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(54) Title: HYDROLYZABLE PRODRUGS FOR DELIVERY OF ANTICANCER DRUGS TO METASTATIC CELLS

(57) Abstract

Hydrolyzable prodrugs according to the present invention are activated by proteases located in the cell membranes of metastatic cells to yield active anticancer drugs that can be taken up by the metastatic cells. In general, a hydrolyzable prodrug according to the present invention comprises an amino-terminal capped peptide that is a substrate for a peptidohydrolase located on the surface of a metastatic cell covalently linked to a therapeutic drug through a self-immolating spacer of sufficient length to prevent the occurrence of steric hindrance. The therapeutic drug is typically an anticancer drug. The anticancer drug is typically doxorubicin, taxol, camptothecin, mitomycin C, or esperamycin. Typically, the peptidohydrolase that hydrolyses the substrate of the hydrolyzable prodrug is cathepsin B.

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HYDROLYZABLE PRODRUGS FOR DELIVERY OF ANTICANCER DRUGS TO METASTATIC CELLS

BACKGROUND OF THE INVENTION

This invention is directed to hydrolyzable prodrugs for delivery of therapeutic drugs to metastatic cells, particularly anticancer drugs.

Metastasis is the hallmark of cancer. A tumor that does not metastasize is termed "benign" because it poses a threat of survival that is small compared to a "malignant" tumor that does metastasize (McGuire, New Eng. J. Med., 320:525 (1989)).

Metastasis involves a sequence of events that few cells can successfully complete (Sanchez, Am. J. Med. Sci., 292:376 (1986); Poste, Nature, 283:139 (1980)). Metastatic cells must break away from the primary tumor, survive attack by the immune system during transit in the blood, lodge somewhere while resisting the shear force of the moving blood stream, penetrate basement membrane to reach a safe haven where they can multiply, and finally create a blood supply of their own when the demand for nourishment of the growing metastatic tumor exceeds what is available locally by diffusion. Metastatic cells are not a representative sample of the tumor (itself highly heterogeneous) (Poste, Ann. New York Acad. Sci., 397:34 (1982); Heppner, Cancer Res., 44:2259 (1984); Fidler, Science, 217:998 (1982)) but rather constitute a small subpopulation that increases with the age of the primary tumor (Fidler, Cancer Res., 50:6130 (1990); Science, 197:873 (1977); Kerbel, Int. J. Cancer, 487:118 (1991)). Each metastasis is clonal but rapidly diversifies (Fidler, Cancer Treat. Rep., 68:193 (1984)).

Although metastases are very small and therefore comparatively accessible to chemotherapy, they are highly resistant to present day drugs, for in spite of heavy medication, survival rates for e.g., phases 2 and 3 breast carcinoma (lymph node involvement signaling spread) are very low compared to phase 1 (no spread). In the absence of

spread, the survival rate is 70% or greater; in the presence of spread, it is less than 10% (McGuire, New Eng. J. Med., 320:525 (1989)). Inhibiting as contrasted with killing metastases can only prolong life a short while because by the time cancer is typically diagnosed, metastasis has already taken place. Therefore, there is a great need in cancer therapy for means of specifically eradicating metastatic foci, either after or before excision of the primary tumor (Fisher, Cancer Res., 483:1488 (1983); Jacquillat, ed., "Neo-Adjuvant Chemotherapy" (Libbey & Co., London, 1986); Bonadonna, J. Nat. Cancer Inst., 82:1539 (1990); Fichtner, Anti-Cancer Res., 7:227 (1987)).

Preferably, such anti-metastatic reagents should be usable against many types of metastases and not depend for their activity on characteristics of the primary tumor cells that might not be shared by the metastases. Preferably, such anti-metastatic reagents should be readily absorbed and lack toxicity, particularly in patients who are already subject to regimens consisting of multiple drug treatment.

SUMMARY

I have developed anti-metastatic reagents that meet these needs in the form of hydrolyzable prodrugs. In general, a hydrolyzable prodrug according to the present invention comprises an amino-terminal capped peptide covalently linked to a therapeutic drug through a self-immolating spacer of sufficient length to prevent the occurrence of steric hindrance. The amino-terminal capped peptide is a substrate for a peptidohydrolase located on the surface of a metastatic cell

Typically, the peptidohydrolase is cathepsin B or collagenase IV. Preferably, the peptidohydrolase is cathepsin B.

Typically, the amino-terminal capped peptide is benzyloxycarbonylphenylalanyllysine, benzyloxycarbonylvalinyllysine, D-phenylalanylphenylalanyllysine, benzyloxycarbonylvalinylcitrulline, t-butyloxycarbonylphenylalanylysine, benzyloxycarbonyl-alanyllarginylarginine, benzyloxycarbonylphenylalanyl-N-tosylarginine,2-

aminoethylthio-succinimidopropionylvalinylcitrulline, 2-aminoethylthio-succinimidopropionyllysylphenylalanyllysine, acetylphenylalanyllysine, or benzyloxycarbonylphenylalanyl-O-benzoylthreonine.

Typically, the therapeutic drug is an anticancer drug. Preferably, the anticancer drug is doxorubicin, mitomycin C, taxol, esperamycin, or camptothecin. A particularly preferred anticancer drug is doxorubicin.

Typically, the spacer is p-aminobenzyl carbonyl ("PABC") or the bis-carbamate p-NH-Ph-CH=($CH_2OCO-)_2$ of bis(hydroxymethyl) styrene or "BHMS".

The hydrolyzable prodrug can further comprise a peptide derived from a protein to which metastatic cells adhere in establishing colonies covalently linked to the therapeutic drug. Typically, the peptide is a RGD-derived active peptide or a YIGSR-derived active peptide. Preferably, the peptide is YIGSR (SEQ ID NO:1) or GRGDS (SEQ ID NO:2).

Preferred hydrolyzable prodrugs according to the present invention include benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, acetylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, acetylphenylalanyllysyl-p-aminobenzyl carbamoylmitomycin C, benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbonyl-7-paclitaxel, acetylphenylalanyllysyl-p-aminobenzyl carbonylcamptothecin, 2-aminoethylthio-succinimidopropionyl-valinylcitrullinyl-BHMS-didoxorubicin, 2-aminoethylthio-succinimidopropionyl-lysylphenylalanyllysyl-BHMS-didoxorubicin, benzyloxycarbonylvalinyllysyl-p-aminobenzyl carbamoyldoxorubicin, D-phenylalanylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, and benzyloxycarbonylvalinylcitrullinyl-p-aminobenzyl carbamoyldoxorubicin.

Another aspect of the present invention is a method for delivering a therapeutic drug to a metastatic cell comprising the steps of:

(1) contacting a hydrolyzable prodrug according to the present invention with a metastatic cell;

(2) allowing the peptidohydrolase located on the surface of the metastatic cell to hydrolyze the hydrolyzable prodrug and release the therapeutic drug from the prodrug; and

(3) allowing the therapeutic drug to enter the metastatic cell.

Yet another aspect of the present invention is a pharmaceutical composition comprising:

(1) a hydrolyzable prodrug according to the present invention; and

(2) a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims and accompanying drawings where:

Figure 1 is a depiction, showing structural formulas and reaction conditions, of the initial stages in the synthesis of the hydrolyzable prodrug Ac-Phe-Lys-PABC-Dox;

Figure 2 is a similar depiction of the final stages in the synthesis of Ac-Phe-Lys-PABC-Dox;

Figure 3 is a similar depiction of the synthesis of the hydrolyzable prodrug Ac-Phe-PABC-MMC, beginning with an intermediate in the synthesis of Ac-Phe-Lys-PABC-Dox prior to the coupling of the doxorubicin residue;

Figure 4 is a similar depiction of the initial stages of the synthesis of the hydrolyzable prodrug Z-Phe-Lys-PABC-Paclitaxel;

Figure 5 is a similar depiction of the final stages of the synthesis of the hydrolyzable prodrug Z-Phe-Lys-PABC-Paclitaxel;

Figure 6 is a similar depiction of the early stages of the synthesis of the hydrolyzable prodrug CA-SP-Lys-Phe-Lys-BHMS-Dox_2;

Figure 7 is a similar depiction of the intermediate stages of the synthesis of CA-SP-Lys-Phe-Lys-BHMS-Dox_2;

Figure 8 is a similar depiction of the final stages of the synthesis of CA-SP-Lys-Phe-Lys-BHMS-Dox_2;

Figure 9 is a table showing the killing of BT-20 tumor cells, which are high-cathepsin B-secreting cells, and MCF-10A tumor cells, which are low-cathepsin B-secreting cells, by several hydrolyzable prodrugs and control compounds in the presence or absence of the cathepsin inhibitor CA-074;

Figure 10 is a table showing the killing of BT-20 tumor cells at various times and prodrug concentrations with several hydrolyzable prodrugs and a control compound;

Figure 11 is a table showing the killing of BT-20 and MCF-10A tumor cells at various times and prodrug concentrations with the hydrolyzable prodrug CA-SP-Lys-Phe-Lys-BHMS-Dox_2; and

Figure 12 is a table showing the stability of hydrolyzable prodrugs according to the present invention under physiological conditions in the presence or absence of cathepsin B.

DESCRIPTION

One new approach to developing reagents specific for metastatic cells takes advantage of the properties of the metastatic cells themselves, particularly those properties possessed by the metastatic cells that allow them to spread through the body and adhere to specific tissues.

One such property is the ability of metastatic cells to penetrate basement membrane (Sanchez, Am. J. Med. Sci, 292:376 (1986); Poste, Nature, 283:139 (1980)), shared only by the peripheral cells of the primary tumor (Poole, Nature, 273:545 (1978); Shamberger, Nature, 213:617 (1967); Graf, Lab. Invest., 45:587 (1981); Baici, Inv. Metas., 4:13 (1984); Duffy, Eur. J. Cancer Clin. Oncol., 23:583 (1987)) and by virtually no normal body cells.

