	ATCC # HTB-55™	1,196,976	41.64
G	HeLa	ATCC # CCL-2™	123,713	291.11
H	IGROV1	NCI 60-cell panel from NCI-DTP	158,418	34.94
I	JIMT-1	DSMZ # ACC-589	850,000	155.46
J	MCF7	ATCC # HTB-22	74,745	13.57
K	MCF7-c18	Gift from Dr. Christopher Bentz, Director, Cancer and Developmental Therapeutics Program, Buck Institute for Age Research, UCSF	1,031,247	115.88
M	MDA-MB-453	ATCC # HBT-131™	393,441	59.97
N	MKN-7	Health Science Research Resource Bank of the Japanese Health Sciences Foundation #JCRB1025	1,217,989	48.24
O	NCI-N87	ATCC # CRL-5822™	1,233,479	47.44
P	OVCAR8	NCI 60-cell panel from NCI-DTP	53,272	90.53
Q	OVCAR8-Her2	NCI 60-cell panel from NCI-DTP, stably transfected with HER2	673,300	37.00
S	SKOV3	ATCC # HTB-77™	1,377,661	43.83
T	ZR75-1	ATCC # CRL-1500™	199,132	26.10
U	MKN-45	DSMZ # ACC-409	152,197	57.02

(Results for AdRr-Her2, HCC1954, and MDA-MB-361 cells were below detection level)

In order to quantify uptake into the different cell populations, MM-302 was prepared to contain a red-fluorescent carbocyanine tracer DiIC18(5)-DS (Invitrogen D12730 – abbreviated DiI5). DiI5 is a lipophilic fluorescent dye that intercalates into the lipid bilayer of the liposome during the extrusion process. 4T1-Her2 cell populations expressing different ranges of human HER2 were incubated with 10μg/ml fluorescently labeled MM-302 for 3hrs, washed and incubated for an additional 21hrs. Cells were harvested, stained for cell surface human HER2 and analyzed for both HER2 levels and liposome binding via flow cytometry. While the 4T1 cell line expresses murine HER2, MM-302 does not bind to the murine receptor. The figure shows that uptake of these liposomes into 4T1 cells was strongly dependent on human HER2 expression (Figure 1C). Similar results were obtained for populations of the HeLa cell lines expressing different ranges of HER2 (Figure1D). These

results further demonstrate that MM-302 is highly effective in binding cells with high HER2 levels but has little or no binding to cells with relatively lower HER2 protein expression.

Example 2: MM-302 is effectively internalized into HER2-overexpressing tumor cells and significantly inhibits tumor growth in a xenograft model: To determine levels of binding and internalization of MM-302 into HER2 over-expressing tumor cells, BT474-M3 cells ($1.7x10^6$ HER2/cell) were incubated with MM-302, PLD or free doxorubicin at 15 μg/ml for up to 3 h (Figure 2A). MM-302 was efficiently taken into tumor cells, as evidenced by total cell binding (Figure 2A) and nuclear doxorubicin accumulation (Figure 2B). By contrast, the untargeted analogue, PLD, did not show any appreciable accumulation demonstrating the requirement of targeting to effectively deliver liposomal doxorubicin *in vitro*. As a control, free doxorubicin was shown to freely enter cells and accumulate in the nucleus. Results showed effective binding and internalization of MM-302 (but not PLD) into HER2-overexpressing tumor cells.

The anti-tumor activity of MM-302 was evaluated in a breast cancer xenograft model. Mice were inoculated with BT474-M3 cells and when the tumor volumes reached an average of 250 mm^3, treatment with PBS (control), MM-302 or PLD (both at 6 mg/kg dox equiv.) was started (q7d, n=3 doses). Both MM-302 and PLD significantly inhibited tumor growth relative to control (t-test at day 55; $p<0.0001$). MM-302 resulted in a stronger inhibition of tumor growth relative to PLD (t-test at day 55; $p=0.0310$) (Figure 2C). At study termination, 3 complete regressions were observed with MM-302 and only 1 with PLD. MM-302 and PLD had similar pharmacokinetic profiles (Figure 2D) indicating that the improved efficacy was as a result of HER2-targeting, rather than prolonged exposure.

Example 3: Impact of HER2 levels on MM-302 uptake *in vivo*: Experiments were conducted to demonstrate HER2-mediated uptake of MM-302 into target tumor cells from xenograft models compared with untargeted liposomal doxorubicin. Mice bearing BT474-M3 xenograft tumors in the mammary fat pad were injected with Dil5-labeled MM-302 (Dil5-F5-PLD) or UT-PLD (Dil5-UT-PL). A tumor single cell suspension was prepared and stained with FITC-HER2 antibodies. Dil5-positive-HER2-positive cells were determined by FACS. A distinct population of cells with elevated doxorubicin levels was identified, indicating that liposomes had not just been deposited in the tumor interstitial space, but had been taken up into the cells themselves (Figure 3A). This was particularly evident in cell samples derived from tumors treated with HER2-targeted liposomes. The percentage of cells with elevated liposome content began to rise in cell subsets expressing on average 100,000 and 200,000 HER2 receptors per cell. Untargeted liposomes did not show any preferential uptake into HER2 positive cells. These results demonstrate that MM-302 uptake in tumor cells *in vivo* is

HER2-dependent and further support a level of at least 100,000-200,000 HER2 receptors per cell necessary to allow significant binding and internalization of MM-302.

The distribution of HER2 membrane intensity was determined on a single-cell basis in full tissue sections and is shown in Figure 3B, representing the variability of expression in the tissue.

Example 4: Human cardiomyocytes do not express sufficient HER2 levels to actively take up MM-302: Human cardiomyocytes have been reported to express low levels of HER2, and therefore were thought to have potential for MM-302 uptake. ESCd and iPSd human cardiomyocytes were obtained to study the effect of MM-302 on human cardiac cells *in vitro*. HER2 receptor levels on cardiomyocytes were determined by qFACS to be approximately 70,000 and 200,000 receptors per cell in human ESCd and iPSd cardiomyocytes, respectively. These results are consistent with the reported low HER2 expression in human cardiac tissue (Fuchs et al., Breast Cancer Res Treat. 2003;82:23-8).

HER2 expression levels on normal and diseased human heart tissue were measured via quantitative immunohistochemistry. A human heart Tissue Microarray (TMA) was stained for HER2 and DAPI and the (Mean HER2 intensity)/core was quantified with Definiens® software. A cell pellet array with cell lines at different HER2 expression levels was stained as above and the (Mean HER2 intensity)/core was quantified and plotted against the correspondent LOG (HER2 receptor #) to generate a standard. Based on the generated standard, the average HER2 receptor #/core for the different human heart TMA cores was interpolated (Table 3).

Table 3: Interpolated HER2 Receptor Number

ID	Pathology Diagnosis	HER2 #
1	Chronic rheumatic valvular disease with calcification	40,000
2-pt1	Chronic rheumatic valvular disease	38,000
2-pt2	Chronic rheumatic valvular disease	38,000
2-pt3	Chronic rheumatic valvular disease	47,000
2-pt4	Chronic rheumatic valvular disease	47,000
2-pt5	Chronic rheumatic valvular disease	39,000
2-pt6	Chronic rheumatic valvular disease	38,000
2-pt7	Chronic rheumatic valvular disease	42,000
2-pt8	Chronic rheumatic valvular disease	42,000
2-pt9	Chronic rheumatic valvular disease	41,000
3	Hepatocellular carcinoma embolus of cardiac atrium	44,000
4	Hypertrophic cardiomyopathy	38,000
5	Normal great arteries tissue	37,000
6	Normal cardiac atrium tissue	37,000
7	Normal myocardial tissue (focal mild hypertrophy)	38,000
8	Normal auricle of heart tissue	48,000
9	Normal myocardial tissue (mild hypertrophy)	38,000
10	Normal myocardial tissue	38,000

To determine if the level of HER2 expression on cardiomyocytes is sufficient to induce uptake of MM-302, total cellular doxorubicin was quantified by HPLC following treatment of ESCd (Figure 4A) and iPSd (Figure 4B) cardiomyocytes. Cardiomyocytes (and cancer cells) treated with free doxorubicin result in doxorubicin accumulation in all cells. Treatment with PLD did not result in an increase in doxorubicin delivery in either cardiomyocyte cell type. In contrast to HER2-overexpressing cancer cells, the HER2 expression level on cardiomyocytes was not sufficient to promote active uptake of MM-302. Taken together, these results demonstrate delivery of doxorubicin via MM-302 does not enhance doxorubicin exposure to low level HER2 expressing non-target cells such as cardiomyocytes as compared to PLD.

Example 5: MM-302 does not reduce human cardiomyocyte viability or stimulate apoptotic responses: Exposure to low levels of doxorubicin can be cytotoxic. To determine if treatment with MM-302 or PLD affected cardiomyocyte viability, ESCd cardiomyocytes were incubated with free dox, PLD or MM-302 for 3 h at the indicated concentration followed by washing and incubation in fresh media for 24 h. Treatment with free doxorubicin resulted in a loss of viability at concentrations as low as 0.2 μg/ml (Figure 4C). Conversely, treatment with MM-302 and PLD did not lead to reductions in viability at any concentration tested, including super-therapeutic concentrations up to 45 μg/ml. To further test whether treatment with MM-302 or PLD affected cardiomyocyte viability, iPSd cardiomyocytes were treated with the indicated concentration of free doxorubicin, PLD or MM-302 for 24 hours. Treatment with doxorubicin resulted in a marked decrease in viability as compared to treatment with PLD and MM-302 (Figure 4D). The presence of elevated levels of cardiac troponins is a clinical indicator of cardiac damage. The supernatant from the iPSd cardiomyocytes in (D) was analyzed for levels of troponin I. As shown in Figure 4E, doxorubicin treatment resulted in a marked increase of Troponin I compared to treatment with PLD or MM-302. These results demonstrate that ESCd and iPSd cardiomyocytes are sensitive to doxorubicin, and that treatment with MM-302 and PLD does not provide sufficient doxorubicin exposure to affect cardiomyocyte viability.

Exposure of cells to low levels of doxorubicin may induce subtle cellular changes not revealed by cell viability measurements, including DNA damage, cell stress and incipient apoptosis. Following treatment with MM-302, PLD and free doxorubicin, cardiomyocytes were stained for proteins in each of these response pathways and imaged using high-content microscopy. Single-cell data were generated by analyzing the resulting images using ImageRail.

In response to double-stranded DNA damage, histone H2AX becomes phosphorylated, forming gamma-H2AX. Treatment of cardiomyocytes with free doxorubicin

resulted in a dose-dependent increase in nuclear gamma-H2AX (Figure 5A). However, treatment with MM-302 and PLD did not increase nuclear gamma-H2AX signal at any concentration tested, indicating that liposomal encapsulation prevented DNA damage to cardiomyocytes in vitro.

In response to cellular stress, HSP27 and p53 can be phosphorylated, leading to cell cycle arrest, followed by DNA repair or apoptosis depending on the extent of injury. Cardiac cells exposed to free doxorubicin demonstrate a dose-dependent increase in phospho-HSP27 and phospho-p53 indicating an induction of cellular stress following treatment (Figure 5B and 5C). However, an increase in phospho-HSP27 was not observed in cells treated with either MM-302 or PLD regardless of concentration. In most cases, there did not appear to be an effect on phospho-p53 in cells treated with MM-302 or PLD, with the exception of a slight increase in phospho-p53 following treatment with 5.0 µg/ml of MM-302. However, treatment at this and higher concentrations did not result in increased cell death.

In cases of severe DNA damage and cell stress, the cell may initiate the apoptotic pathway including activation of a caspase cascade, ultimately resulting in the cleavage of the DNA repair protein PARP. Treatment with 5.0 µg/ml of free doxorubicin led to an increase in nuclear cleaved PARP (cPARP) (Figure 5D), correlating with the observed increase in cell death. However, treatment with MM-302 or PLD did not result in increased nuclear cPARP suggesting that treatment under these conditions is not sufficient to induce apoptosis.

Example 6: Impacts of HER2-targeted agents on intracellular signaling in cardiomyocytes: The concurrent use of doxorubicin and trastuzumab is contraindicated due to an unacceptably high incidence of cardiac events observed in patients treated with the combination. The mechanism of action for the cardiotoxicity associated with this combination is believed to be the simultaneous induction of cellular stress by doxorubicin and by trastuzumab-mediated inhibition of HER2 signaling pathways that is necessary to respond to the cellular stress induced by doxorubicin.

To determine if pretreatment with MM-302 alters HER2-mediated signaling (an essential pathway in cardiomyocytes), iPDd cardiomyocytes were pretreated for 24 hours with trastuzumab, lapatinib (a small molecule HER2 tyrosine kinase inhibitor), or the MM-302 antibody (F5-scFv) and an empty liposome identical to MM-302 except that it does not contain doxorubicin) (F5-lipo). After stimulation with 10nM (Figure 6A) and 5nM (Figure 6B) of heregulin (HRG) for 10 min, the levels of phospho-AKT (pAKT, Figure 6A) and phospho-ERK (pERK, Figure 6B) were measured by high content microscopy as described above. Pretreatment with trastuzumab for 24 h resulted in a reduction in HRG-mediated phosphorylation of both proteins. Pretreatment with lapatinib led to a reduction in basal phosphorylation of AKT and ERK as well as a complete inhibition of HRG-induced

phosphorylation of these proteins. Pre-treatment F5-lipo did not inhibit HRG-induced phosphorylation of AKT or ERK. These results suggest that, despite targeting HER2, MM-302 does not inhibit ligand-induced phospho-AKT and phospho-ERK signaling in cardiomyocytes, leaving these critical signaling pathways functional.

These results also show that trastuzumab and lapatinib have a significantly greater negative impact on this signaling pathway in cardiomyocytes than do F5 scFv or F5 lipo. This in turn is an indication that the anti-HER2 antibody component of MM-302 is less cardiotoxic than the anti-HER2 antibody trastuzumab. The results show that F5, either alone or linked to the exterior of an MM-302 liposome, does not interfere with heregulin (ligand)-stimulated HER2/HER3 heterodimer-mediated signaling in cardiomyocytes which is an essential intracellular signaling modality, inhibition of which is believed to be a key mechanism mediating trastuzumab-induced cardiotoxicity.

Example 7: MM-302 has a lower accumulation in mouse heart tissue compared to free doxorubicin: Liposome targeting with a highly specific antibody fragment such as F5 generally does not alter the total tissue deposition of liposomes, but rather alters their microdistribution following extravasation. The macro-level biodistributions of MM-302 (square), PLD (UT-PLD, triangle) and doxorubicin (free dox, circle) were compared in mouse heart tissue (Figure 7A), human xenograft tumor tissue (Figure 7B) and paw tissue (Figure 7C) in NCI-N87 tumor bearing mice inoculated as described above. Mice (n=4/time point/group) were injected i.v. with MM-302, PLD, or free doxorubicin (all at 3 mg/kg dox equiv.) and hearts were collected at 0.5, 4, 24, and 96 h (for dox) or 168 h (for MM-302 and PLD) post injection and doxorubicin quantified by HPLC (Figure 7A). Injection of free doxorubicin resulted in a high peak exposure in the heart at 0.5 h (10% of injected dose (i.d.)/g tissue) compared to the two liposomal formulations. The clearance of doxorubicin from the heart tissue after free doxorubicin injection was faster compared to MM-302 and PLD and at 24 h the amount of detected doxorubicin (0.77% of i.d./g tissue) was close to background. Both MM-302 and PLD had a sustained accumulation profile that peaked at 24 h (2.8% and 2.6% for MM-302 and PLD, respectively) while values returned to background at 168 h. These results are in a range similar to that of previously reported data on the heart biodistribution of other PLD formulations. No significant differences were observed between the heart biodistribution of MM-302 and PLD.

Example 8: MM-302 results in lower nuclear doxorubicin accumulation in mouse tissue compared to free doxorubicin.

The microdistribution of doxorubicin (naturally fluorescent) and liposomes (DiI5-labelled) was analyzed in cryosections generated from heart tissues of mice injected with

either free doxorubicin, MM-302-DiI5 or PLD-DiI5 (all at 3 mg/kg dox equiv) at 0.5, 4 and 24 h post injection. In order to visualize the heart vasculature, mice were injected i.v. with FITC-lectin 5 min before sacrificing. Heart slices were imaged by fluorescence confocal microscopy. Representative fields for the different treatment groups at the three time points analyzed (0.5, 4 and 24 h) are shown in Figure 7D. Untreated hearts were also imaged and a representative image is shown in Figure 7D (left panels). Co-localization of doxorubicin with the nuclear signal is shown in the bottom panels of Figure 7D. Higher magnification images of the nuclear doxorubicin signal are shown in Figure 7E, for both doxorubicin and MM-302 at the 0.5 h time point. While with MM-302 no doxorubicin signal is visible in the nuclei, with free doxorubicin the majority of the nuclei are doxorubicin-positive. The images were analyzed as described above and the percent of doxorubicin-positive nuclei determined. Injection of free doxorubicin resulted in a prominent nuclear accumulation of doxorubicin at 0.5 h, with about 50% of the nuclei positive for doxorubicin. By 4 h, however, only 23% of the nuclei were positive for doxorubicin and the signal returned to basal levels at 24 h.

With the liposomal formulations, little to no signal was detected for the majority of fields of view. Occasional signal in the DiI5 channel (liposome) was detected. In these cases, the liposome signal predominantly co-localized with the FITC-lectin signal, indicating liposomes that had not extravasated into the heart tissue but still remained in the vascular compartment. Upon MM-302-DiI5 or PLD-DiI5 treatment, doxorubicin was not detected in the nucleus in the majority of the heart fields analyzed, independent of time point. In one of the four MM-302-DiI5 hearts collected at 0.5 h and in one of the four MM-302-DiI5 hearts collected at 4 h, a liposomal signal was detected in the extravascular space and doxorubicin was found in a small percentage of the nuclei. Similarly, in one of four PLD hearts collected at 0.5 h and in two of four PLD heart collected at 24 h, images revealed the extravascular liposomal signal and presence of nuclear doxorubicin. These fields are not presented as representative images, however their values were considered for the quantification shown in Figure 7F. The area under the curves of both MM-302 and PLD were statistically significantly lower than the free doxorubicin AUC ($p<0.001$). No significant differences were observed between the AUCs of MM-302 and PLD.

In order to get a broader visualization of the distribution of doxorubicin and of the liposomes in the heart tissue, full heart section scans were taken. The full section heart scans visually confirmed the results of the confocal microscopy, showing a broad doxorubicin distribution with nuclear localization upon free doxorubicin injection, and only rare liposome and doxorubicin signals in the hearts of mice injected with either DiI5 MM-302 or DiI5 PLD.

In summary, treatment with either MM-302 or PLD showed significantly lower nuclear doxorubicin accumulation than was seen following treatment with free doxorubicin, while this did not differ significantly between MM-302 and PLD.

Equivalents

Those skilled in the art will recognize, or be able to ascertain and implement using no more than routine experimentation, many equivalents of the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims. Any combinations of the embodiments disclosed in the dependent claims are contemplated to be within the scope of the disclosure.

Incorporation by reference

The disclosure of each and every U.S. and foreign patent and pending patent application and publication referred to herein is specifically incorporated by reference herein in its entirety.

WHAT IS CLAIMED IS:

1. A method for determining a safe and effective dosage for use in treating a human cancer patient by administration of anthracycline-comprising anti-HER2 immunoliposomes, the patient being diagnosed with a cancer characterized by expression of HER2 receptor,

the method comprising determining a first dosage, such a dosage indicating a dose magnitude and frequency of dosing, for a patient diagnosed with a cancer characterized by expression of HER2 receptor, the first dosage being for a liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome, which dosage is determined to provide to the patient a safe and effective amount of the liposomal anthracycline chemotherapeutic agent; and

determining a dosage for the administration of the anthracycline-comprising anti-HER2 immunoliposomes, a plurality of which immunoliposomes is each bearing a plurality of anti-HER2 antibody molecules on its surface and each containing the anthracycline chemotherapeutic agent,

wherein the safe and effective dosage for the administration of the anthracycline-comprising anti-HER2 immunoliposomes is the first dosage.

2. A method of treating a human cancer patient by administration of anthracycline-comprising anti-HER2 immunoliposomes,

the method comprising determining a first dosage, such a dosage indicating a dose magnitude and frequency of dosing, for a patient diagnosed with a cancer characterized by expression of HER2 receptor, the first dosage being for a liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome, which dosage is determined to provide to the patient a safe and effective amount of the liposomal formulation, and

administering anthracycline-comprising anti-HER2 immunoliposomes, a plurality of which immunoliposomes is each bearing a plurality of anti-HER2 antibody molecules on its surface and each containing the anthracycline chemotherapeutic agent,

wherein the anthracycline-comprising anti-HER2 immunoliposomes are administered to the patient at the first dosage.

3. The method of claim 1 or claim 2, wherein the anthracycline is doxorubicin.

4. The method of claim 3, wherein the liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome is doxorubicin HCl liposome injection and the HER2-targeted immunoliposomes are MM-302.

5. The method of any one of claims 1-4, wherein the cancer is breast cancer, Kaposi's sarcoma, ovarian cancer, or multiple myeloma.

6. The method of any one of claims 1-5, wherein the first dosage is 50 mg/m^2, 40 mg/m^2, 30 mg/m^2, 20 mg/m^2, or 10 mg/m^2 every two weeks or every three weeks or every four weeks.

7. The method of any one of claims 1-6, wherein the cancer characterized by expression of HER2 receptor is further characterized as being HER2^{2+}, HER2^{3+}, or HER2 FISH positive.

8. The method of any one of claims 1-7, wherein the cancer characterized by expression of ErbB2 receptor further characterized as expressing an average of at least 200,000 cell surface ErbB2 receptors per cell.

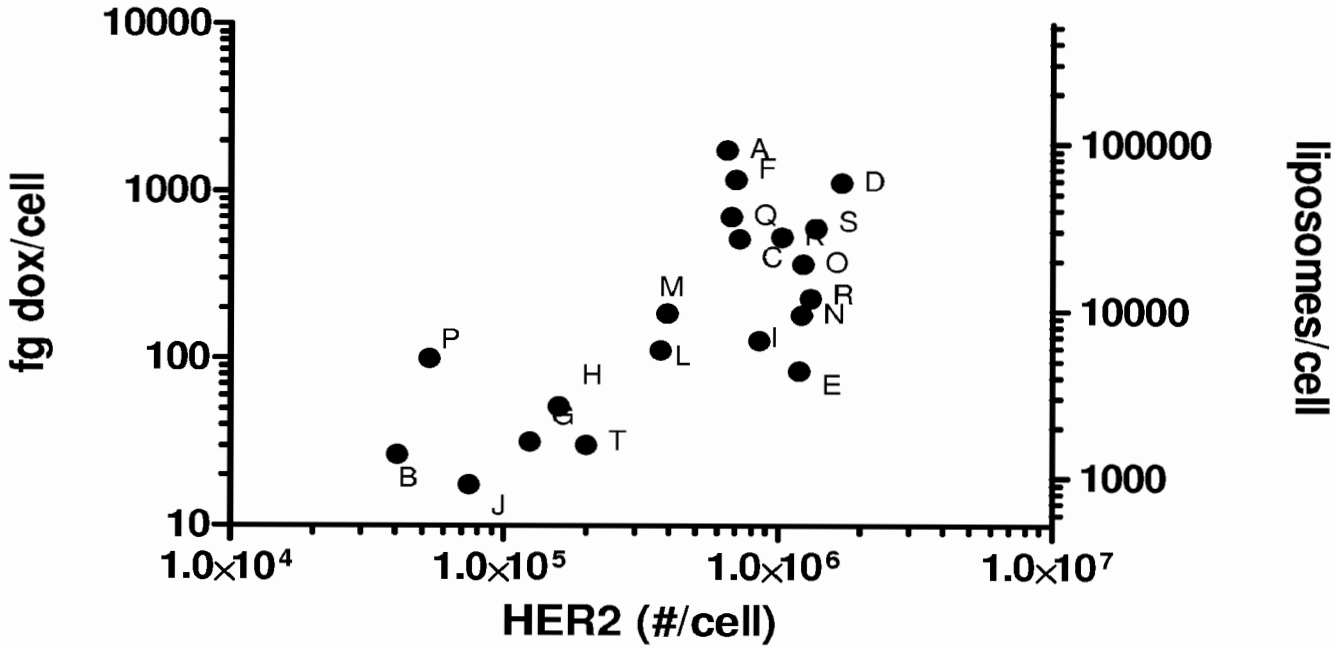
9. The method of any one of claims 1-8, wherein the administration of the immunoliposomes at the first dosage is effective to treat the cancer.

10. The method of any one of claims 1-9, wherein the administration of the immunoliposomes at the first dosage does not result in increased cardiotoxicity as compared to administration at the first dosage of the liposomal anthracycline chemotherapeutic agent that does not comprise an immunoliposome.

11. The method of any one of claims 1-10, wherein the administration of the immunoliposomes to the patient at the first dosage results in a peak concentration of the immunoliposome in the patient's bloodstream and wherein treating human cardiomyocytes in vitro by culturing in medium comprising the immunoliposomes at about the peak concentration does not reduce, or reduces by no more than 5%, heregulin-stimulated increase of pERK or pAKT in the cultured cardiomyocytes as compared to in control human cardiomyocytes cultured in medium free of the immunoliposomes.

12. The method of claim 11, wherein the immunoliposome concentration in the patient's bloodstream is measured as a serum immunoliposome concentration.

13. The method of any one of claims 1-12, wherein each of the HER2 immunoliposomes bears on its surface, on average, 45 anti-HER2 antibody molecules.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100000
10000
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1.0×10⁵
1.0×10⁶
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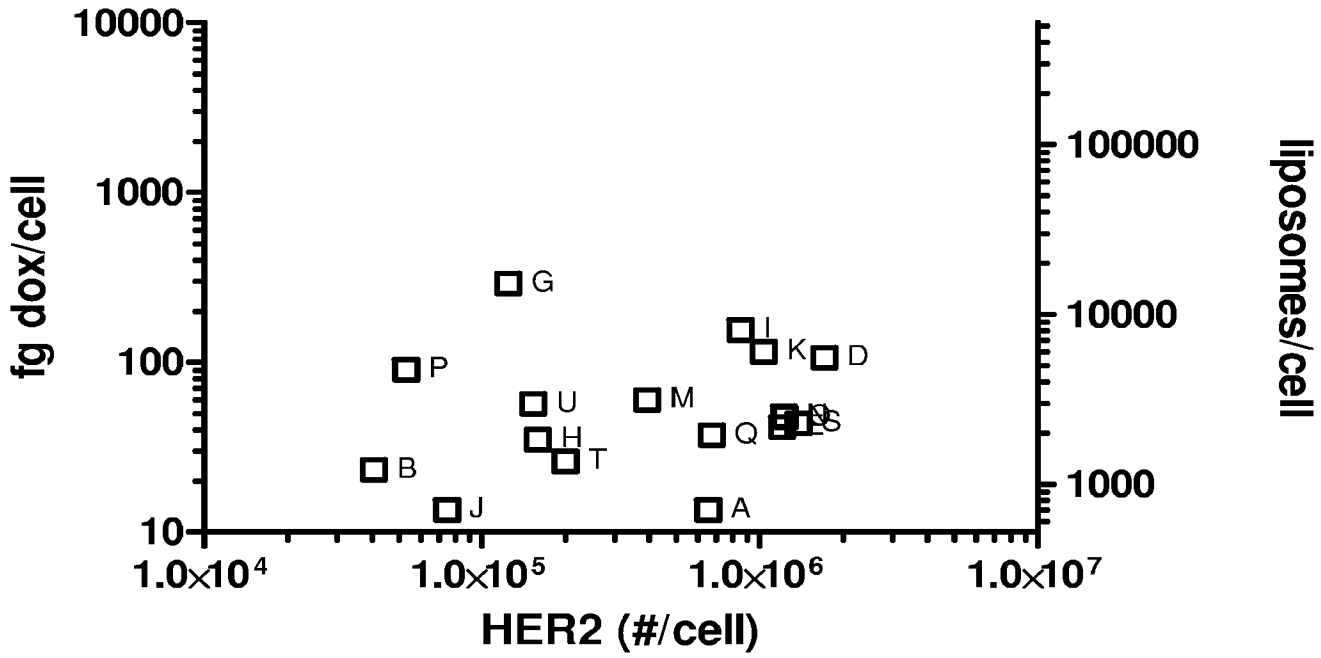
HER2 (#/cell)
A
F
D
Q
S
C
O
M
R
N
P
I
L
H
E
T
B
J