Metastatic cells do this by means of hydrolytic enzymes which they secrete into the medium (Duffy, Eur. J. Can. Clin. Oncol., 23:583 (1987); MacKay, Cancer Res., 50:5997 (1990); Goldfarb, Sem. Thromb. Hemostas., 12:294 (1986); Pietras, Gynec. Oncol., 7:1 (1979)) or in their plasma membranes (Sylven, Virchows Arch. B., 17:97 (1974); Sloane, Biomed. Biochim. Acta, 50:549 (1991); Keren, Cancer Res., 48:1416 (1989); Pietras, J. Biol. Chem., 256:8536 (1981); Weiss, Proc. Am. Assoc. Cancer Res., 31:73 (1990); Sloane, Bio. Chem. Hoppe-Seyler, 371 Suppl.:193 (1990); Rozhin, Cancer Res., 47:6620 (1987); Sloane, Proc. Natl. Acad. Sci., 83:2483 (1986)). Tumors are proteolytic (Fischer, Arch. Entw. Mech. Arg., 104:210 (1925); Duffy, Eur. J. Cancer Clin. Oncol., 23:583 (1987); Strauli et al., ed., "Proteinases and Tumor Invasion" (Raven Press, New York, 1980)), and this power is correlated with metastatic propensity (Duffy, Eur. J. Cancer Clin. Oncol., 23:583 (1987); Sylven, Virchows Arch. B., 17:97 (1974); Sloane, Biomed. Biochim. Acta, 50:549 (1991); Keren, Cancer Res., 48:1416 (1989); Pietras, J. Biol. Chem., 256:8536 (1981); Weiss, Proc. Am. Assoc. Cancer Res., 31:73 (1990); Sloane, Bio. Chem. Hoppe-Seyler, 371 Suppl.:193 (1990); Rozhin, Cancer Res., 47:6620 (1987); Sloane, Proc. Natl. Acad. Sci., 83:2483 (1986); Sheahan, Cancer Res., 49:3809 (1989); Koppel, Exp. Cell Biol., 52:293 (1984); Sloane, Science, 212:1151 (1981); Sloane, Cancer Res., 45:3636 (1985); Nakajima, Cell. Biochem., 36:157 (1988); Nakajima, Science, 220:611 (1983); Qian, Cancer Res., 49:4870 (1989); Hendrix, Molec. Cell Probes, 6:59 (1992)). Enzymes known to be secreted by metastasizing cells include cathepsin B (Sloane, Biomed. Biochim. Acta, 50:549 (1991); Keren, Cancer Res., 48:1416 (1989); Pietras, J. Biol. Chem., 256:8536 (1981); Weiss, Proc. Am. Assoc. Cancer Res., 31:73 (1990); Sloane, Bio. Chem. Hoppe-Seyler, 371 Suppl.:193 (1990); Rozhin, Cancer

Res., 47:6620 (1987); Sloane, Proc. Natl. Acad. Sci., 83:2483 (1986); Sheahan, Cancer Res., 49:3809 (1989); Koppel, Exp. Cell Biol., 52:293 (1984); Sloane, Science, 212:1151 (1981); Sloane, Cancer Res., 45:3636 (1985); Nakajima, Cell. Biochem., 36:157 (1988); Nakajima, Science, 220:611 (1983); Qian, Cancer Res., 49:4870 (1989)), cathepsin D (Montcourrier, Cancer Res., 50:6045 (1990); Vasishta, Brit. J. Surg., 72:386 (1985)), cathepsin L (Rozhin, Biochem. Biophys. Res. Comm., 164:556 (1989); Vasishta, Brit. J. Surg., 72:386 (1985)), cathepsin H (Vasishta, Brit. J. Surg., 72:386 (1985)), collagenase IV (Goldfarb, Sem. Thromb. Hemostas., 12:294 (1986); Hendrix, Molec. Cell. Probes, 6:59 (1992)), urokinase-type plasminogen activator (Goldfarb, Sem. Throm. Hemostas., 12:294 (1986); Marutsuka, Inv. Metas. 11:181 (1991)), β-glucuronidase (heparanase) (Rozhin, Cancer Res., 47:6620 (1987); Nakajima, Cell. Biochem., 36:157 (1988); Nakajima, Science, 220:611 (1983)), gelatinase (Aoyama, Proc. Natl. Acad. Sci., 87:8996 (1990)), guanidinobenzoatase (Steven, Anti-Cancer Res., 11:143 (1991)); and undefined tryptic enzymes (Goldfarb, Sem. Thromb. Hemostas., 12:294 (1986)). Many studies have reported greatly increased enzyme secretion by malignant cells vis-a-vis nearby normal cells from the same patient (Poole, Nature, 273:545 (1978); Sheahan, Cancer Res., 49:3809 (1989); Rozhin, Biochem. Biophys. Res. Comm., 164:556 (1989); Watanabe, Hepato-Gastro-Enterology, 34:126 (1987); Murnane, Cancer Res., 51:1137 (1991); Chauhan, Cancer Res., 51:1478 (1991); Durdey, Brit. J. Surg., 72:378 (1985); Sedo, J. Cancer Res. Clin. Oncol., 117:249 (1991); Lah, Int. J. Cancer, 50:36 (1992); Dengler, Biomed. Biochim. Acta, 50:555 (1991)).

A potential application of these enzymes is to unmask cytotoxic drugs at the sites of metastatic foci. Preferred drugs are those that are readily ingested by cells such as doxorubicin (Dox) (also known as adriamycin (ADM)), mitomycin C (MMC), taxol, camptothecin (CPT), and esperamycin, as well as derivatives of these drugs. Typically, such anticancer drugs have substantial hydrophobic moieties so that they can pass through the plasma membrane of metastatic cells. Other anticancer drugs can be derivatized with appropriate hydrophobic moieties to improve

their permeability to the lipid bilayer of the plasma membrane of the metastatic cell.

A particularly useful enzyme with prodrugs according to the present invention is cathepsin B, principally because it is an exopeptidase (Takahashi, J. Biol. Chem., 261:9375 (1986); Koga, J. Biochem., 110:179 (1991)) for which numerous peptide substrates are already known (Dingle, ed., "Lysosomes" (North-Holland, Amsterdam, 1977); Strauli et al., eds., "Proteinases and Tumor Invasion" (Raven Press, New York, 1980); Neuberger, ed., "Hydrolytic Enzymes" (Elsevier, Amsterdam, 1987)). Cathepsin B is a lysosomal enzyme that is ubiquitous within cells (Dingle, ed., "Lysosomes" (North-Holland, Amsterdam, 1977); Strauli et al., eds., "Proteinases and Tumor Invasion" (Raven Press, New York, 1980); Neuberger, ed., "Hydrolytic Enzymes" (Elsevier, Amsterdam, 1987)), but almost never secreted normally. If small amounts of cathepsin B do escape during exocytosis or unprogrammed cell death, it loses all activity within 15 minutes at neutral pH (Sheahan, Cancer Res., 49:3809 (1989); Dingle, ed., "Lysosomes" (North Holland Publishing Co., Amsterdam, 1977); Buck, Biochem. J., 282:273 (1992)). There is a strong association of cathepsin B with cancer as opposed to normal cells (Duffy, Eur. J. Cancer Clin. Oncol., 23:583 (1987); Goldfarb, Sem. Thromb. Hemostas., 12:294 (1986); Pietras, Gynecol. Oncol., 7:1 (1979)), often within the same patient (Poole, Nature, 273:545 (1978); Watanabe, Hepato-Gastro-Enterology, 34:126 (1987); Murnane, Cancer Res., 51:1137 (1991); Durdey, Brit. J. Surg., 72:378 (1985); Sedo, J. Cancer Res. Coin. Oncol., 117:249 (1991); Lah, Int. J. Cancer, 50:36 (1992); Dengler, Biomed. Biochim. Acta, 50:555 (1991)), and its secretion correlates with the metastatic propensity of cells from patients and animals, and with the degree of malignancy of their disease (Duffy, Eur. J. Cancer Clin. Oncol., 23:583 (1987); Goldfarb, Sem. Thromb. Hemostas., 12:294 (1986); Pietras, Gynecol. Oncol., 7:1 (1979); Sloane, Biomed. Biochim. Acta, 50:549 (1991); Keren, Cancer Res., 48:1416 (1989); Pietras, J. Biol. Chem., 256:8536 (1981); Weiss, Proc. Am. Assoc. Cancer Res., 31:73 (1990); Sloane, Bio. Chem. Hoppe-Seyler, 371 Suppl.:193 (1990); Rozhin, Cancer Res., 47:6620 (1987); Sloane, Proc. Natl. Acad. Sci., 83:2483

(1986); Sheahan, Cancer Res., 49:3809 (1989); Koppel, Exp. Cell Biol., 52:293 (1984); Sloane, Science, 212:1151 (1981); Sloane, Cancer Res., 45:3636 (1985); Nakajima, Cell. Biochem., 36:157 (1988); Nakajima, Science, 220:611 (1983); Qian, Cancer Res., 49:4870 (1989) Lah, Int. J. Cancer, 50:36 (1992)). At pH 7, cathepsin B degrades a number of extracellular matrix components including type IV collagen (Buck, Biochem. J., 282:273 (1992); Maciejewicz, Biomed. Biochim. Acta, 50:561 (1991); Maciejewicz, Int. J. Cancer, 43:478 (1989)), laminin (Buck, Biochem. J., 282:273 (1992)), and fibronectin (Buck, Biochem. J., 282:273 (1992)). Although not normally stable at pH 7 as opposed to pH 5, cathepsin B from tumors is stable at pH 7 (Sheahan, Cancer Res., 49:3809 (1989); Buck, Biochem. J., 282:273 (1992); Sloane, Cancer Metastas. Rev., 3:249 (1984)), especially when kept within the cells' plasma membranes (Rozhin, Cancer Res., 47:6620 (1987)). In fact, malignant cells do secrete cathepsin B into their plasma membranes.

The plasma membrane is obviously a highly desirable place to activate a latent cytotoxic drug which should be delivered, not only as directly as possible to the target cells, but also to neighboring cancer cells that might not display as much cathepsin B as the majority, owing to the high genetic instability (Fidler, Cancer Treat. Rep., 68:193 (1984)) of metastasizing cells.

I. HYDROLYZABLE PRODRUGS

A preferred reagent for delivering therapeutic drugs to metastasizing cells according to the present invention is a hydrolyzable prodrug comprising an amino-terminal capped peptide covalently linked to a therapeutic drug through a self-immolating spacer of sufficient length to prevent the occurrence of steric hindrance. The amino-terminal capped peptide is a substrate for a peptidohydrolase located on the surface of a metastatic cell.

Typically, the peptidohydrolase is cathepsin B or collagenase IV; preferably, the peptidohydrolase is cathepsin B.