Figure 1A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100
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100000
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liposomes/cell
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HER2 (#/cell)
G
P
U
M
I
K
D
H
Q
T
B
J
A

Figure 1B

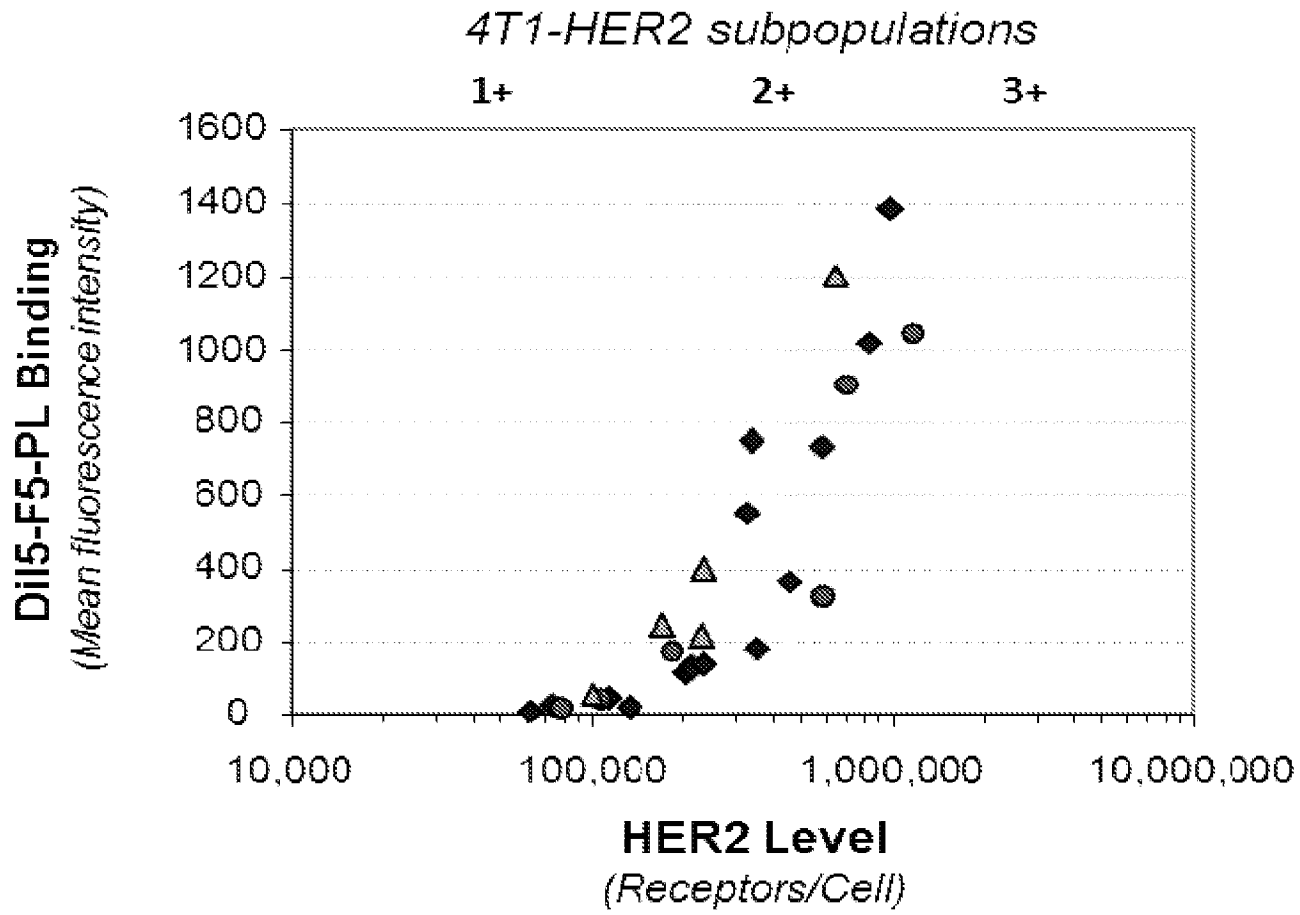
4T1-HER2 subpopulations
1+
2+
3+
DiI5-F5-PL Binding
(Mean fluorescence intensity)
1600
1400
1200
1000
800
600
400
200
0
10,000
100,000
1,000,000
10,000,000
HER2 Level
(Receptors/Cell)

Figure 1C

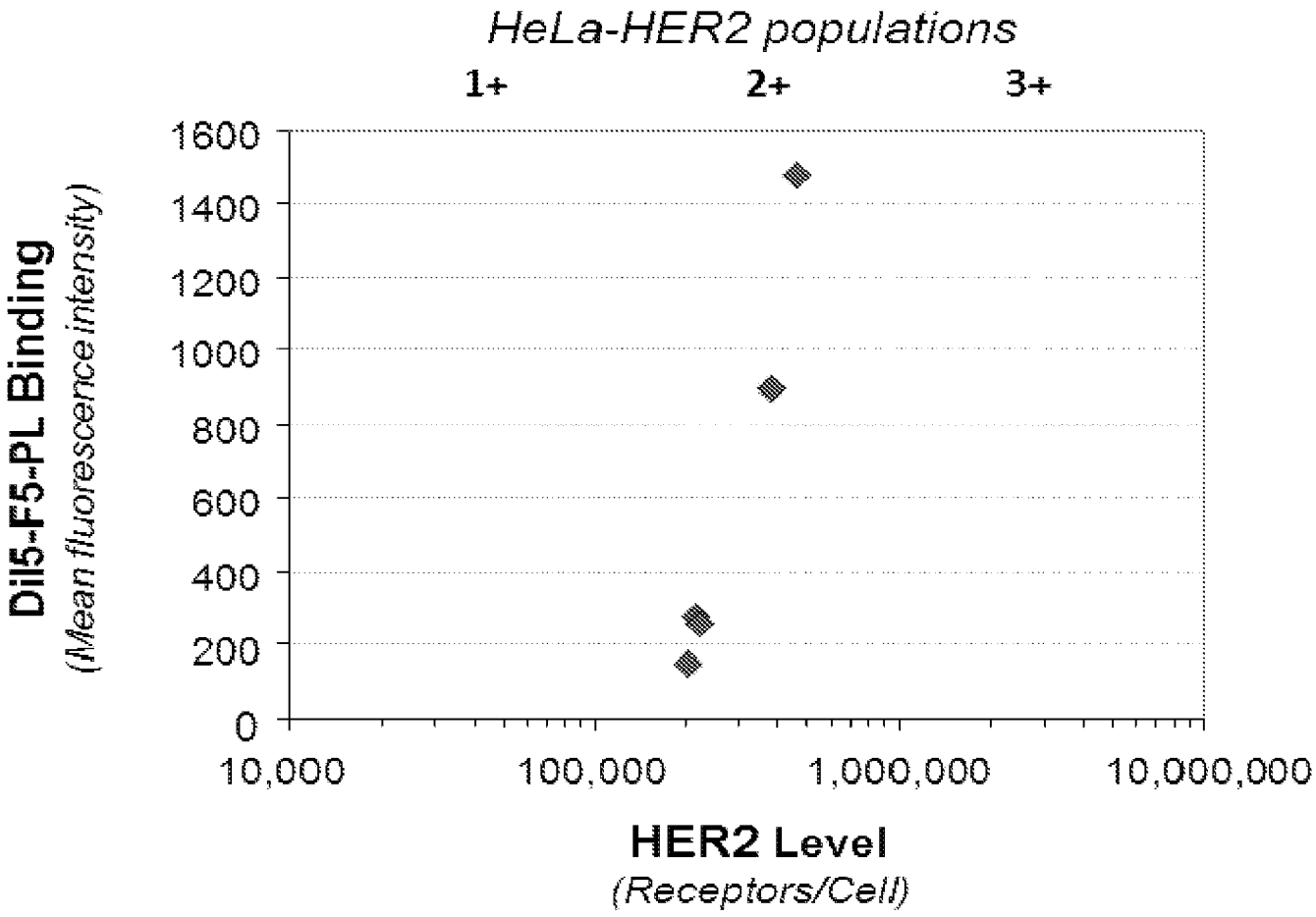
HeLa-HER2 populations
1+
2+
3+
1600
1400
1200
1000
800
600
400
200
0
Dil5-F5-PL Binding
(Mean fluorescence intensity)
10,000
100,000
1,000,000
10,000,000
HER2 Level
(Receptors/Cell)

Figure 1D

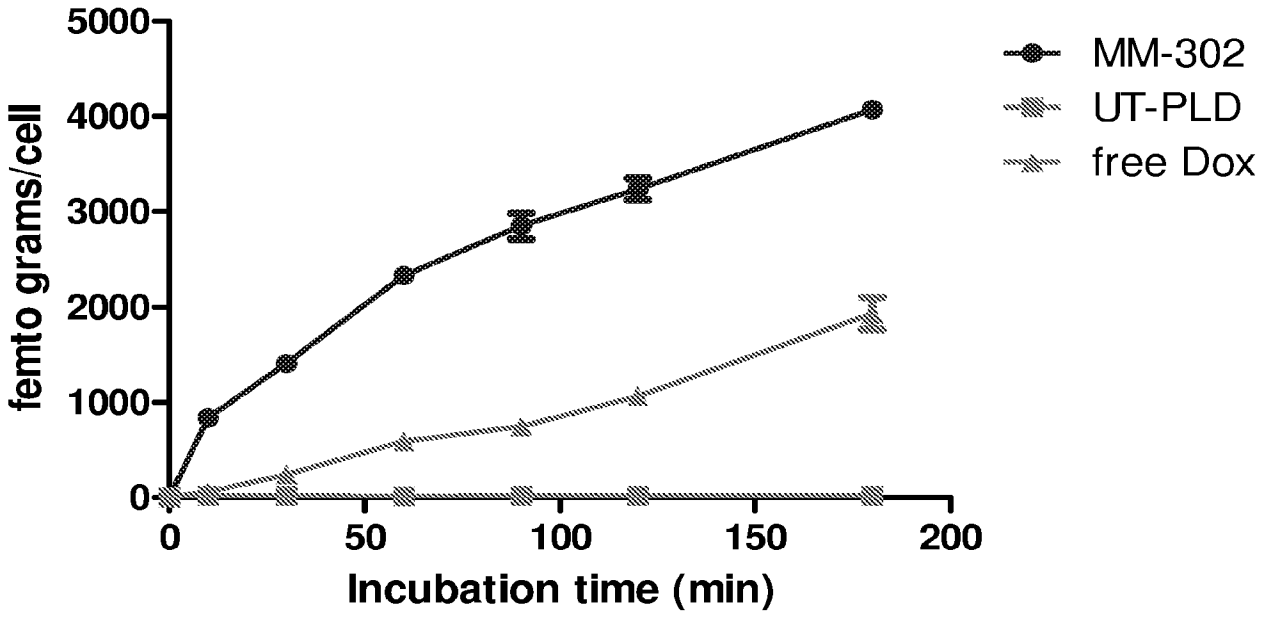

Figure 2A

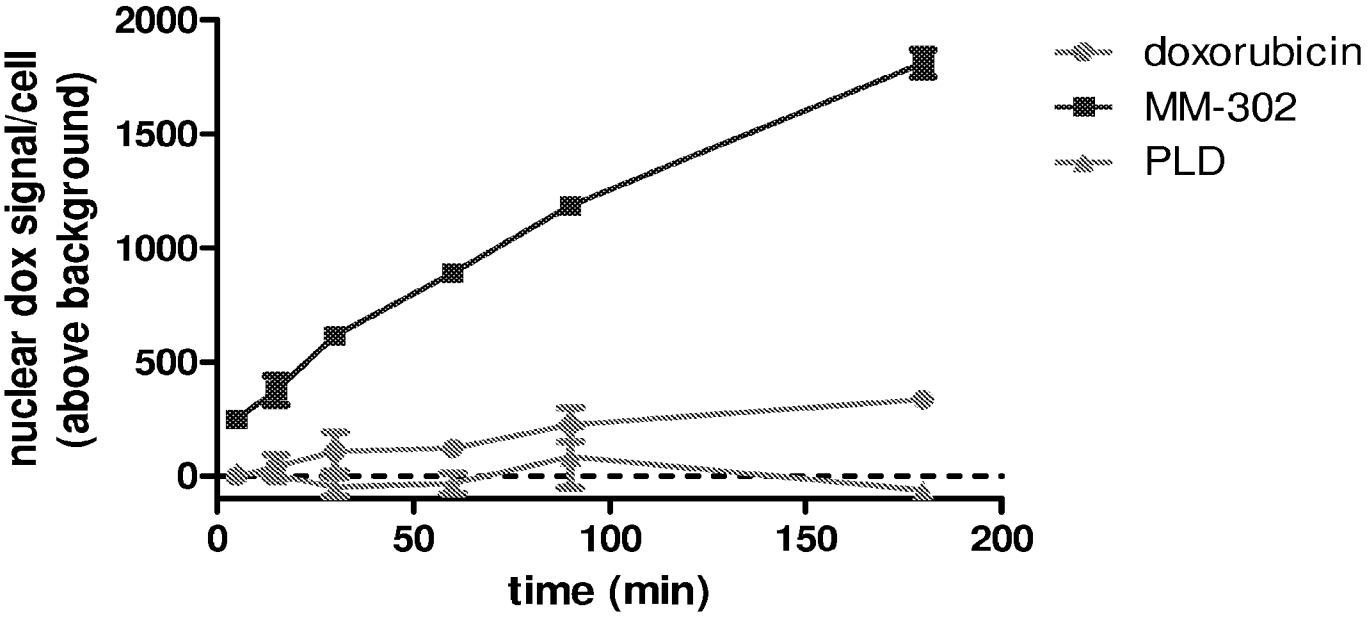

Figure 2B

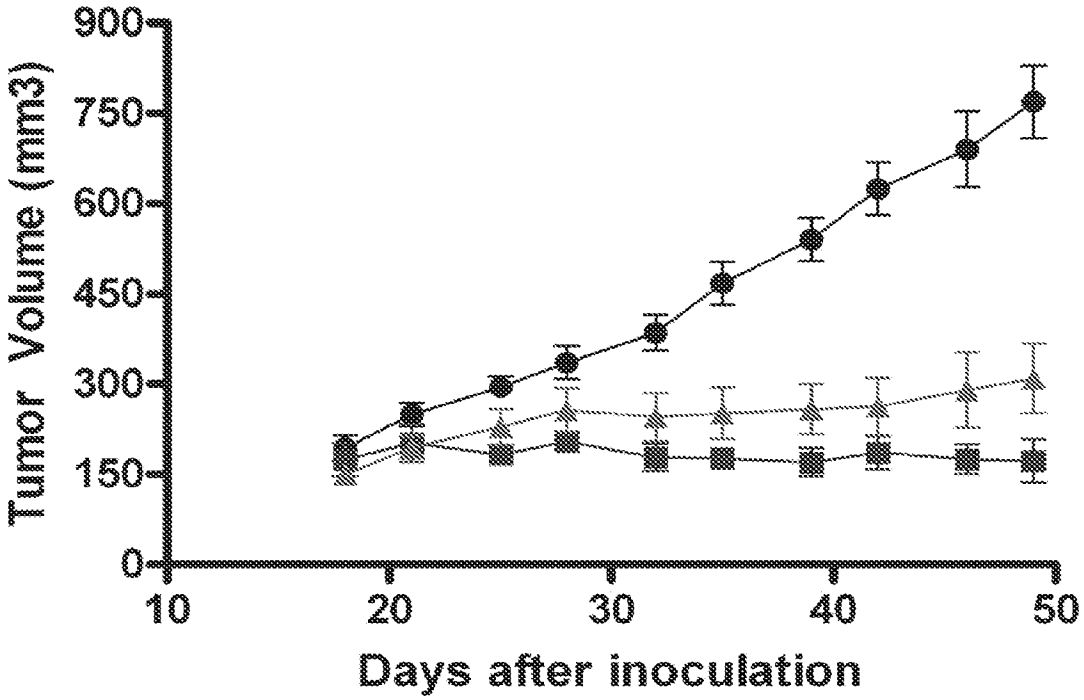
900
750
600
450
300
150
0
Tumor Volume (mm3)
10
20
30
40
50
Days after inoculation

Figure 2C

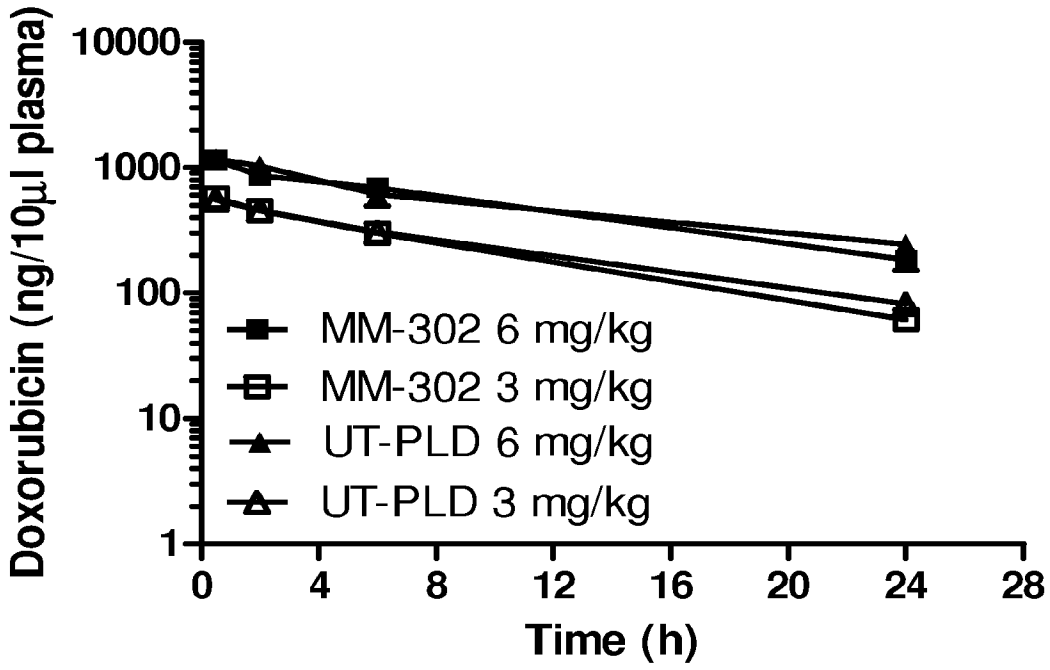
Doxorubicin (ng/10µl plasma)
10000
1000
100
10
1
0
4
8
12
16
20
24
28
Time (h)
MM-302 6 mg/kg
MM-302 3 mg/kg
UT-PLD 6 mg/kg
UT-PLD 3 mg/kg

Figure 2D

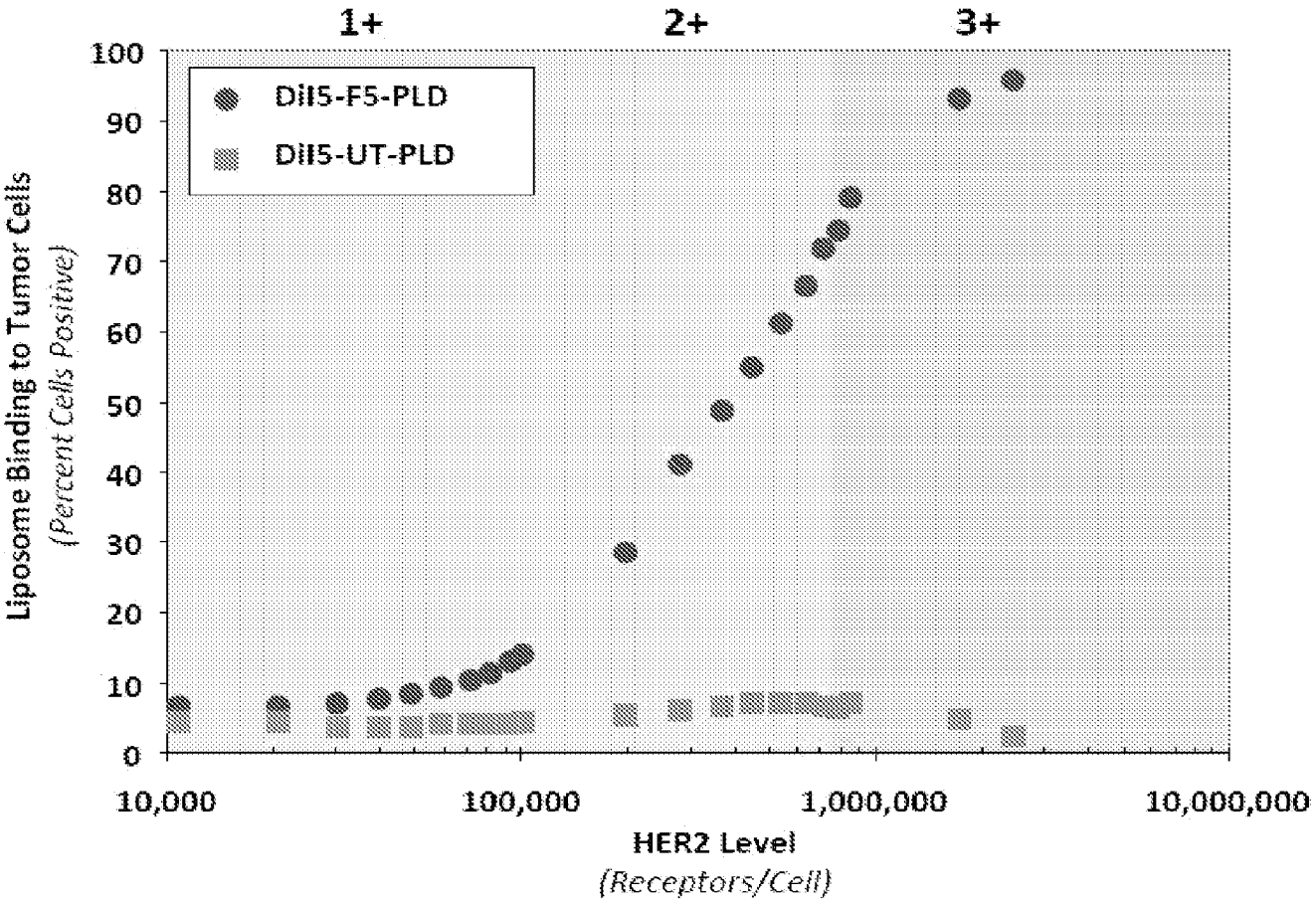
1+
2+
3+
Dil5-F5-PLD
Dil5-UT-PLD
Liposome Binding to Tumor Cells
(Percent Cells Positive)
100
90
80
70
60
50
40
30
20
10
0
10,000
100,000
1,000,000
10,000,000
HER2 Level
(Receptors/Cell)

Figure 3A

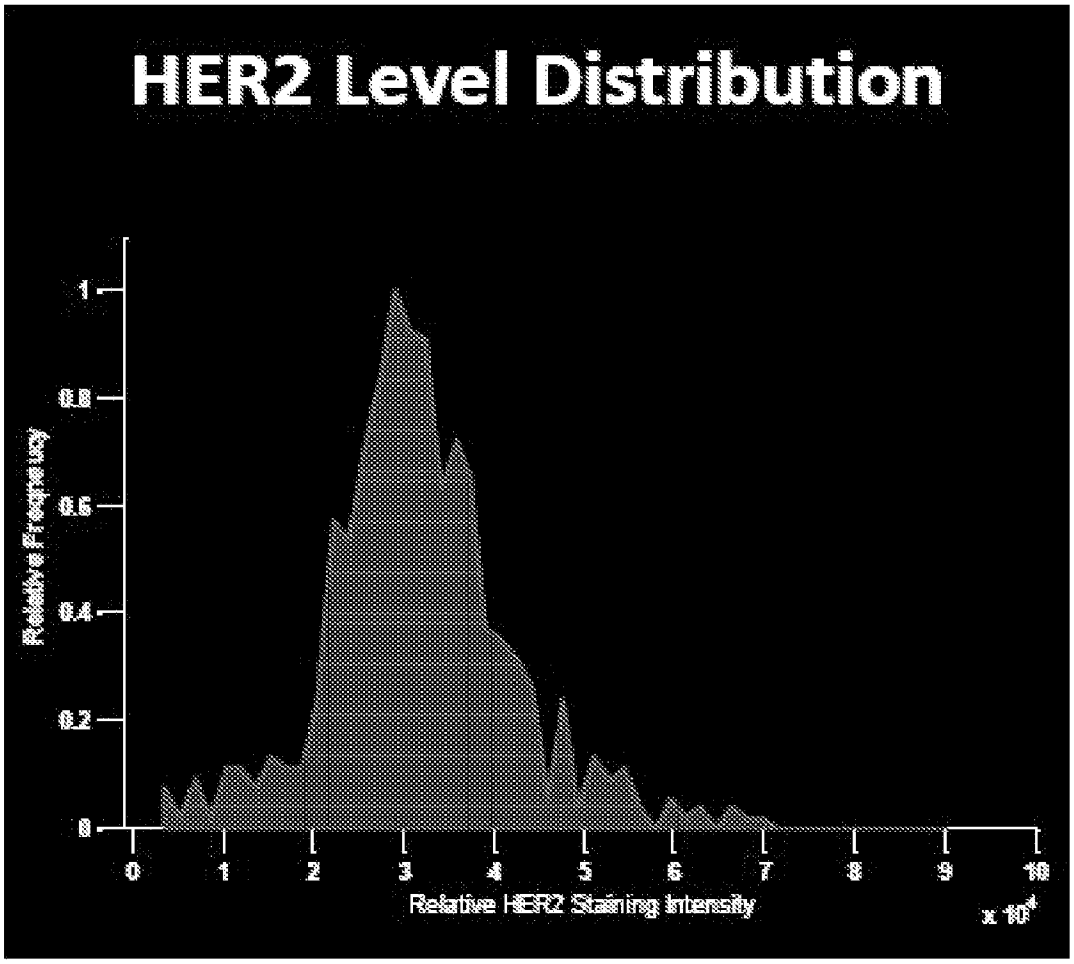
HER2 Level Distribution
Relative Frequency
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
9
10
Relative HER2 Staining Intensity
x 10⁴