If the peptidohydrolase is cathepsin B, the amino-terminal capped peptide that acts as a substrate for the peptidohydrolase is typically one of benzyloxycarbonylphenylalanyllysine, benzyloxycarbonylvalinyllysine, D-phenylalanylphenylalanyllysine, benzyloxycarbonylvalinylcitrulline, t-butyloxycarbonylphenylalanylysine, benzyloxycarbonylalanyllarginylarginine, benzyloxycarbonylphenylalanyl-N-tosylarginine,2-aminoethylthio-succinimidopropionylvalinylcitrulline, 2-aminoethylthio-succinimidopropionyllysylphenylalanyllysine, acetylphenylalanyllysine, and benzyloxycarbonylphenylalanyl-O-benzoylthreonine. Derivatives of these peptides in which the derivatizing groups do not interfere with the cleavage of the peptide by cathepsin B can also be used. Alternatively, the amino-terminal capping groups of these peptides can be replaced by others known in the art.

Preferably, the amino-terminal capped peptide is benzyloxycarbonylphenylalanyllysine or acetylphenylalanyllysine.

Other suitable substrates for cathepsin B are known. For example, substrates containing paired basic residues can be hydrolyzed by cathepsin B (J.K. McDonald & S. Ellis, Life Sci., 17:1269-1276 (1975)).

For hydrolyzable prodrugs according to the present invention, the amino-terminal residue must be "capped" or protected with a protecting group. Such protecting groups are well-known in peptide chemistry and include, for example, benzyloxycarbonyl (also known as carbobenzoxy and generally abbreviated as Z), acetyl, 2-aminoethylthio-succinimidopropionyl, t-butyloxycarbonyl, and other amino-terminal protecting groups such as those disclosed in M. Bodanszky, "Principles of Peptide Synthesis" (2d Ed., Springer-Verlag, Berlin, 1993). These groups include triphenylmethyl, p-methoxybenzyloxycarbonyl, adamantyloxycarbonyl, biphenylylisopropyloxycarbonyl, formyl, isonicotinyloxycarbonyl, o-nitrophenylsulfenyl, 9-fluorenylmethyloxycarbonyl, derivatives of benzyloxycarbonyl substituted on the aromatic ring of the benzyl group, or, in some cases, in which the phenyl moiety of the benzyl group is replaced with

another fully aromatic moiety such as furan or pyridine, phthaloyl, dithiasuccinyl, p-toluenesulfonyl (tosyl), and other groups.

Alternatively, the amino-terminal protecting group can be a D-amino acid such as D-phenylalanine. When the amino-terminal protecting group is a D-amino acid, the carboxyl group of the D-amino acid forms a peptide bond with the amino-terminal residue of the amino-terminal protected peptide.

Preferably, when the peptide portion of the amino-terminal protected peptide is phenylalanyllysine, the protecting group is benzyloxycarbonyl or acetyl, so that the amino-terminal protected peptide is benzyloxycarbonylphenylalanyllysine or acetylphenylalanyllysine.

The hydrolyzable prodrug includes a spacer of sufficient length to prevent the occurrence of steric hindrance between the amino-terminal protected peptide and the therapeutic drug. If the spacer is too short, the therapeutic drug may prevent the binding of the substrate for the peptidohydrolase to the active site of the peptidohydrolase by steric hindrance. A particularly suitable spacer is p-aminobenzyl carbonyl ("PABC"). This has an approximate length of 10 angstroms.

Derivatives of p-aminobenzyl carbonyl can also be used, such as compounds substituted on the aromatic moiety of the benzyl group.

Alternatively, other spacer groups can be used. The length of the spacer should be greater than about 10 angstroms; spacers of significantly greater length can be used, and can incorporate, for example, additional aliphatic or aromatic moieties. In general, such spacers should be relatively unbranched so as not to introduce steric hindrance of their own. The chemical functionality terminating the spacer can vary but one end is able to react with the carboxyl-terminal residue of the substrate for the peptidohydrolase. Typically, this is an amino group. The other functionality terminating the spacer is capable of reacting with the therapeutic drug. In

one preferred embodiment, this functionality reacts at an amino group of the drug to form a carbamate or urethane linkage as part of the spacer.

Another preferred spacer is the bis-carbamate of bis(hydroxymethyl)styrene ("BHMS"), which has the structure p-NH-Ph-CH=(CH_2OCO-)$_2$. This spacer also reacts at an amino group of the drug to form a carbamate or urethane linkage. This spacer is bifunctional and can bind two drug moieties such as doxorubicin.

Preferably, such spacers have the property of self-immolation. A self-immolating spacer is one in which the residual portion of the spacer attached to the therapeutic drug subsequent to the hydrolysis of the peptide bond by the peptidohydrolase is then further cleaved by spontaneous, nonenzymatic hydrolysis in an aqueous medium to restore the original unconjugated drug. Both PABC and BHMS are self-immolating. For example, if the spacer is p-aminobenzyl carbonyl ("PABC") and the therapeutic drug is doxorubicin, the portion of the spacer remaining attached to the drug after hydrolysis of the peptide bond by cathepsin B, then subsequently undergoes spontaneous hydrolysis to p-aminobenzyl alcohol, carbon dioxide, and doxorubicin.

Typically, the therapeutic drug is an anticancer drug. However, other therapeutic drugs can be incorporated into hydrolyzable prodrugs according to the present invention and can be delivered to metastatic cells. Preferred anticancer drugs incorporated into hydrolyzable prodrugs according to the present invention include doxorubicin, taxol, camptothecin, mitomycin C, and esperamycin, as well as derivatives thereof. Other anticancer drugs that have hydrophobic moieties that allow them to be taken up efficiently by metastatic cells or that can be derivatized with such moieties can also be used.

Therefore, particularly preferred hydrolyzable prodrugs according to the present invention include benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, acetylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin,

acetylphenylalanyllysyl-p-aminobenzyl carbamoylmitomycin C, benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbonyl-7-paclitaxel, acetylphenylalanyllysyl-p-aminobenzyl carbonylcamptothecin, 2-aminoethylthio-succinimidopropionyl-valinylcitrullinyl-bis(hydroxymethyl)-styryl-bisdoxorubicin, 2-aminoethylthio-succinimidopropionyl-lysylphenylalanyllysyl-bis(hydroxymethyl)styryl-bisdoxorubicin, benzyloxycarbonylvalinyllysyl-p-aminobenzyl carbamoyldoxorubicin, D-phenylalanylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, and benzyloxycarbonylvalinylcitrullinyl-p-aminobenzyl carbamoyldoxorubicin.

Synthesis of hydrolyzable prodrugs according to the present invention can be accomplished by condensation reactions that are well known in the art. Examples of syntheses are given below in Examples 1-6. In general, using the spacer PABC, the synthetic procedure comprises: (1) synthesizing the peptide that is the substrate of the peptidohydrolase by conventional peptide synthetic techniques, with the α-amino group of the amino-terminal amino acid residue protected and appropriate protection for reactive side chains of the amino acids; (2) linking the p-aminobenzyl moiety to the carboxyl group of the carboxyl-terminal amino acid; (3) activating the p-aminobenzyl moiety for covalent linkage of the therapeutic drug; (4) covalently linking the therapeutic drug, which may have certain reactive side chains protected as well; and (5) removing the remaining protecting groups on the peptide and the therapeutic drug.

In general, using the spacer BHMS, the synthetic procedure comprises: (1) synthesizing a peptide that is the substrate of the peptidohydrolase by conventional peptide synthetic techniques, with appropriate protective groups as above; (2) linking the BHMS moiety to the carboxyl group of the carboxyl-terminal amino acid of the peptide; (3) activating the BHMS moiety for coupling of the therapeutic drug; (4) coupling the therapeutic drug to the activated BHMS moiety; (5) removing any protecting groups on amino acid side chains, such as the ε-amino group of lysine; and (6) modifying the amino-terminal blocking group so that the desired capping group is present.

As another alternative, if the substrate for cleavage by the peptidohydrolase is a tripeptide or peptide with more than three amino acids, the peptide can be extended at its amino-terminus after the coupling of the carboxyl-terminus to the spacer. This involves removing the amino-terminal protecting group of the peptide, activating the carboxyl group of the amino acid to be added, and coupling it to the deblocked amino group to form a peptide bond. This step can be repeated if a longer peptide is desired. Then, the therapeutic drug is coupled to the completed peptide and synthesis of the hydrolyzable prodrug is completed as above.

In one embodiment according to the present invention, the hydrolyzable prodrug further comprises a peptide derived from a protein that adheres to metastatic cells. Such peptides include peptides derived from the protein fibronectin (GRGDS)(SEQ ID NO:1)(Humphries, Science 233:467 (1986); Olden, Ann. New York Acad. Sci. 421 (1989); Dedhar, Bio Essays, 12:583 (1990)) and laminin (YIGSR)(SEQ ID NO:2)(Iwamoto, Science, 238:1132 (1987); Saiki, Brit. J. Cancer 59:194 (1989)).

Typically, the peptide is covalently linked to the amino side of the amino-terminal capped peptide that is the substrate for the peptidohydrolase, either directly as an amide, or indirectly via attachment to the capping group,benzyloxycarbonyl, acetyl, maleimidopropionyl or the like, which has been modified to accept the peptide. The linkage can be through either the amino or carboxyl group of the peptide, or, in some cases, through functional groups of the peptide such as the carboxyl of aspartic acid, the hydroxyl of serine or other functional groups of other residues. Depending upon the peptide chosen and the therapeutic drug, various cross-linking reagents can be used. For example, carbodiimides, such as dicyclohexylcarbodiimide, can form an amide linkage between a carboxyl group and an amine. Other reactive groups are known and are described, for example, in G.T. Hermanson, "Bioconjugate Techniques" (Academic Press, San Diego, 1996), in S.S. Wong, "Chemistry of Protein Conjugation and Crosslinking" (CRC Press, Boca Raton, Fla. 1991), and in T.E. Creighton, Ed., "Protein Function: A Practical Approach" (IRL Press, Oxford

1989). The peptide can be linked, for example, to a maleimidopropionyl cap via a cysteine whose thiol group is added to the maleimido group, or to a glycine or other amino acid cap (replacing the acetyl cap) via acylation of the amino group of the glycine, or by attaching the peptide to the p-position of the benzyloxycarbonyl cap, or by other methods known to the art. The linkage of these peptides such as YIGSR (SEQ ID NO: 2) to the prodrug may be with or without intervening links which might or might not consist of other amino acids.

Other conjugation or crosslinking methods can also be used to attach the peptides such as the peptides from laminin or fibronectin to other portions of the hydrolyzable prodrug,. In most cases, attaching the peptide to the self-immolating spacer would either prevent hydrolysis of the substrate by the peptidohydrolase or result in steric hindrance.

A number of peptides derived from the fibronectin and laminin peptides can be linked to the hydrolyzable prodrugs. These peptides can be classified in terms of their structure and homology to the fibronectin or laminin sequence as follows:

Fibronectin-derived peptides include: (1) the GRDGS (SEQ ID NO: 1) pentapeptide derived from the fibronectin sequence (I. Hardan et al., "Inhibition of Metastatic Cell Colonization in Murine Lungs and Tumor-Induced Morbidity by Non-Peptidic Arg-Gly-Asp Mimetics," Int. J. Cancer 55: 1023-1028 (1993)); (2) derivatives of the fibronectin pentapeptide sequence with conservative amino acid substitutions, such as GRGES (SEQ ID NO: 3) (R.J. Tressler et al., "Correlation of Inhibition of Adhesion of Large Cell Lymphoma and Hepatic Sinusoidal Endothelial Cells by RGD-Containing Peptide Polymers with Metastatic Potential: Role of Integrin-Dependent and -Independent Adhesion Mechanisms," Cancer Commun. 1: 55-63 (1989)); (3) truncated peptides derived from this sequence, such as RGD and RGDS (SEQ ID NO: 4) (I. Saiki et al., "Anti-Metastatic and Anti-Invasive Effects of Polymeric Arg-Gly-Asp (RGD) Peptide, Poly(RGD), and Its Analogues," Japan J. Cancer Res. 81: 660-667 (1990)); (4) truncated peptides with amino

acid substitutions, such as RGDT (SEQ ID NO: 5) (I. Saiki et al. (1990), supra), as well as RGE and RGET (SEQ ID NO: 6); (5) extended peptides derived from the fibronectin sequence, such as GRGDSP (SEQ ID NO: 7) (M.D. Pierschbacher & E. Ruoslahti, "Influence of Stereochemistry of the Sequence Arg-Gly-Asp-Xaa on Binding Specificity in Cell Adhesion," J. Biol. Chem. 262: 17294-17298 (1987)) and GRGDSPA (SEQ ID NO: 8) (H. Kumagai et al., "Effect of Cyclic RGD Peptide on Cell Adhesion and Tumor Metastasis," Biochem. Biophys. Res. Commun. 177: 74-82 (1991); (5) extended peptides with amino acid substitutions, such as GRGDXPC, where X is a naturally-occurring L-amino acid other than M, C, H, Y, G, or P (M.D. Pierschbacher & E. Ruoslahti (1987), supra), GRGDNPC (SEQ ID NO: 9) (A. Hautanen et al., "Effects of Modification of the RGD Sequence and Its Context on Recognition by the Fibronectin Receptor," J. Biol. Chem. 264: 1437-1442 (1989)), GRGDAPC (SEQ ID NO: 10) (M.D. Pierschbacher & E. Ruoslahti, "Variants of the Cell Recognition Site of Fibronectin that Retain Attachment-Promoting Activity," Proc. Natl. Acad. Sci. USA 81: 5985-5988 (1984)), GRGDXPA, where X is a naturally-occurring L-amino acid other than M, C, H, Y, G, or P (by analogy to results reported in M.D. Pierschbacher & E. Ruoslahti (1987), supra, and H. Kumagai et al. (1991), supra), GRGDSG (SEQ ID NO: 11) (by analogy to results with branched peptides reported in M. Nomizu et al., "Multimeric Forms of Tyr-Ile-Gly-Ser-Arg (YIGSR) Peptide Enhance the Inhibition of Tumor Growth and Metastasis," Cancer Res. 53: 3459-3461 (1993)), GRGDXG, where X is a naturally-occurring L-amino acid other than M, C, H, Y, G, or P (by analogy to results reported in M.D. Pierschbacher & E. Ruoslahti (1987), supra, and M. Nomizu et al. (1993), supra), and GRDGXPA, where X is a naturally-occurring L-amino acid other than M, C, H, Y, G, or P (by analogy to results reported in M.D. Pierschbacher & E. Ruoslahti (1987), supra, and in H. Kumagai et al. (1991), supra), as well as analogous peptides in which the D residue in the fourth position is replaced by an E (by analogy to results of R.J. Tressler et al. (1989), supra); (6) extended substituted peptides with a D-amino acid replacing one of the naturally-occurring L-amino acids, such as G(dR)GDSP and GRGD(dS)P (M.D. Pierschbacher & E. Ruoslahti (1987), supra); (7) cyclized peptides,

including c(GRGDSPA), c(GRGDSP), c(GRGDS), c(GRGD), and c(RGDS) (H. Kumagai et al. (1991), supra); (8) cyclized peptides with a D-amino acid replacing one of the naturally-occurring L-amino acids, such as c(RGD(dF)V) and c(RGDF(dV) (M. Aumailley et al., "Arg-Gly-Asp Constrained Within Cyclic Pentapeptides," FEBS Lett. 291: 50-54 (1991)); (9) oligomers of peptides, including oligo (RGD) (1.5 kDa molecular weight) (J. Murata et al., "Molecular Properties of Poly(RGD) and Its Binding Capacities to Metastatic Melanoma Cells," Int J. Peptide Protein Res. 38: 212-217 (1991), $(GRGDS)_4$, (SEQ ID NO: 12) $(GRGES)_4$, (SEQ ID NO: 13) and $(GRGDS)(GRGES)_2(GRGDS)$ (SEQ ID NO: 14)(R.J. Tressler et al. (1989), supra); (10) polymers of peptides, including poly (RGD) (10 kDa molecular weight) (J. Murata et al. (1991), supra), poly (RGDT) (10 kDa molecular weight) (I. Saiki et al. (1990), supra), and copoly (RGD, YIGSR) (10 kDa molecular weight) (I. Saiki et al. (1989), supra); (11) branched peptides, such as $(AcGRGDSG)_{16}K_8K_4K_2KG$-OH (M. Nomizu et al. (1993), supra), and (12) cyclic peptides incorporating penicillamine, such as G(Pen)GRGDSPC (M.D. Pierschbacher & E. Ruoslahti (1987), supra). Derivatives of the RGD sequence that possess activity in blocking adhesion of metastatic cells, including derivatives in categories (1)-(12), are referred to generically herein as "RGD-derived active peptides" and are within the scope of the invention.

Laminin-derived peptides include: (1) the YIGSR (SEQ ID NO: 2) pentapeptide derived from the laminin sequence (J. Murata et al. (1989), supra); (2) derivatives of the laminin pentapeptide sequence with conservative amino acid substitutions such as YCGSR (SEQ ID NO: 15) (K. Kawasaki et al., "Amino Acids and Peptides. XXI. Laminin-Related Peptide Analogs Including Poly(Ethylene Glycol) Hybrids and Their Inhibitory Effect on Experimental Metastasis ," Chem. Pharm. Bull. 42:917-921 (1994); (3) truncated peptides derived from this sequence, such as YIGS (SEQ ID NO: 16) (K. Kawasaki et al. (1994), supra); (4) extended sequences such as CDPGYIGSR (SEQ ID NO: 17) (K. Kawasaki et al. (1994), supra) (5) peptides, including substituted peptides and extended sequences with amino acid substitutions, in which a D-amino acid replaces one of the naturally occurring L-amino acids, such as CDPGYI(dA)SR and YIG(dA)SR

(G.J. Ostheimer et al., "NMR Constrained Solution Structures for Laminin Peptide 11," J. Biol. Chem. 267: 25120-25125 (1992)); (6) branched peptides such as (Ac-YIGSRG)$_{16}$K$_8$K$_4$K$_2$KG-OH, (YIGSRG)$_{16}$K$_8$K$_4$K$_2$KG-OH, (Ac-YIGSRG)$_8$K$_4$K$_2$KG-OH, and (Ac-YIGSRG)$_4$K$_2$KG-OH (M. Nomizu et al. (1992), supra); and (7) polymers of peptides including poly (YIGSR) (10 kDa molecular weight) (I. Saiki et al. (1989), supra). Derivatives of the YIGSR sequence that possess activity in blocking adhesion of metastatic cells, including derivatives in categories (1)-(7), are referred to generically herein as "YIGSR-derived active peptides" and are within the scope of the invention.

II. METHODS FOR DELIVERING THERAPEUTIC DRUGS TO METASTATIC CELLS

An additional aspect of the present invention is a method for delivery of a therapeutic drug to a metastatic cell. Typically, such a method comprises the steps of:

(1) contacting a hydrolyzable prodrug comprising an amino-terminal capped peptide covalently linked to a therapeutic drug through a self-immolating spacer of sufficient length to prevent the occurrence of steric hindrance with a metastatic cell, the amino-terminal capped peptide being a substrate for a peptidohydrolase located on the surface of the metastatic cell;

(2) allowing the peptidohydrolase located on the surface of the metastatic cell to hydrolyze the hydrolyzable prodrug and release the therapeutic drug from the prodrug; and

(3) allowing the therapeutic drug to enter the metastatic cell.

Typically, as above, the therapeutic drug is an anticancer drug.

Typically, the hydrolyzable prodrug is delivered to the metastatic cells under conditions under which the prodrug is stable in the absence of enzymatic hydrolysis. Typically, such prodrugs are stable in plasma at pH 7.4 at 37º C. for at least 6 days in the absence of a

peptidohydrolase such as cathepsin B. In some cases, the prodrugs are stable for 16 days or more or 20 days or more in the absence of cathepsin B. Thus, the prodrug can be delivered to the metastatic cells either *in vivo* or *in vitro*. Typically, the hydrolyzable prodrugs of the present invention are administered in a quantity sufficient to kill at least a fraction of the metastatic cells.

The hydrolyzable prodrugs of the present invention can be administered *in vivo* using conventional modes of administration including, but not limited to, intravenous, intraperitoneal, oral or intralymphatic. Other routes of injection can alternatively be used. Oral or intraperitoneal administration is generally preferred. The composition can be administered in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred dosage form depends on the mode of administration and the quantity administered.