Figure 3B

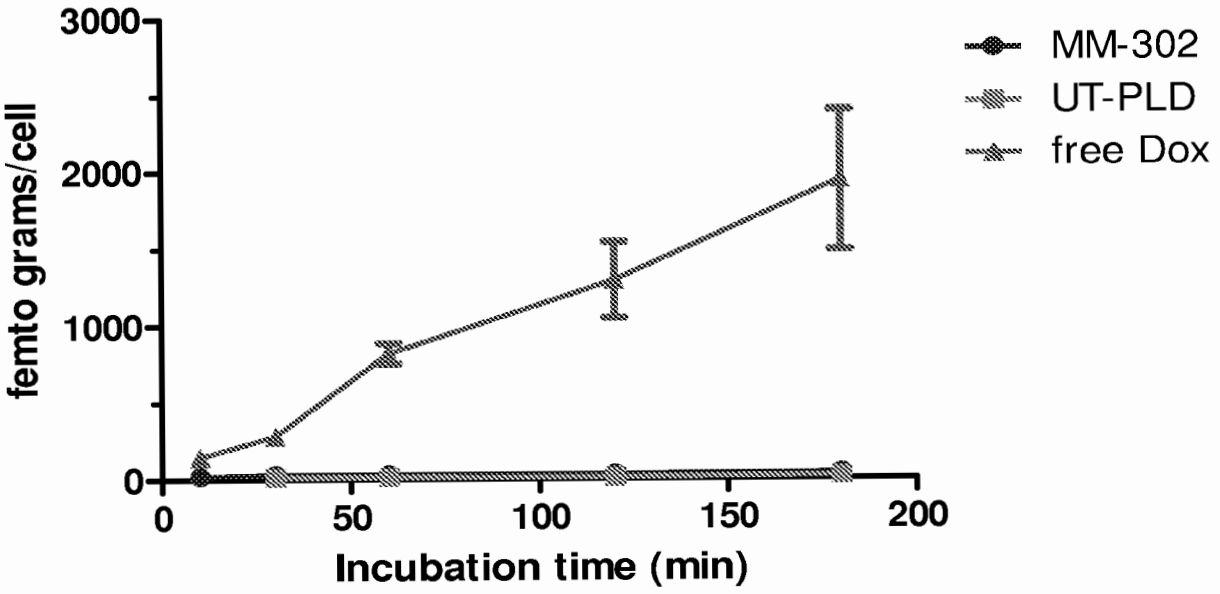

Figure 4A

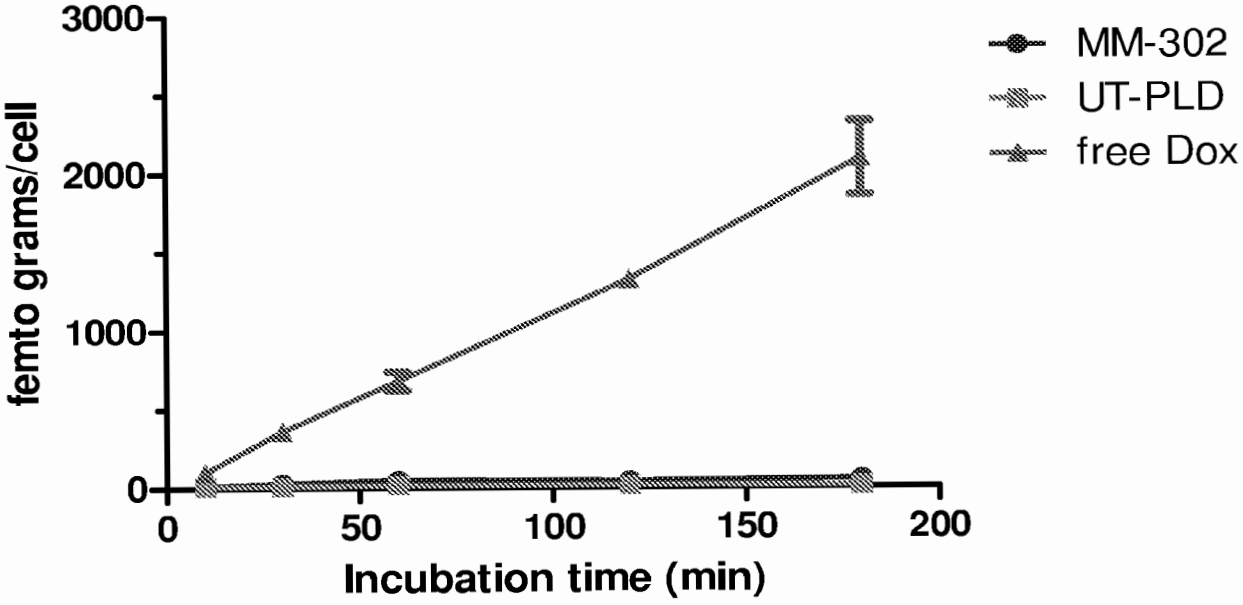

Figure 4B

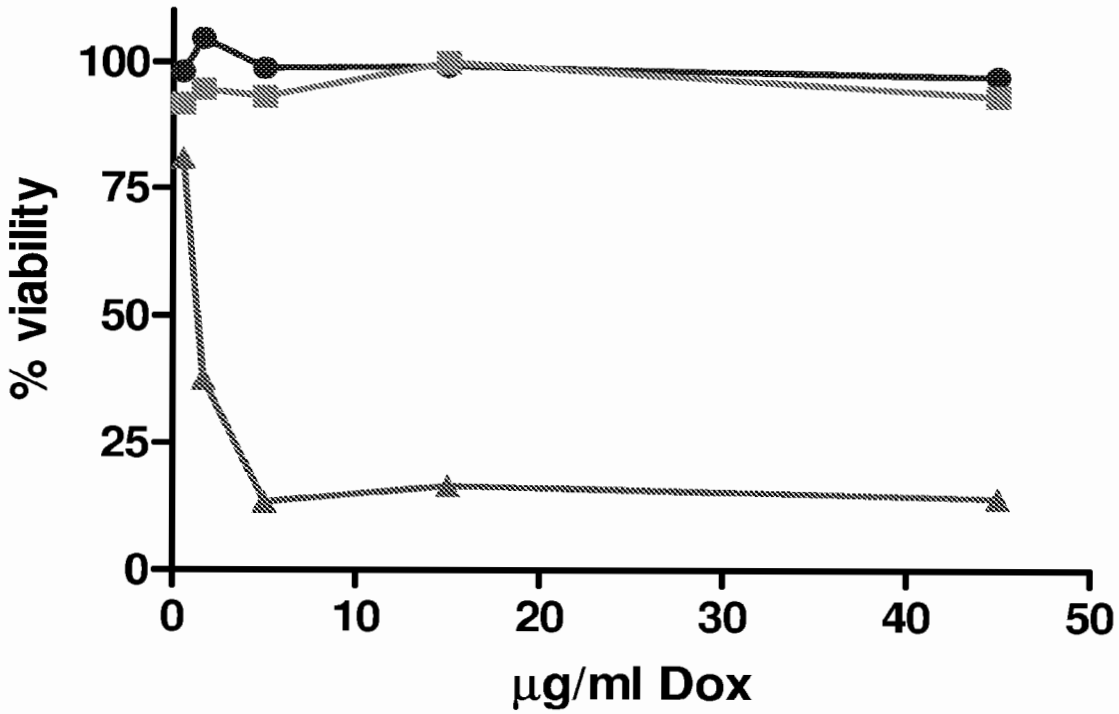

Figure 4C

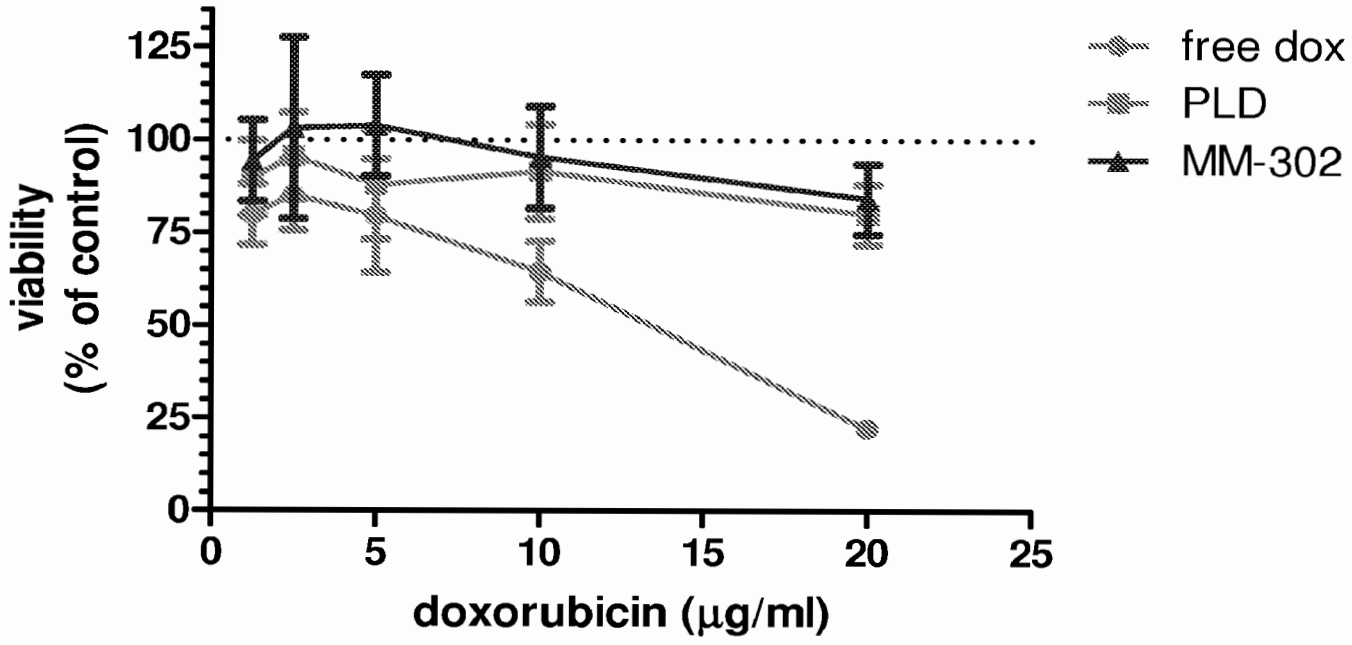

Figure 4D

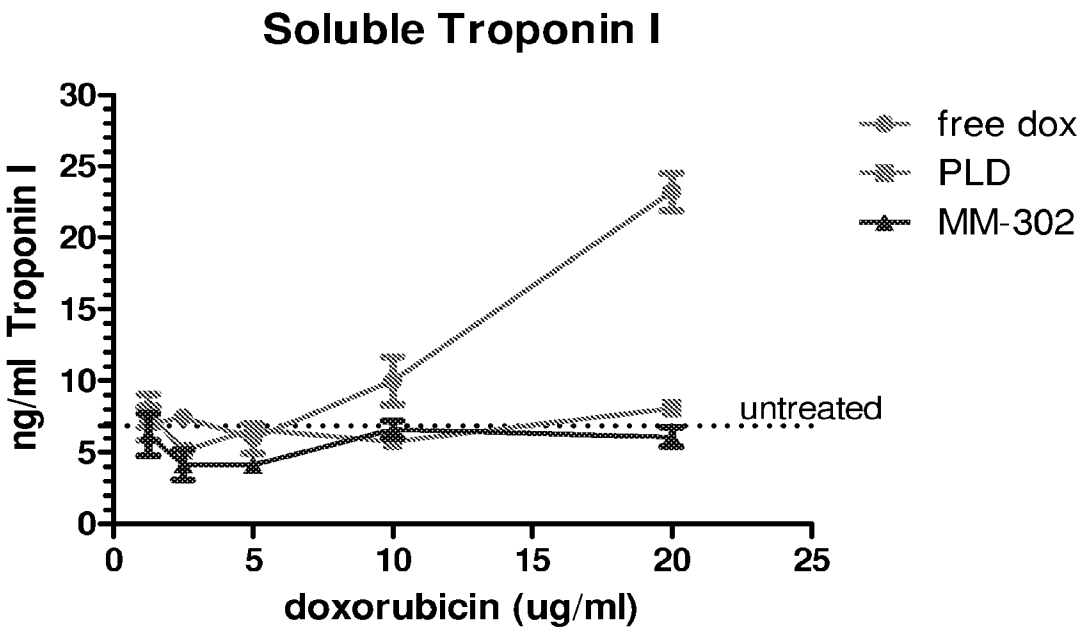
Soluble Troponin I
ng/ml Troponin I
30
25
20
15
10
5
0
0
5
10
15
20
25
doxorubicin (ug/ml)
free dox
PLD
MM-302
untreated