Pharmaceutical compositions for administration according to the present can include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate. The most effective modes of administration and dosage regimen for the hydrolyzable prodrugs as used in the methods of the present invention depend on the severity and course of the disease, the patient's health, the response to treatment, the particular type of metastatic cells characteristic of the particular primary tumor, the location of the metastases, pharmacokinetic considerations such as the condition of the patient's liver and/or kidneys that can affect the metabolism and/or excretion of the administered hydrolyzable prodrugs, and the judgment of the treating physician. Accordingly, the dosages should be titrated to the individual patient.

The invention is further exemplified by the following Examples. These examples are for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1

Synthesis of Ac-Phe-Lys-PABC-CPT

One example of a hydrolyzable prodrug according to the present invention is acetylphenylalanyllysyl-p-aminobenzyl carbonylcamptothecin (Ac-Phe-Lys-PABC-CPT).

Synthesis of Fmoc-Phe-Lys-Boc-PABC-CPT. Camptothecin (534 mg, 1.53 mmol) was suspended in 14 ml of methylene chloride. To this was added 3.09 ml. of 1.93 M Cl_2CO (in toluene) and pyridine (0.14 ml, 1.68 mmol). The resulting slurry was stirred overnight after which the solvent and reagents were removed under vacuum. The resulting solid was resuspended in methylene chloride and the solvent removed under vacuum to ensure complete removal of excess Cl_2CO. This step was repeated two more times. The material was suspended a final time in 3.0 ml of methylene chloride and then added by pipet to a 5.0 ml suspension of Fmoc-Phe-Lys(Boc)-PABOH (1.61 g, 2.27 mmol). (In this intermediate, Fmoc refers to the 9-fluorenylmethyloxycarbonyl protecting group and Boc refers to the t-butyloxycarbonyl group.) The reaction was stirred for 4 hours then transferred to a separatory funnel and diluted with additional methylene chloride. The organic layer was washed with saturated aqueous sodium bicarbonate, and saturated sodium chloride, then dried over sodium sulfate. The solution was filtered and the solvent removed under vacuum. The product was isolated by column chromatography (2 x 12 in silica, 98:2 methylene chloride/ethanol) and isolated as a solid.

Synthesis of Ac-Phe-Lys (Boc)-PABC-CPT. The compound isolated from the first stage of the reaction, Fmoc-Phe-Lys(Boc)-PABC-CPT, has, in place of the acetyl group, a 9-fluorenylmethyloxycarbonyl ("Fmoc") group and has the ε-amino group of the lysine protected with a butyloxycarbonyl ("Boc") blocking group. The first step is the conversion of the 9-fluorenylmethyloxycarbonyl amino-terminal blocking group to the acetyl blocking group. To perform this conversion, Fmoc-Phe-Lys-(Boc)-PABC-CPT (200 mg, 0.18 mmol) was suspended in 6.0 ml of methylene

chloride and 1.0 ml of diethylamine added. The suspension gradually dissolved as the solution and was stirred for 3 h. The solvent and diethylamine were removed under vacuum. The resulting foamy solid was redissolved in methylene chloride and then acetic anhydride (0.068 ml, 0.72 mmol) and diisopropylethylamine (0.13 mol) added. The reaction mixture was stirred overnight, transferred to a separatory funnel, and washed with pH 7 buffer. The organic layer was dried over sodium sulfate, filtered, and the solvent removed under vacuum. The product was purified by column chromatography (2 x 3 in, silica, with a gradient of 7:3-95:5 methylene chloride/ethanol) to yield 110 mg (67 % yield) of the material as a yellow solid.

Synthesis of Ac-Phe-Lys-PABC-CPT. The final step in the synthesis was the removal of the protecting butyloxycarbonyl group on the ε-amino group of the lysine in the hydrolyzable substrate. Ac-Phe-Lys-(Boc)-PABC-CPT (20 mg, 0.02 mmol) was suspended in 0.5 ml of dichloromethane to which was added 0.5 ml of dichloroacetic acid. The resulting bright yellow solution was stirred for 3 h after which it was added to 50 ml of diethyl ether to precipitate the product. The solid was filtered to give 15 mg (83% yield) of a light yellow solid. The mass spectroscopy-electrospray ionization (MS-ESI) molecular weight calculated for MH^+ ($C_{45}H_{46}N_6O_9$) was 815.3. The measured value was 815.5.

Example 2

Synthesis of CA-SP-Val-Cit-BHMS-Dox_2

Another example of a hydrolyzable prodrug according to the present invention uses the bifunctional self-immolating spacer BHMS (p-NH-Ph-CH=(CH_2OCO-)$_2$). This bifunctional spacer can bind two doxorubicin molecules. This compound also has a capping group of 2-aminoethylthio (CA) linked to the valine residue through a succinimidopropionyl (SP) group.

Synthesis of MP-Val-Cit-BHMS(OTES)$_2$. The first step in the synthesis of this compound is the synthesis of MP-Val-Cit-BHMS-(OTES)$_2$,

where MP is maleimidopropionyl and TES is triethylsilyl. The starting material, Val-Cit-BHMS-$(OTES)_2$ (500 mg, 0.76 mmol) was dissolved in 2.0 ml of dimethylformamide followed by the addition of diisopropylethylamine (0.20 ml, 1.14 mmol) and N-succinimidyl-3-maleimidopropionate (303 mg, 1.14 mmol). After stirring for 2 h, the reaction mixture was transferred to a separatory funnel, diluted with methylene chloride (200 ml) and washed with water (2 x 200 ml). The organic layer was dried over sodium sulfate with the addition of methanol to solubilize the product, filtered, and the solvent removed under vacuum. The product was purified by column chromatography with a 1.5 x 10 cm column of silica, 95:5 methylene chloride/methanol to yield 250 mg (41% yield) product as a solid. The ESI-MS value calculated for M-H($C_{40}H_{66}N_6O_8SI_2$) was 813.4. The value found was 813.4.

Synthesis of MP-Val-Cit-BHMS-$(OPNP)_2$. The next step in the synthesis was the conversion of the triethylsilyl (TES) groups to p-nitrophenyl (PNP) groups. For this step, MP-Val-Cit-BHMS-$(OTES)_2$ (500 mg, 0.61 mmol) and di(p-nitrophenyl) carbonate were placed in a 10 ml flask and dissolved in 2.0 ml of dimethylformamide. Cesium fluoride (220 mg, 1.4 mmol) was added and the reaction mixture stirred for 1 h. The solution was then transferred to a separatory funnel, diluted with 100 ml of methylene chloride and washed with water (3 x 200 ml). The organic layer was dried over sodium sulfate, filtered, and the solvent removed yielding 420 mg (75% yield) of product as a solid.

Synthesis of MP-Val-Cit-BHMS-$(Dox)_2$. The next step is the coupling of the doxorubicin molecules. For this step, MP-Val-Cit-BHMS-$(OPNP)_2$ (420 mg, 0.46 mmol), was dissolved in 4.0 ml of dimethylformamide to which was added diisopropylethylamine (0.40 ml, 2.3 mmol) and doxorubicin hydrochloride (665 mg, 1.15 mmol). The reaction was stirred 1 h followed by precipitation from methanol. The resulting solid was filtered and purified by column chromatography on a 4.8 x 10 cm column of silica with a gradient of 90:10 to 85:15 trichloromethane/methanol to isolate 315 mg (32% yield) of product as a red

solid. The ESI-MS calculated for M-H ($C_{84}H_{92}N_8O_{32}$) was 1723.6 the value found was 1723.6.

Synthesis of CA-SP-Val-Cat-BHMS-$(Dox)_2$. The final step in the synthesis was the reaction of cysteamine with the maleimidopropionyl moiety to yield a 2-aminoethylthio-succinimidopropionyl capping group at the amino-terminus of the peptide. MP-Val-BHMS-$(Dox)_2$ (74 mg, 0.043 mmol) was suspended in 1.0 ml methanol. Dimethylformamide (15 drops) was added followed by cysteamine hydrochloride (8 mg, 0.07 mmol). After stirring for 0.5 h, the product was precipitated by the addition of approximately 10 ml of diethyl ether to yield 80 mg of red solid. The material was redissolved in dimethylformamide and precipitated from dichloromethane to yield 56 mg (73% yield) of product as a red solid. The ESI-MS calculated for MH^+ ($C_{86}H_{100}N_9O_{32}S$) was 1802.8. The value found was 1802.7.

Example 3

Synthesis of Ac-Phe-Lys-PABC-Dox

Synthesis of Fmoc-Lys (MMT). The first step in the synthesis of the hydrolyzable prodrug Ac-Phe-Lys-PABC-Dox is the synthesis of Fmoc-Lys (MMT). This lysine derivative has its α-amino group protected with the protecting group 9-fluorenylmethoxycarbonyl and its ε-amino group protected with the blocking group monomethoxytrityl. A stirred suspension of Fmoc-Lys hydrochloride (23.78 g, 56.42 mmol) and dry methylene chloride (250 ml) under argon at room temperature was treated with trimethylsilyl chloride (15 ml, 2.1 equiv.) and diisopropylethylamine (10.3 ml, 1.05 equiv.). The mixture was heated at reflux for 1 h, during which time it became homogenous, and then cooled to 0ºC. Diisopropylethylamine (31 ml, 3.1 equiv.) was added, followed by p-anisyldiphenylmethyl chloride (18.29 g, 1.05 equiv.). The reaction was stirred at room temperature for 14 h. The solvent was evaporated and the residue partitioned between ethyl acetate and pH 5 buffer (0.05 M biphthalate). The organic phase was washed with more pH 5 buffer, water, and brine, dried over sodium sulfate, and

evaporated to give a pale-yellow foam (34.71 g, 96% yield). Proton nuclear magnetic resonance (NMR) was performed in $CDCl_3$ to yield: δ 1.26 and 1.68 (m, 2H and 4H), 2.45 (m, 2H), 3.71 (s, 3H), 4.05-4.40 (m, 4H), 6.81 (d, 2H), and 7.15-7.77 (m, 20H). MS-FAB yielded peaks at 641 $(MH)^+$, 663 $(M+Na)^+$, and 679 $(M+K)^+$. The HRMS calculated was 641.3015. The value found was 641.3001. The structure is shown in Figure 1.