Figure 4E

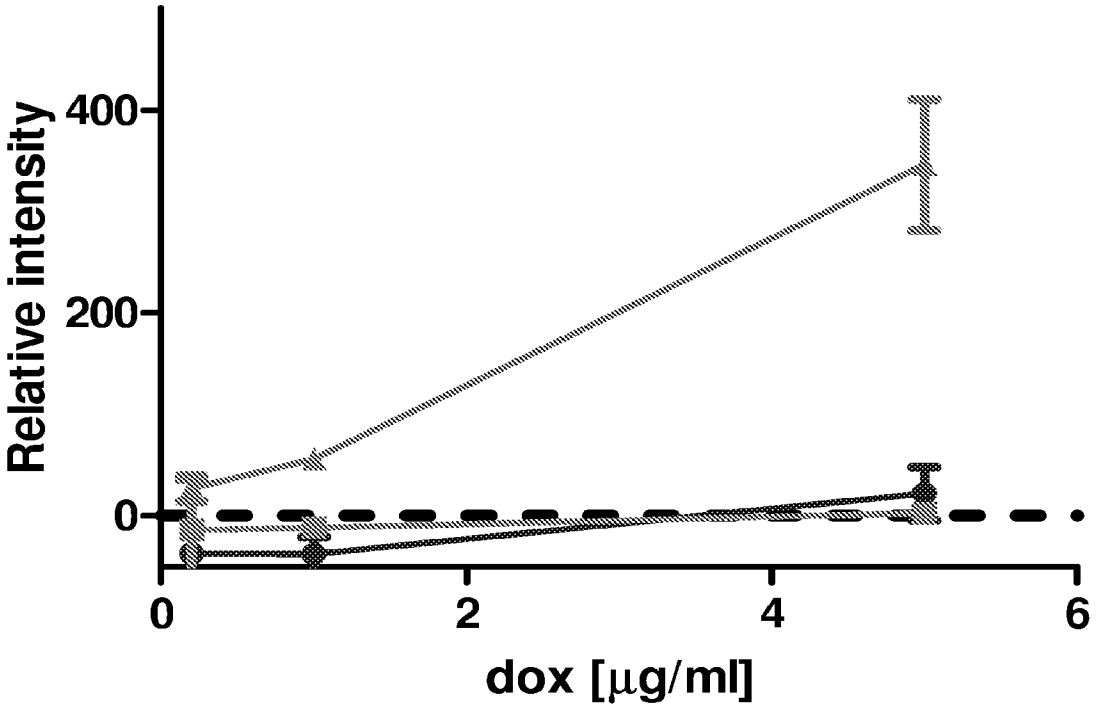

Figure 5A

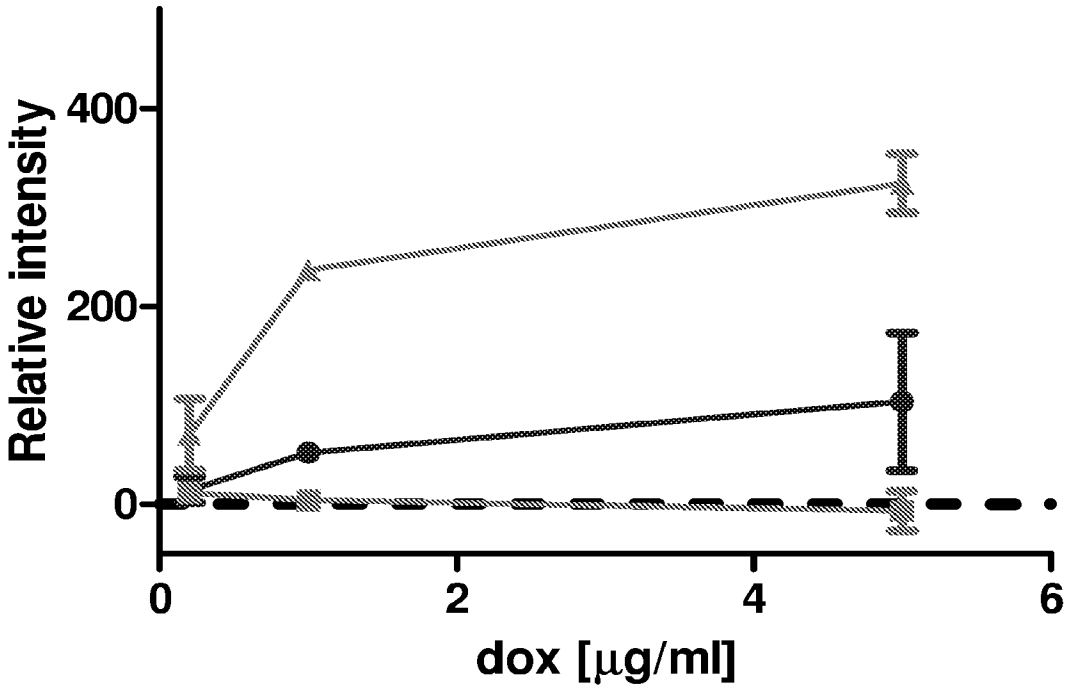

Figure 5B

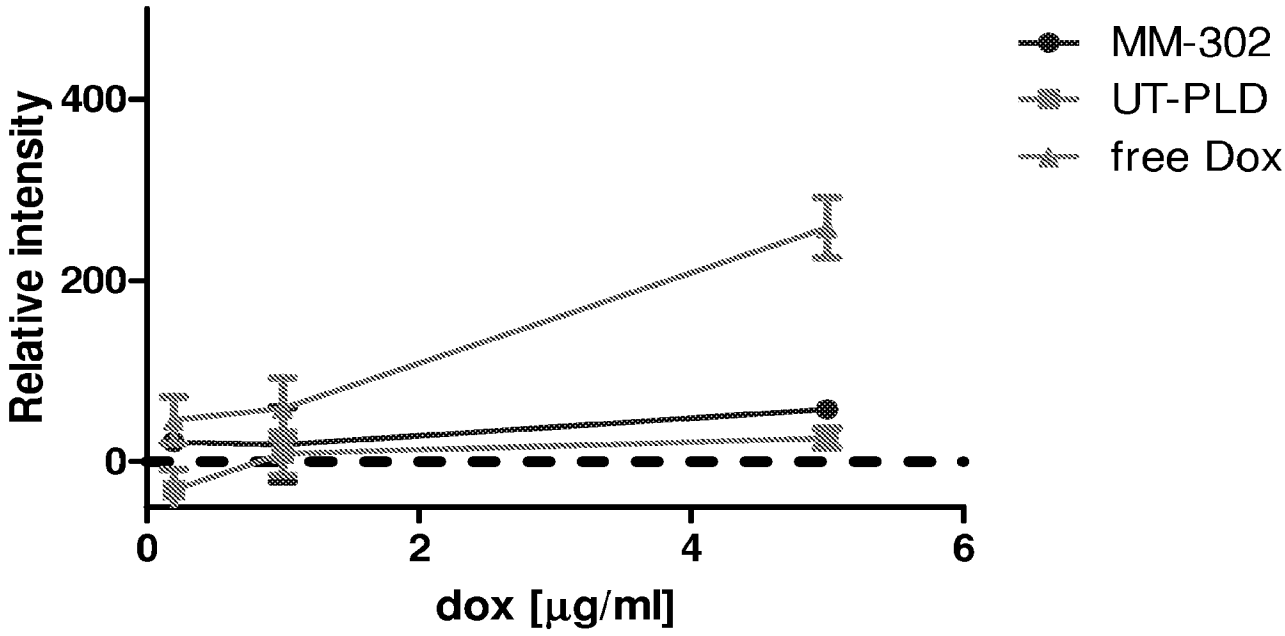

Figure 5C

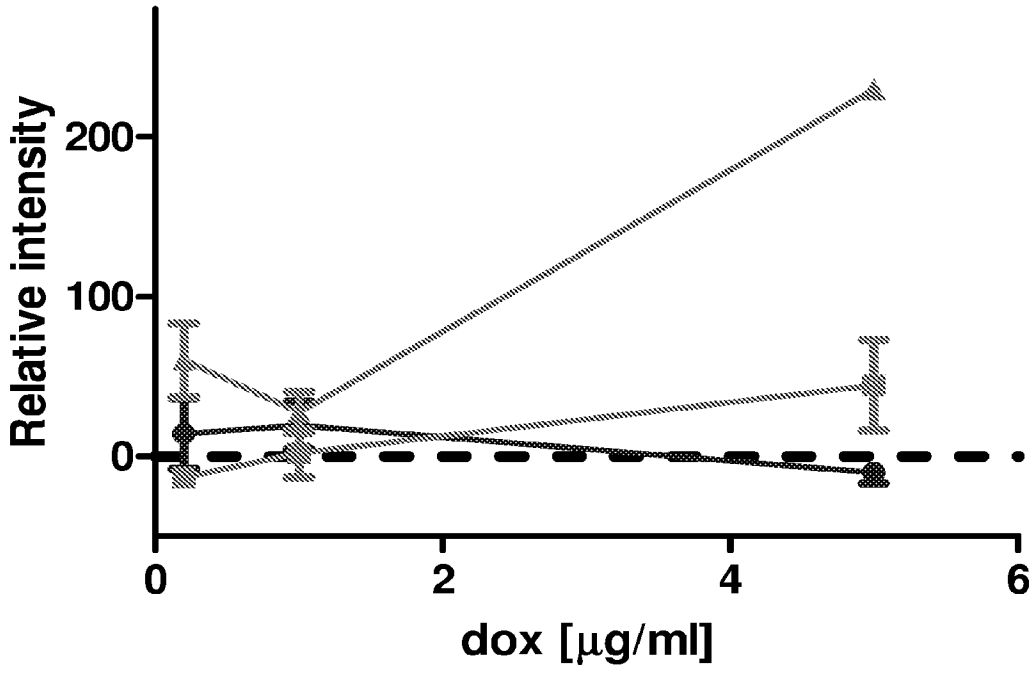

Figure 5D

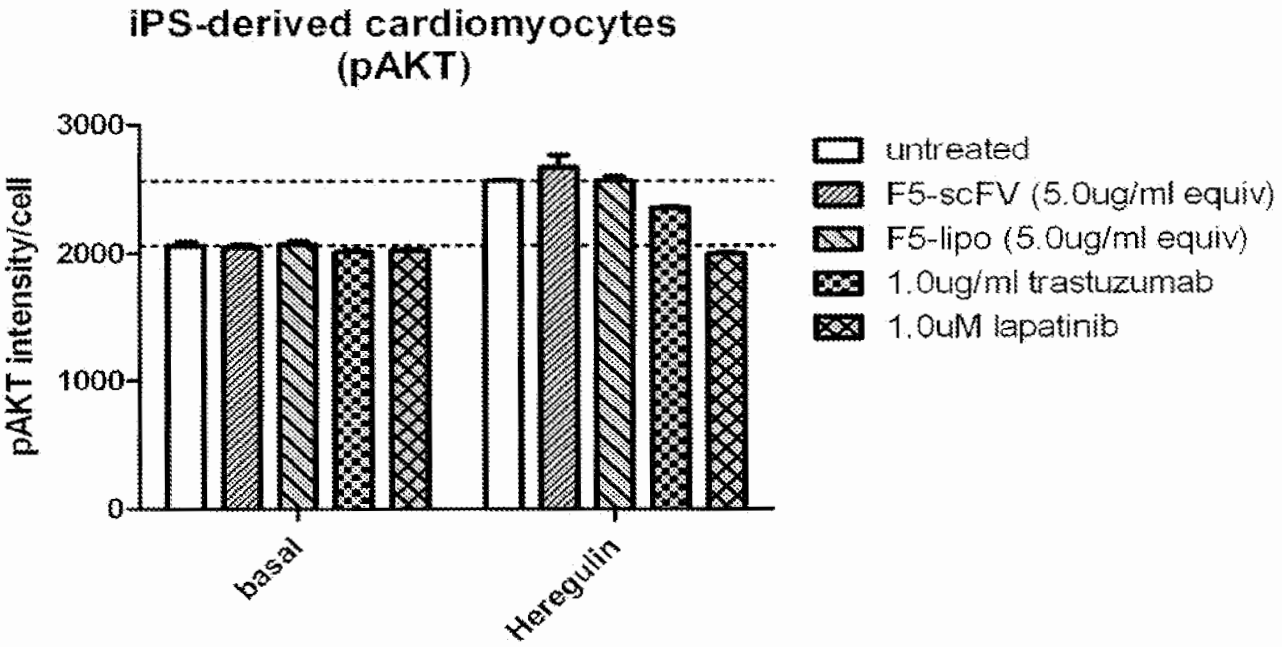
iPS-derived cardiomyocytes
(pAKT)
pAKT intensity/cell
3000
2000
1000
0
basal
Heregulin
untreated
F5-scFV (5.0ug/ml equiv)
F5-lipo (5.0ug/ml equiv)
1.0ug/ml trastuzumab
1.0uM lapatinib

Figure 6A

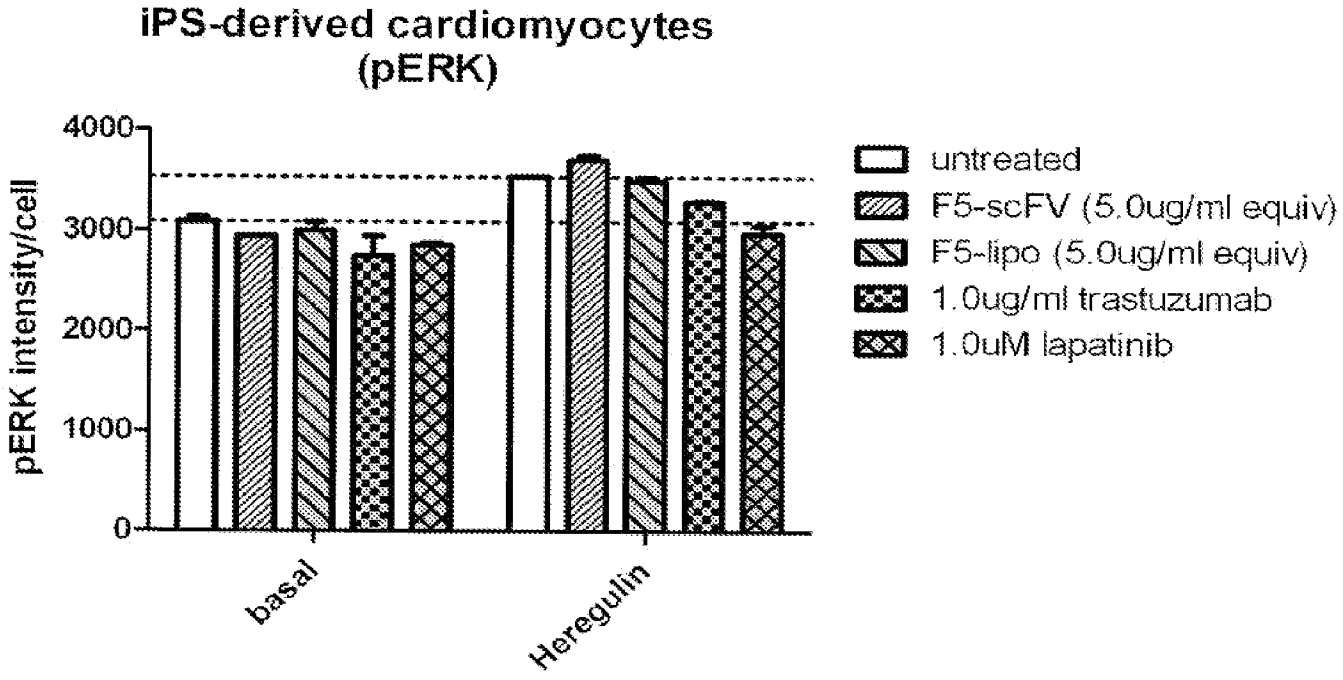
iPS-derived cardiomyocytes
(pERK)
pERK intensity/cell
4000
3000
2000
1000
0
basal
Heregulin
untreated
F5-scFV (5.0ug/ml equiv)
F5-lipo (5.0ug/ml equiv)
1.0ug/ml trastuzumab
1.0uM lapatinib

Figure 6B

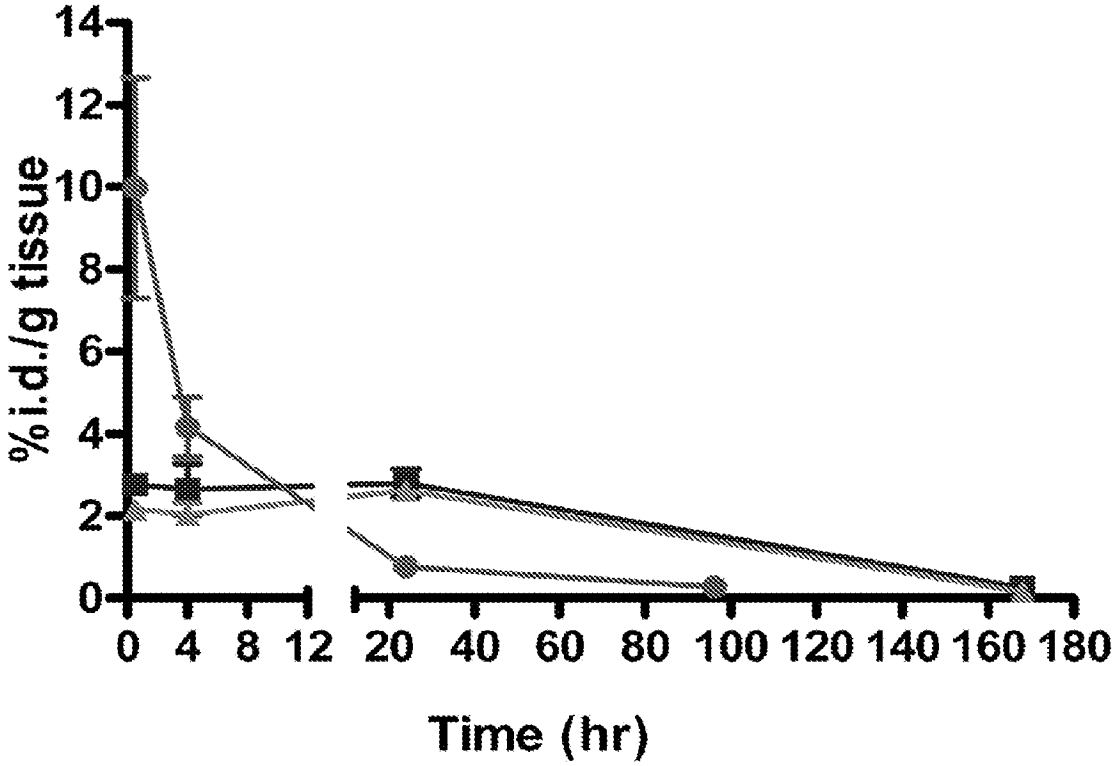
14
12
10
8
6
4
2
0
%i.d./g tissue
0 4 8 12 20 40 60 80 100 120 140 160 180
Time (hr)

Figure 7A

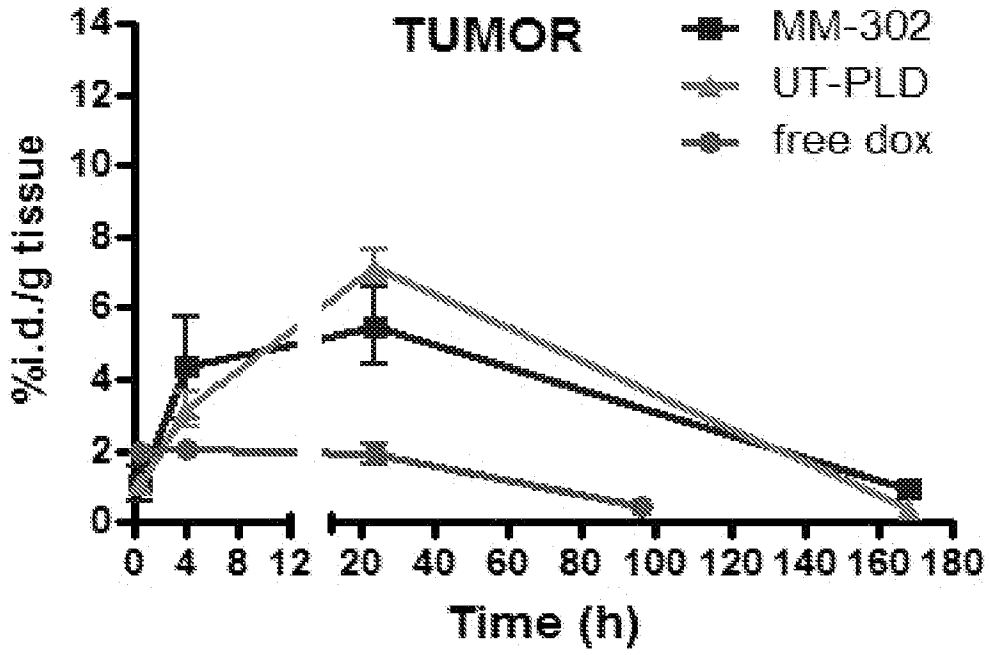
TUMOR
MM-302
UT-PLD
free dox
%i.d./g tissue
0
2
4
6
8
10
12
14
0 4 8 12 20 40 60 80 100 120 140 160 180
Time (h)

Figure 7B

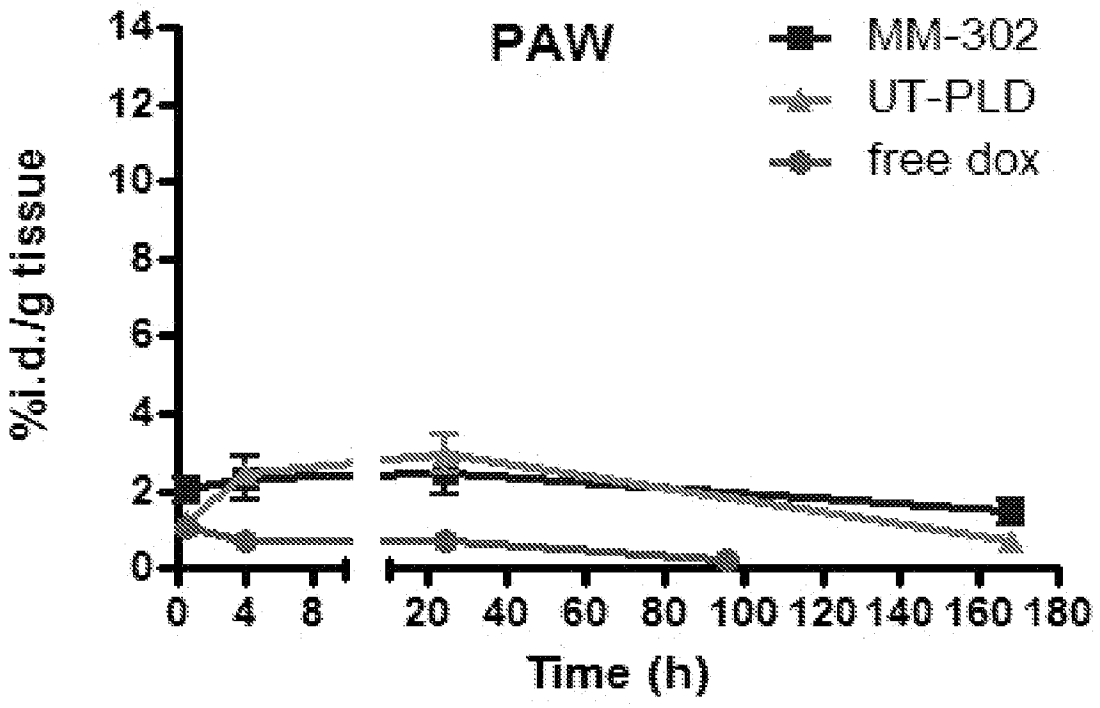
PAW
MM-302
UT-PLD
free dox
%i.d./g tissue
14
12
10
8
6
4
2
0
0
4
8
20
40
60
80
100
120
140
160
180
Time (h)

Figure 7C

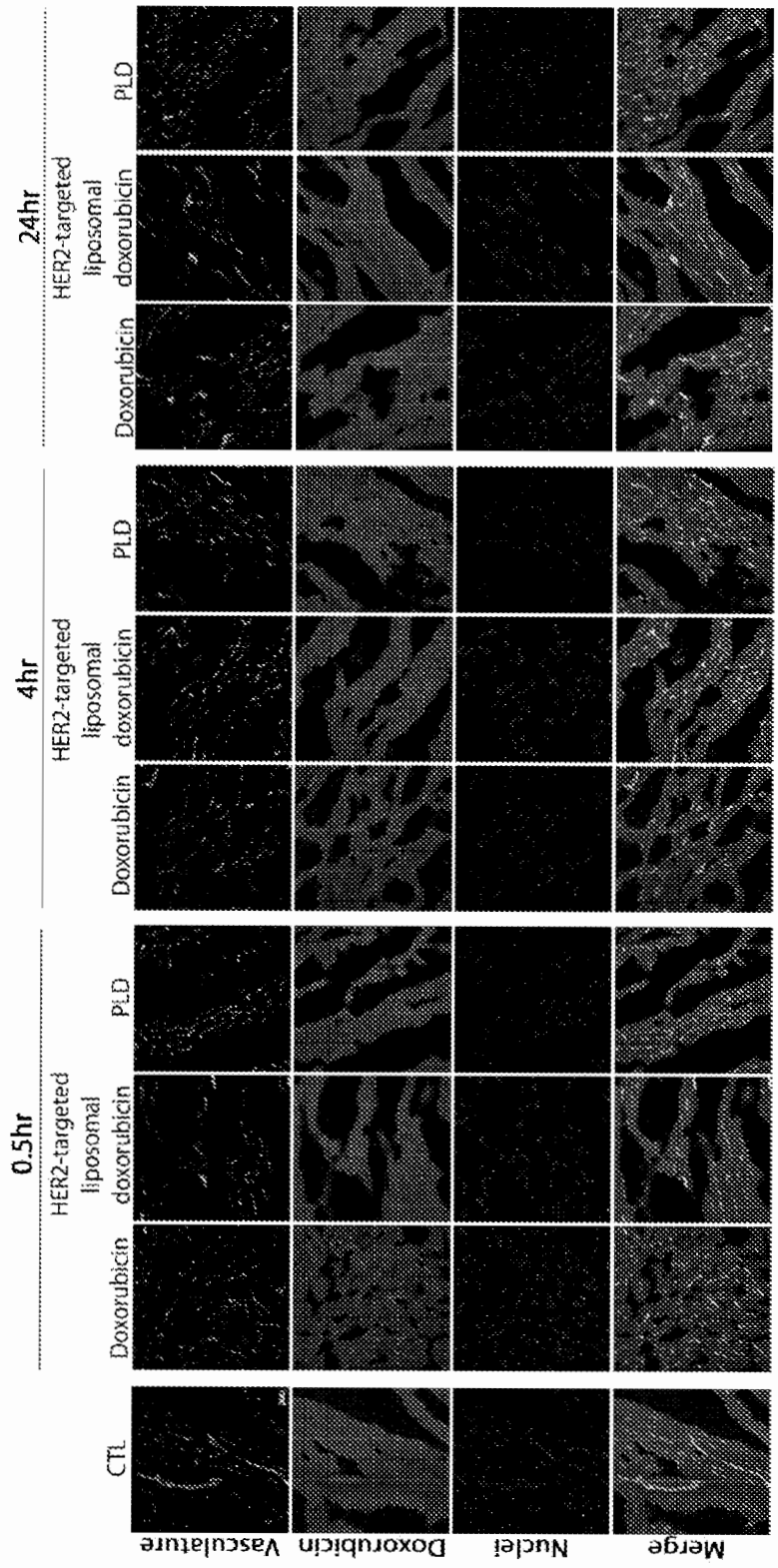
0.5hr
4hr
24hr
HER2-targeted liposomal doxorubicin
Doxorubicin
PLD
CTL
Vasculature
Doxorubicin
Nuclei
Merge