Synthesis of Lys(MMT). The next step is the removal of the 9-fluorenylmethoxycarbonyl group on the ε-amino residue of the lysine so that this group is available for condensation. Fmoc-Lys (MMT) (5.25 g, 8.19 mmol) in 1:1 methylene chloride/acetonitrile (80 ml) at room temperature was treated with diethylamine. After 1.5 h, the solvents were evaporated. The residue was flushed with acetonitrile (2x 50 ml) at 60ºC, and then triturated with diethyl ether (80 ml). The resulting solid was collected by filtration, washed with diethyl ether, and then dissolved as far as possible in 1.1 methylene chloride/methanol. Some solid by-product was removed by filtration and the filtrate was concentrated in vacuo. The resulting light-tan solid was dried in vacuo for 4 h (3.221 g, 94% yield). Proton NMR in DMSO-d_6 gave: δ 1.34, 1.57, and 1.72 (m, 6H), 2.05 (m, 2H), 3.38 (m, 1H), 3.68 (s, 3H), 3.71 (d, 2H), 7.03, 7.40) (m, 12H). MS-FAB yielded peaks at 419.2 $(MH)^+$, 441.4 $(M+Na)^+$, and 457.4 $(M+K)^+$. The analytical data calculated for $C_{26}H_{30}N_2O_3 \bullet 0.5H_2O$ was C-73.04, H-7.31, N-6.55. The values found were N-73.62, H-7.59, N-6.56. The resulting structure is shown in Figure 1.

Synthesis of Ac-Phe-Lys (MMT). The next step in the synthesis is the condensation of a phenylalanyl residue with the α-amino-terminus of the lysine. The phenylalanyl residue has its own α-amino-terminus protected with an acetyl group. A stirred solution of Lys (MMT) (4.0940 g, 9.781 mmol) and lithium hydroxide monohydrate (410.4 mg, 1 equiv.) in water (25 ml) and dimethoxyethane (70 ml) at room temperature was treated with a solution of Ac-Phe-OSu (2.9763 g, 1 equiv.) in dimethoxyethane (70 ml), where Ac refers to the acetyl group blocking the α-amino group and Su refers to the succinimidyl group. The stirred mixture gradually became homogeneous within a few hours. After 16 h, as much dimethoxyethane was

removed on the rotary evaporator as possible. The residue was partitioned between ethyl acetate and pH 4 buffer. The organic phase was washed with more pH 4 buffer, water, and brine, dried over sodium sulfate, and evaporated to give a pale-yellow solid (5.347 g, 90% yield). Proton NMR in $CDCl_3/CD_3OD$ gave: δ 1.22 (m, 2H), 1.58 (m, 3H), 1.71 (m, 1H), 1.82 (s, 3H), 2.49 (m, 2H), 3.00 (m, 2H), 3.75 (s, 3H), 4.26 (t, 1H), 4.63 (t, 1H), 6.82 (d, 2H), 7.10-7.43 (m, 17H). MS-ESI yielded a peak at 608.5 $(MH)^+$. The analytical values calculated for $C_{37}H_{41}N_3O_5 \bullet 2.5H_2O$ was: C-68.08, H-7.10, N-6.44. The values found were: C-68.39, H-7.10, N-6.23. This structure is shown in Figure 1.

Synthesis of Ac-Phe-Lys (MMT)-PABOH. The next step in the synthesis is the addition of the p-aminobenzyl moiety to introduce the self-immolating linker. A stirred mixture of Ac-Phe-Lys (MMT) (5.3096 g, 8.736 mmol) and di-t-butylpyrocarbonate (2.8601 g, 1.5 equiv.) in methylene chloride (120 ml) at room temperature was treated with pyridine (0.741 ml, 1.05 equiv.). After 15 min, p-aminobenzyl alcohol (1.6140 g, 1.5 equiv.) was added. Stirring was continued for 16 h, and then the solvent was evaporated. The residue was dried in vacuo for 1 h, and then triturated with diethyl ether. The resulting solid was collected by filtration, washed repeatedly with diethyl ether, and air-dried (5.2416 g, 84% yield). Proton NMR ($CDCl_3/CD_3OD$) yielded: δ 1.31 (m, 1H), 1.50 (m, 1H), 1.89 (m and s, 7H), 2.18 (m, 2H), 3.00 (m, 2H), 3.74 (s, 3H), 4.40 (t, 1H), 4.61 (s, 2H), 4.68 (m, 1H), 6.67 (d, 1H), 6.77 (d, 2H), 7.00-7.55 (m, 21H), 8.92 (br, 1H). MS-ESI yielded peaks at 713.6 $(MH)^+$, 735.7 $(M+Na)^+$. The analytical values calculated for $C_{44}H_{48}N_4O_5 \bullet 0.5H_2O$ were C-73.21, H-6.84, N-7.76. The values found were: C-73.48, H-7.07,N-7.77. This compound is shown in Figure 1.

Synthesis of Ac-Phe-Lys(MMT)-PABC-PNP. The next step is the activation of the hydroxyl of the benzyl alcohol moiety of PABOH by converting it into a p-nitrophenyl ester. A mixture of Ac-Phe-Lys(MMT)-PABOH (5.861 g, 7.134 mmol), bis-p-nitrophenyl carbonate (6.511 g, 3 equiv.), and freshly activated powdered sieves (10 g) under argon at room temperature was treated with dry methylene chloride (120 ml) and then

diisopropylethylamine (3.71 ml, 3 equiv.). The mixture was stirred at room temperature for 16 h and then filtered. The filtrate was evaporated and the residue dried in vacuo for several hours and then dissolved in methylene chloride (20 ml). To this was added diethyl ether (40 ml) with moderate stirring. The resulting solid was collected by filtration, washed repeatedly with 2:1 ether/methylene chloride, and air dried (4.1966 g, 67% yield). Proton NMR in DMF-d_7 yielded: δ 1.43 (m, 2H), 1.58 (m, 2H), 1.72 (m, 1H), 1.87 (m and s, 4H), 2.09 (m, 2H), 2.4 (brt, 1H), 3.05 (m, 2H), 3.78 (s, 3H), 4.52 (m, 1H), 4.72 (m, 1H), 5.36 (s, 2H), 6.90 (d, 2H), 7.29 (m, 16H), 7.41 (d, 2H), 7.50 (d, 4H), 7.68 (d, 2H), 8.13 (d, 1H), 8.19 (d, 1H), 8.41 (d, 2H), 10.11 (s, 1H). MS-ESI yielded peaks at 878.5 $(MH)^+$ and 900 $(M+Na)^+$. The analytical values calculated for $C_{51}H_{51}N_5O_9 \bullet H_sO$ were C-68.36, H-5.96, N-7.82. The values found were: C-68.35, H-5.98, N-8.26. This compound is shown in Figure 1.

Synthesis of Ac-Phe-Lys(MMT)-PABC-Dox. The next step in the synthesis is the coupling of the doxorubicin moiety, which is the anticancer drug. A stirred mixture of Ac-Phe-Lys(MMT)-PABC-PNP (1.1006 g, 1.253 mmol) and doxorubicin hydrochloride (7.634 mg, 1.05 equiv.) in dimethylformamide (60 ml) at room temperature with treated with diisopropylethylamine (0.23 ml, 1.05 equiv.). After 2 d, the mixture was poured into ethyl acetate (400 ml). This solution was washed 4x with water, and then evaporated to give an orange solid that was chromatographed on silica, eluting with (1) 20:1 and (2) 15:1 methylene chloride/methanol, to give the product as an orange solid (959.8 mg, 60% yield). Proton NMR in DMF-d_7 gave: δ 1.25 (d, 3H), 1.41 (m, 2H), 1.56 (m, 2H), 1.87 (m and s, 4H), 2.09 (m, 4H), 2.34 (m, 4H), 3.12 (m, 4H), 3.63 (b, r, s, 1H), 3.78 (s, 3H), 3.92 (m, 1H), 4.11 (s, 3H), 4.33 (m, 1H), 4.51 (m, 1H), 4.68 (m, 1H), 4.81 (s, 2H), 4.90 (m, 1H), 5.00 (brs, 2H), 5.13 (brs, 1H), 5.40 (brs, 1H), 5.61 (s, 1H), 6.78 (d, 1H), 6.89 (d, 2H), 7.28 (m, 17H), 7.50 (d, 4H), 7.71 (m, 3H), 8.05 (m, 3H), 9.98 (s, 1H). MS-ESI yielded peaks at 1280.3 $(M-H)^-$ and 1282.4 $(MH)^+$. Analytical values calculated for $C_{72}H_{75}N_5O_{17} \bullet 2H_2O$ were: C-65.59, H-6.04, N-5.31. The values found were: C-65.46, H-5.99, N-5.25. This compound is shown in Figure 2.

Synthesis of Ac-Phe-Lys-PABC-Dox•HCl. The final step is the removal of the monomethoxytrityl group protecting the ε-amino group of the lysine residue to yield the final hydrolyzable prodrug according to the present invention. A stirred suspension of Ac-Phe-Lys(MMT)-PABC-Dox (1.8932 g, 1.476 mmol), and anisole (16 ml, 100 equiv.) in methylene chloride (50 ml) at room temperature was treated with dichloroacetic acid (1.22 ml, 10 equiv.). After 1.5 h, the mixture was poured in ethyl acetate (400 ml) and the resulting suspension was stirred for 1 h. The orange solid was collected by filtration, washed repeatedly with ethyl acetate, and then dissolved in methanol (80 ml). The solution was slowly eluted through a column of AG2-X8 ion exchange resin (50 g, chloride form). The orange fractions were collected and the solvent evaporated. The residue was triturated with methylene chloride and the resulting solid collected by filtration, washed with methylene chloride and dried in vacuo (1.5138 g, 98% yield). Proton NMR in DMF-d_7 gave: δ 1.25 (d, 3H), 1.41 (m, 2H), 1.56 (m, 2H), 1.87 (m and s, 4H), 2.09 (m, 4H), 2.34 (m, 4H), 3.12 (m, 4H), 3.63 (brs, 1H), 3.78 (s, 3H), 3.92 (m, 1H), 4.11 (s, 3H), 4.33 (m, 1H), 4.51 (m, 1H), 4.68 (m, 1H), 4.81 (s, 2H), 4.90 (m, 1H), 5.00 (brs, 2H), 5.13 (brs, 1H), 5.40 (brs, 1H), 5.61 (s, 1H), 6.78 (d, 1H), 6.89 (d, 2H), 7.28 (m, 17H), 7.50 (d, 4H), 7.71 (m, 3H), 7.91 (m, 1H), 8.32 (d, 1H), 8.45 (br, 3H), 10.21 (brs, 1H). MS-ESI yielded a peak at 1010.5 $(MH)^+$. Analytical values calculated for $C_{52}H_{60}N_5O_{16}Cl•2.5H_2O$ were: C-57.22, H-6.00, N-6.41. Values found were: C-57.16, H-6.03, N-6.34. The structure of this compound is shown in Figure 2.