Figure 7D

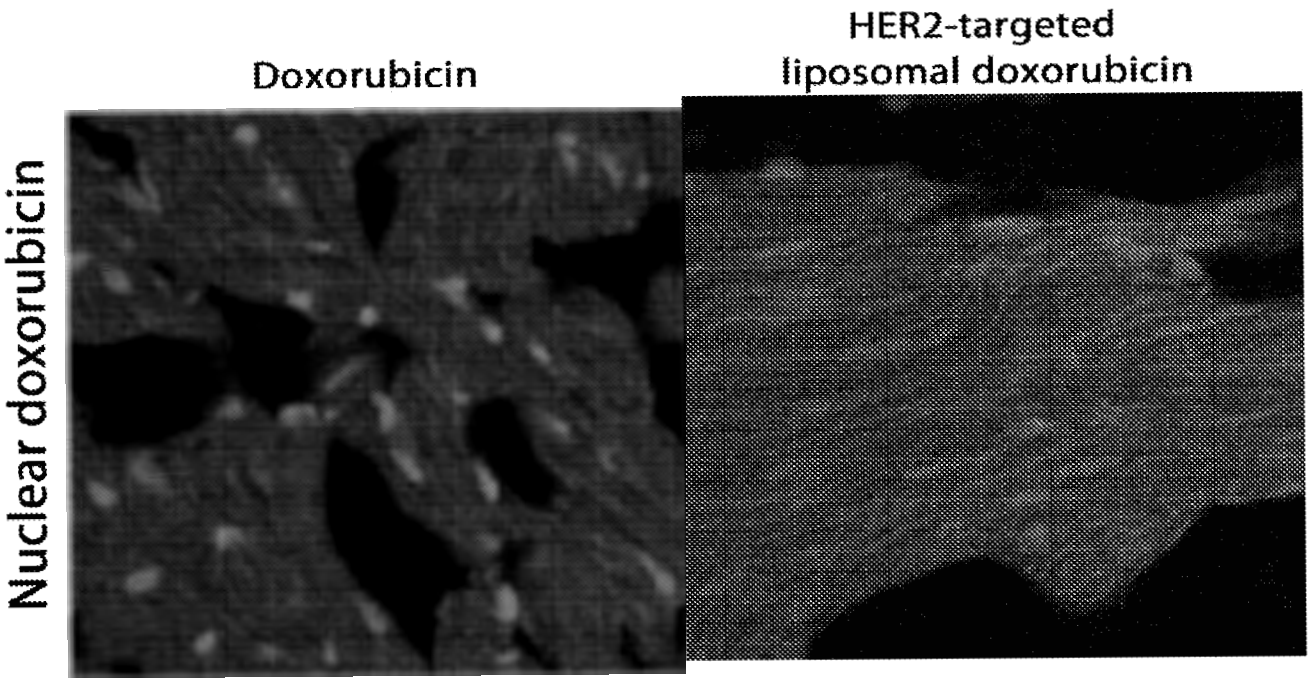
Doxorubicin
HER2-targeted
liposomal doxorubicin
Nuclear doxorubicin

Figure 7E

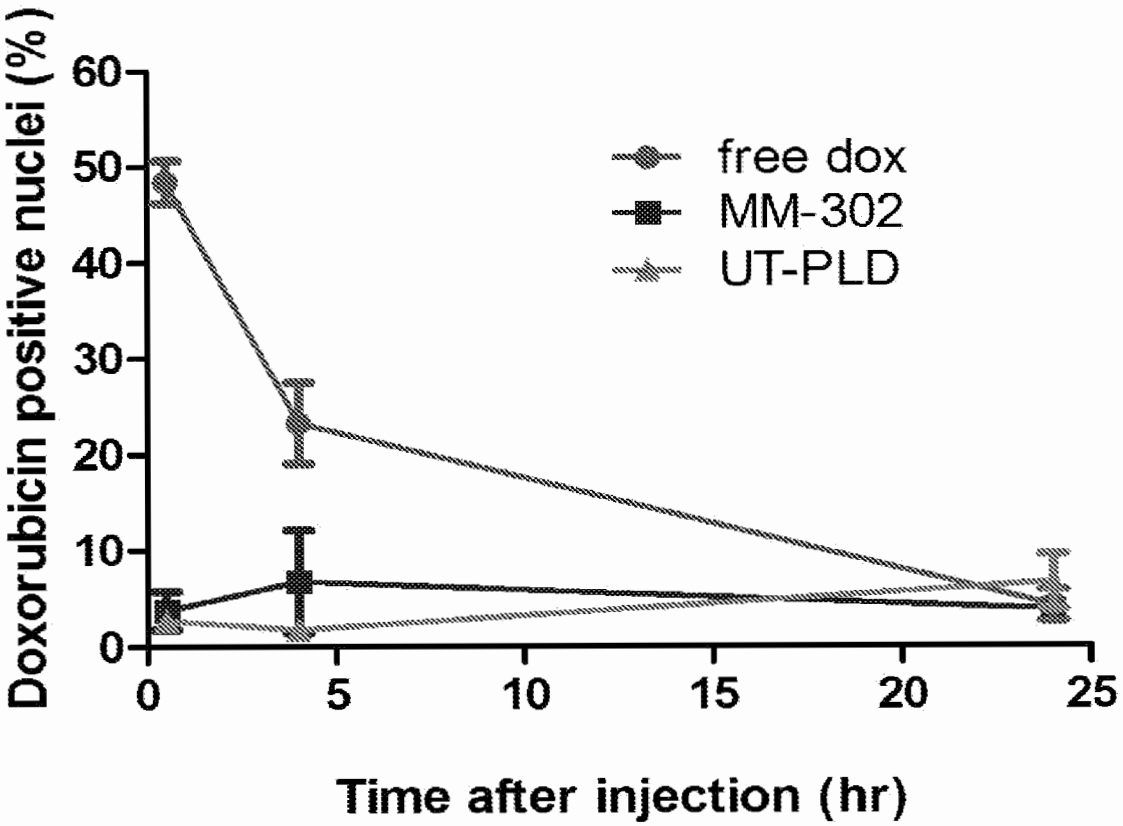
Doxorubicin positive nuclei (%)
60
50
40
30
20
10
0
0
5
10
15
20
25
Time after injection (hr)
free dox
MM-302
UT-PLD

Figure 7F

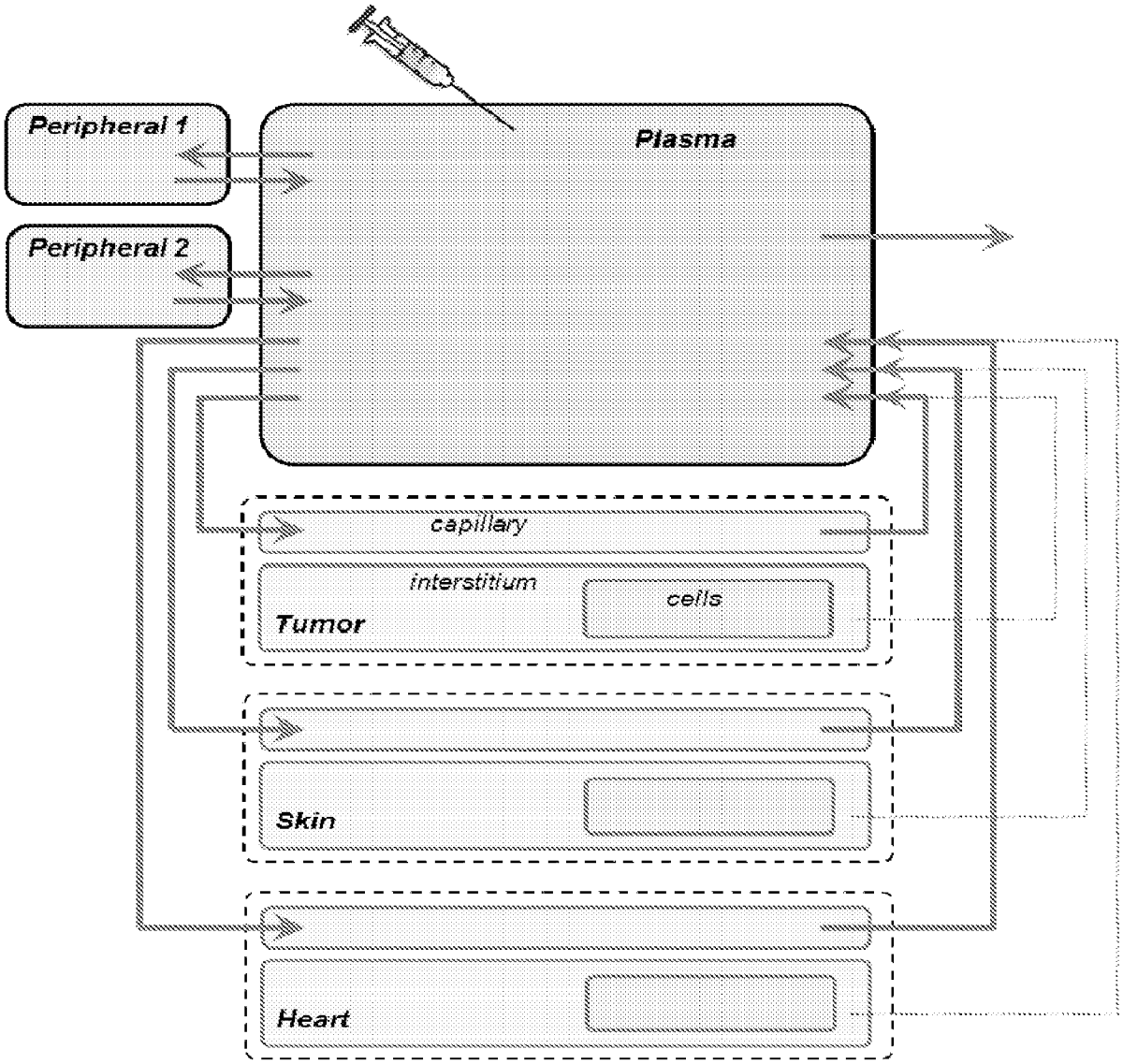
Peripheral 1
Peripheral 2
Plasma
capillary
interstitium
cells
Tumor
Skin
Heart

Figure 8A

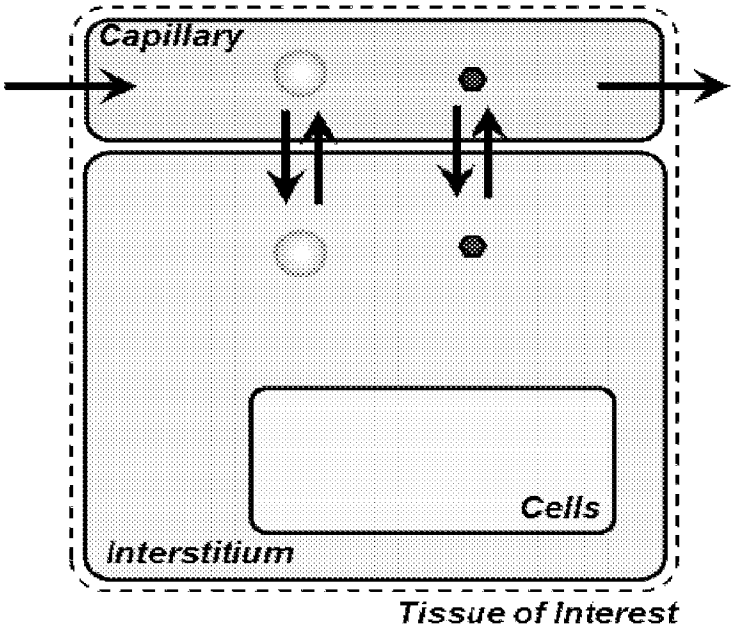

Figure 8B

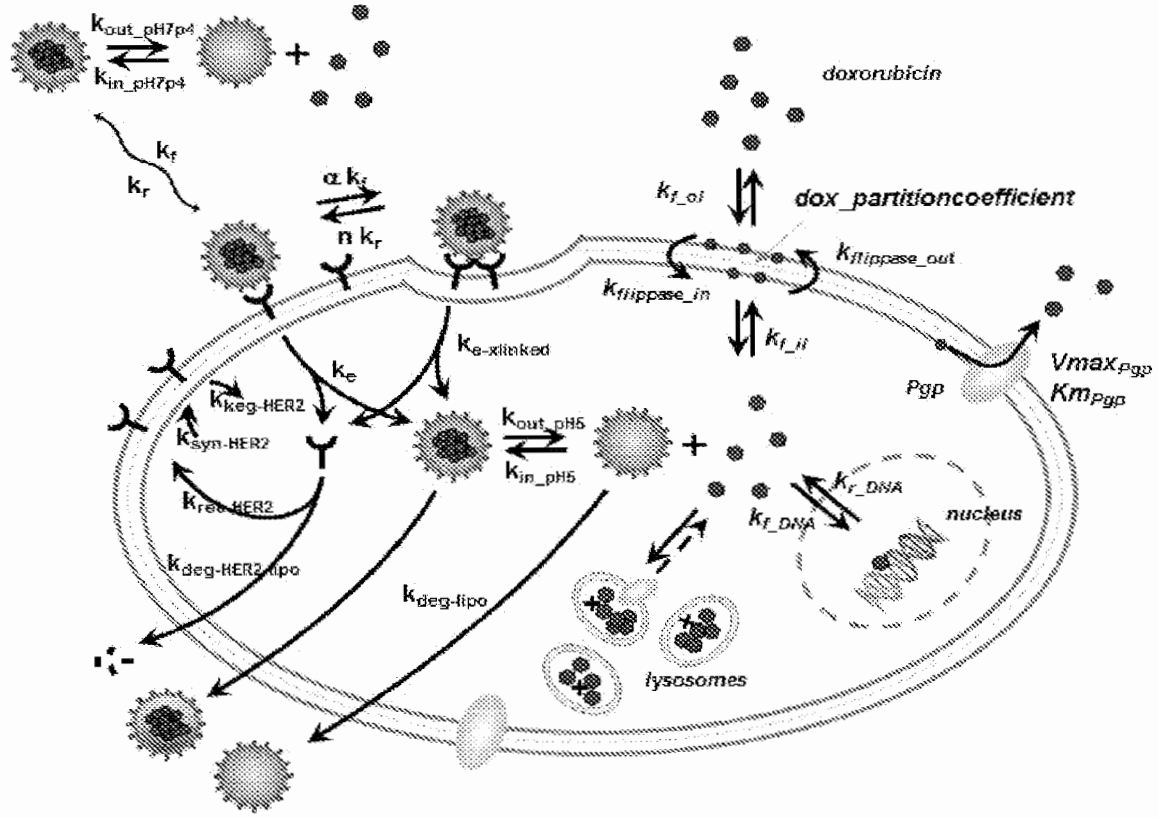
k_out_pH7p4
k_in_pH7p4
k_f
k_r
α k_r
n k_r
k_e
k_e-xlinked
k_keg-HER2
k_syn-HER2
k_rec-HER2
k_deg-HER2-lipo
k_deg-lipo
k_out_pH5
k_in_pH5
doxorubicin
k_f_oi
dox_partitioncoefficient
k_flippase_out
k_flippase_in
k_f_ii
Pgp
Vmax_Pgp
Km_Pgp
k_f_DNA
k_f_DNA
nucleus
lysosomes

Figure 8C