Example 4

Synthesis of Ac-Phe-Lys-PABC-MMC

By analogous methods, a hydrolyzable prodrug according to the present invention with an acetyl capping group and mitomycin C (MMC) as the anticancer drug was synthesized. The peptide substrate for cathepsin B is Phe-Lys.

Synthesis of Ac-Phe-Lys(MMT)-PABC-MMC. A mixture of the previously prepared activated intermediate Ac-Phe-Lys(MMT)-PABC-PNP (614.1 mg, 0.6994 mmol), mitomycin C (245.5 mg, 1.05 equiv.), hydroxybenzotriazole (945.1 mg, 10 equiv.), and freshly activated powdered sieves (4 g) under argon at room temperature were treated with dimethyl formamide (15 ml) and diisopropylethylamine (1.22 ml, 10 equiv.). After 2 d, the mixture was diluted with ethyl acetate (150 ml) and the solution washed with water (4x) and brine, dried over sodium sulfate and evaporated. The residue was chromatographed on silica, eluting with 15:1 methylene chloride/methanol to give the product as a purple solid (501.0 mg, 67% yield). Proton magnetic resonance in DMF-d_7 gave: δ 1.41 (m, 2H), 1.56 (m, 2H), 1.69 (m, 1H), 1.77 (s, 3H), 1.84 (m and s, 4H), 2.08 (m, 2H), 2.39 (brt, 1H), 3.04 (m, 2H), 3.25 (s, 3H), 3.67 (m, 3H), 3.78 (s, 3H), 4.12 (t, 1H), 4.39 (d, 1H), 4.49 (m, 1H), 4.68 (m, 1H), 4.91 (ABq, 1H), 5.04 (ABq, 2H), 6.68 (br, 2H), 6.89 (d, 2H), 7.05 (br, 1H), 7.29 (m, 15H), 7.49 (d, 4H), 7.70 (d, 2H), 8.13 (t, 2H), 9.99 (s, 1H). MS-ESI yielded peaks at 1.071.6 $(M-H)^-$, 1.073.5 $(MH)^+$, 1.095.6 $(M+Na)^+$. Analytical data calculated for $C_{60}H_{64}N_8O_{11}\bullet 2H_2O$ was: C-64.97, H-6.18, N-10.10. The values found were: C-65.01, H-6.11, N-10.12. This compound is shown in Figure 3.

Synthesis of Ac-Phe-Lys-PABC-MMC•$ClCH_2CO_2H$. The final step in the synthesis of the hydrolyzable prodrug is the removal of the monomethoxytrityl group blocking the ε-amino group of the lysine residue. A stirred suspension of Ac-Phe-Lys(MMT)-PABC-MMC (269.5 mg, 0.2511 mmol) and anisole (2.73 ml, 100 equiv.) in methylene chloride (18 ml) at room temperature was treated with 1 M chloroacetic acid in methylene chloride (2.5 ml, 10 equiv.). After 3.5 h, diethyl ether (30 ml) was added. The resulting suspension was stirred for 1 h, and then the purple solid was collected by filtration, washed with diethyl ether and dried in vacuo (218.3 mg, 97% yield). Proton nuclear magnetic resonance in DMF-d_7 gave: δ 1.51 (m, 2H), 1.73 (s and m, 5H), 1.88 (s and m, 5H), 3.07 (m, 4H), 3.24 (s, 3H), 4.09 (s, 2H), 4.12 (t, 1H), 4.39 (d, 1H), 4.51 (m, 1H), 4.68 (m, 1H), 4.92 (ABq, 1H), 5.05 (ABq, 2H), 6.69 (br, 2H), 7.04 (brs, 2H), 7.30 (m, 7H), 7.78 (d, 2H), 8.49 (d, 1H), 8.70 (d, 1H), 10.30 (s, 1H). MS-ESI yielded peaks at: 801.6

$(MH)^+$, 823.8 $(M+Na)^+$. Analytical data calculated for $C_{42}H_{51}N_8O_{12}Cl \bullet 2.5H_2O$ was: C-53.64, H-6.00, N-11.91. The values found were: C-53.58, H-5.95, N-11.51. This structure is shown in Figure 3.

Example 5

Synthesis of Z-Phe-Lys-PABC-7-Paclitaxel

Synthesis of Z-Phe-Lys(MMT). The first step in the synthesis is the coupling of the phenylalanyl moiety with the lysine moiety, whose ε-amino group is protected with a monomethoxytrityl (MMT) residue. Coupling is accomplished with the use of a succinimidyl derivative of the phenylalanine, whose carboxyl group is thereby activated. A stirred mixture of Lys(MMT) (7.4001 g, 17.68 mmol) and Z-Phe-OSu (7.0084 g, 1 equiv.) ("Su" refers to the succinimidyl group) in dimethylformamide (80 ml) at room temperature was treated with diisopropylethylamine (9.2 ml, 3 equiv.). After 3 d, the reaction was diluted with ethyl acetate (400 ml) and the solution was washed with pH 4 buffer (2x), water (2x), brine, dried over sodium sulfate, and evaporated to give a yellow foam. This was flushed several times with methylene chloride until the foam was solid enough to be broken up with a spatula (11.2597 g, 91% yield). Proton NMR in DMF-d_7 gave: δ 1.35-1.95 (m, 6H), 2.17 (m, 2H), 3.09 (m, 2H), 3.81 (s, 3H), 4.42 (m, 1H), 4.52 (m, 1H), 4.99 (ABq, 2h), 6.91 (d, 2H), 7.41 (m, 22H), 8.27 (d, 1H). MS-ESI yielded a peak of 700.5 $(MH)^+$. Analytical values calculated for $C_{43}H_{45}N_3O_6 \bullet 1.5H_20$ were: C-71.05, H-6.65, N-5.78. The values found were: C-71.21, H-6.43, N-5.57. The compound resulting from this synthetic step is shown in Figure 4.

Synthesis of Z-Phe-Lys(MMT)-PABOH. The next step is the addition of the p-aminobenzyl moiety at the carboxyl group of the lysine residue for the linkage to paclitaxel. A stirred mixture of Z-Phe-Lys(MMT) (7.8703 g, 11.24 mmol) and di-t-butylpyrocarbonate (3.681.6 g, 1.5 equiv.) in methylene chloride (400 ml) at room temperature was treated with pyridine (0.955 ml, 1.05 equiv.). After 20 min, p-aminobenzyl alcohol (2.0775 g, 1.5 equiv.) was added. The mixture was stirred overnight at room temperature and then the solvent was evaporated. The residue was dried in vacuo and

then triturated with diethyl ether. The resulting solid was collected by filtration and washed repeatedly with diethyl ether (6.2674 g, 69% yield). Proton NMR in DMF-d_7 gave: δ 1.35-1.95 (m, 6H), 2.09 (m, 2H), 2.37 (m, 1H), 3.08 (m, 2H), 3.77 (s, 3H), 4.50 (m, 2H), 4.55 (d, 2H), 4.99 (ABq, 2H), 5.12 (t, 1H), 6.88 (d, 2H), 7.30 (m, 24H), 7.69 (d, 2H), 8.22 (d, 1H), 10.04 (s, 1H). MS-ESI yielded peaks at 803.4 $(M-H)^-$, 805.7 $(MH)^+$, 827.4 $(M+Na)^+$. Analytical data calculated for $C_{50}H_{52}N_4O_6 \bullet 0.5H_2O$ were: C-73.78, H-6.56, N-6.88. The data found were: C-73.99, H-6.81, N-7.10. The resulting structure is shown in Figure 4.

Synthesis of 2'-Monomethoxytrityl-Paclitaxel. The next step is the synthesis of the protected paclitaxel derivative, 2'-monomethoxytrityl-paclitaxel. A stirred solution of paclitaxel (0.51 g, 0.597 mmol), and p-anisyldiphenylmethyl chloride (4.63 g, 25 equiv.) in methylene chloride (14 ml) under nitrogen at room temperature was treated with pyridine (1.23 ml, 25 equiv.). After 16 h at room temperature, the solvent was evaporated, and the residue dissolved in ethyl acetate. The solution was washed with cold pH 5 buffer (2x 100 ml), water, and brine, dried, and evaporated. The residue was chromatographed on silica, eluting with 3% methanol/methylene chloride to give the product as a white solid (482 mg, 72% yield). Proton NMR in $CDCl_3$ gave: δ 1.11 (s, 3H), 1.17 (s, 3H), 1.55 (s, 3H), 1.67 (s, 3H), 1.90 and 2.54 (m, 2H), 2.26 (s, 3H), 2.51 (s, 3H), 2.54 (m, 2H), 3.66 (d, 1H), 3.78 (s, 3H), 4.21 (ABq, 2H), 4.41 (m, 1H), 4.63 (d, 1H), 4.92 (d, 1H), 5.62 (d, 1H), 5.70(m, 2H), 6.22(s, 1H), 6.74(d, 2H), 7.09-7.60(m, 23H), 7.80(d, 2H), 8.09(d, 2H). MS-FAB yielded peaks at 1148$(M+Na)^+$, 1164$(M+K)^+$. Analytical data calculated for $C_{67}H_{67}NO_{15} \bullet 0.5H_2O$: was C-70.88, H-6.04, N-1.23. The values found were: C-70.58, H-6.20, N-1.25. The product is shown in Figure 5.

Synthesis of Z-Phe-Lys(MMT)-PABC-7-Paclitaxel-2'-OMMT. The next step is the linkage of the paclitaxel moiety to the protected peptide. A stirred solution of 2' monomethoxytrityl-paclitaxel (1.5251 g, 1.354 mmol) in methylene chloride (10 ml) at 0º C. under argon was treated with diisopropylethylamine (0.236 ml, 1 equiv.), pyridine, and diphosgene (0.09

ml, 0.55 equiv.). After 10 min. the ice bath was removed and the mixture was stirred at room temperature for 4 h. The crude chloroformate solution was then added to a stirred solution of Z-Phe-Lys (MMT)-PABOH (1.1560 g, 1.06 equiv.) and diisopropylethylamine (0.236 ml, 1 equiv.) in methylene chloride (20 ml). The mixture was stirred at room temperature for 2 d and then the solvent was evaporated. The residue was partitioned between ethyl acetate and pH 5 buffer. The organic phase was washed with water and brine, dried over sodium sulfate, and evaporated. The residue was chromatographed on silica. (the column was prepared in solvent containing 0.1% triethylamine), eluting with 4:1 methylene chloride/ethyl acetate, to give the product as a colorless glass (1.9307 g, 73% yield). Proton NMR in $CDCl_3/CD_3OD$) gave: δ 1.11(s, 6H), 1.23 (m, 2H), 1.47(m, 2H), 1.79(s, 3H), 1.96(m, 2H), 2.11(m, 2H), 2.18(s, 3H), 2.29(s, 3H), 2.58(m, 2H), 3.05(m, 2H), 3.77(s, 3H), 3.82(s, 3H), 4.22(ABq, 2H), 4.40(m, 2H), 4.70(d, 1H), 4.94(brd, 1H), 5.04(brs, 2H), 5.18(ABq, 2H), 5.46(m, 2H), 5.67(brt, 1H), 5.73(m, 1H), 6.31(s, 1H), 6.79(m, 4H), 7.00-7.60(m, 49H), 7.81(d, 2H), 8.08(d, 2H), 8.73(br, 1H). Analytical data calculated for $C_{118}H_{117}N_5O_{22}\bullet H_2O$ is: C-71.75, H-6.07, N-3.54. The values found were: C-71.77, H-6.14, N-3.45. The product is shown in Figure 5.

Synthesis of Z-Phe-Lys-PABC-7-Paclitaxel•Cl_2CHCO_2H. The final step is the removal of the protecting monomethoxytrityl residues on the ε-amino group of the lysine and the paclitaxel moiety. A stirred solution of Z-Phe-Lys (MMT)-PABC-7-Paclitaxel-2'-OMMT (1.192 g, 0.6086 mmol) and anisole (13.2 ml, 100 equiv.) in methylene chloride (50 ml) at room temperature was treated with dichloroacetic acid (100 ml, 20 equiv.). After 1.75 h diethyl ether (80 ml) was added and the resulting suspension was stirred for 2 h and stored at 4º C. overnight. The white solid was then collected by filtration, washed repeatedly with diethyl ether, and air-dried. (894.1 mg, 95% yield).

Proton NMR in DMF-d_7 gave: δ 1.15(s, 3H), 1.18(s, 3H), 1.47(m, 2H), 1.98(m, 2H), 2.15(s, 3H), 2.22(m, 2H), 2.33(s, 3H), 2.54(m, 1H), 2.85(m, 2H), 3.00(m, 2H), 3.86(m, 1H), 4.21(ABq, 2H), 4.47(m, 2H), 4.81(d, 1H),

4.91(d, 1H), 4.98(d, 2H), 5.13(ABq, 2H) 5.42(m, 1H), 5.65(d, 1H), 5.72(m, 1H), 5.87(s, 1H), 6.11(brt, 1H), 6.31(s, 1H), 7.05-7.70(m, 25H), 7.75(d, 2H), 8.08(d, 2H), 9.30(br, 1H). MS-ESI yielded peaks at 1410.7(M-H)$^-$, 1412.8(MH)$^+$. Analytical data calculated for $C_{80}H_{87}N_5O_{22}Cl_2 \bullet H_2O$ was: C-60.91, H-5.81, N-4.44. The values found were: C-60.77, H-5.77, N-4.31. The product is shown in Figure 5.

Example 6

Synthesis of CA-SP-Lys-Phe-Lys-BHMS-Dox$_2$

Synthesis of Bis(hydroxymethyl)p-aminostyrene(BHMS). The first step in the synthesis of the bifunctional hydrolyzable prodrug CA-SP-Lys-Phe-Lys-BHMS-Dox$_2$ is a synthesis of the bifunctional linker bis(hydroxymethyl)p-aminostyrene (BHMS). Raney nickel (5.28 ml, 50% slurry in H_2O) and hydrazine monohydrate (21 ml, 1.5 equiv.) were added to a stirred solution of 2-(p-nitrobenzylidene)-propane-1,3-diol (P. Vanelle et al., Eur. J. Med. Chem. 26:709 (1991)) (60.29 g, 0.2885 mol) in a mixture of tetrahydrofuran (950 ml) and methanol (950 ml) at 30°C under a N_2 atmosphere. Vigorous gas evolution was observed and the reaction temperature rose to 47°C. Additional quantities of hydrazine monohydrate (21 ml, 1.5 equiv.) were added after 30 min and 1.5 h while maintaining the reaction temperature between 45-50°C. The reaction was then allowed to cool to room temperature and the catalyst was removed by filtration through a celite pad. Removal of the solvent from the filtrate left the product as a yellow solid (51.43 g, 99.6% yield). The melting point was 119°C with a sample crystallized from ethyl acetate. Infrared spectroscopy (KBr) yielded peaks at 3368, 1628, 1510, and 1022 cm^{-1}. Proton nuclear magnetic resonance (NMR) (300 Hz) in DMSO-d_6 gave: δ 4.03 (s, 2H, CH_2), 4.05 (s, 2H, CH_2), 4.65 (m, 2H, OH, exchanges with D_2O), 5.09 (s, 2H, NH_2, exchanges with D_2O), 6.33 (s, 1H, vinylic H), 6.50 (d, 2H, J = 8.4Hz aromatic H), 6.99 (d, 2H, J =8.4 Hz aromatic H). ^{13}C NMR in DMSO-d_6 yielded peaks at 57.5, 63.8, 113.6, 124.5, 126.2, 129.7, 136.0, and 147.6. Mass spectroscopy gave a *m/z* of 179 (M$^+$). High resolution mass spectroscopy (HRMS) calculated for $C_{10}H_{14}NO_2$ (MH$^+$) was 180.1024. The value found was

180.1017. The analytical values calculated for $C_{10}H_{14}NO_2$ were: C-67.02, H-7.31, N-7.82. The values found were: C-66.82, H-7.31, N-7.80. This structure is shown in Figure 6.

Synthesis of Fmoc-Phe-Lys(MMT)BHMS. The next step in the synthesis is the coupling of the BHMS that is part of the linker moiety with a peptide, Phe-Lys, which has its α-amino terminus blocked with a 9-fluorenylmethoxycarbonyl (Fmoc) group and which has the ε-amino group of the lysyl residue blocked with the monomethoxytrityl (MMT) group. A stirred solution of Fmoc-Phe-Lys(MMT)-OH (62.0 g, 78.3 mmol), bis(hydroxymethyl)p-aminostyrene (14.4 g, 1.1 equiv.) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (28.9 g, 1.5 equiv.) in a mixture of methylene chloride (400 ml) and methanol (100 ml) was left stirring at room temperature overnight. The solvents were removed and the residue was triturated with diethyl ether to leave the product as a light solid (47.3 g, 50% yield). IR (KBr) gave 3284, 1692, 1644, and 1510 cm^{-1}. Proton NMR (300 Hz) in DMSO-d_6 gave: δ 1.20-1.75 (m, 6H), 1.90 (t, 2H), 2.39 (t, 1H), 2.74-2.99 (m, 2H), 3.64 (s, 3H), 4.00-4.16 (m, 7H), 4.26-4.42 (m, 2H), 4.79 (t, J = 5.0 Hz, 1H), 4.84 (t, J = 5.6 Hz, 1H), 6.48 (s, 1H), 6.77 (d, J =8.7 Hz), 7.06-7.57 (m, 27H), 7.84 (d, J = 7.6 Hz, 2H), 8.18 (d, J = 7.6 Hz, 1H), 10.07 (s, 1H). Mass spectroscopy gave a *m/z* of 948 (M^+). HRMS calculated for $C_{60}H_{60}N_4O_7$ (M Na^+) was 971.4360. The value found was 971.4375. This structure is shown in Figure 6.

Synthesis of H-Phe-Lys(MMT)-BHMS. The next step in the synthesis was the removal of the blocking Fmoc group at the amino-terminus of the peptide to yield an amino group that is available for further coupling. A stirred solution of Fmoc-Phe-Lys(MMT)-BHMS (2.66 g, 2.81 mmol) and diethylamine (7 ml) in dimethylformamide (15 ml) was stirred at room temperature for 5 min after which the solvent was removed. The residue was chromatographed on silica gel (gradient elution with methylene chloride/methanol 98:2 to 92:8) to give the product as a tan solid (0.77 g, 38% yield). Proton NMR in DMSO-d_6 plus D_2O gave:δ 1.20-1.91 (m, 6H), 2.59, 2.96 (m, 2H), 3.44 (m, 1H), 3.67 (s, 3H), 4.04 (s, 2H), 4.10 (s, 2H), 4.43

(m, 1H), 6.47 (s, 1H), 6.79-7.56 (m, 23H). Mass spectroscopy by electrospray ionization (MS-ESI) yielded a *m/z* of 725($M-H^+$). HRMS calculated for $C_{45}H_{50}N_4O_5$ (MNa^+) was 749.3679. The value found was 749.3678. This structure is shown in Figure 6.

Synthesis of H-Lys(MMT)-Phe-Lys(MMT)-BHMS. The next step is the addition of the amino-terminal lysyl residue. This is accomplished by reacting a lysine with its α-amino terminus blocked with Fmoc, its ε-amino group blocked with a monomethoxytrityl group, and its carboxyl group activated with a succinimidyl (Su) moiety. A solution of H-Phe-Lys(MMT)-BHMS (0.770 g, 1.06 mmol) and Fmoc-Lys(MMT)-OSu (0.860 g, 1.1 equiv.) in dry dimethylformamide (5 ml) was left stirring at room temperature for 2h. This was then diluted with ethyl acetate and washed with water. The organic phase was filtered through celite, washed with brine and then dried over sodium sulfate. Removal of the solvents left a solid that was dissolved in a mixture of methylene chloride (10 ml) and diethylamine (10 ml). After 3h at room temperature, the solvents were removed and the residue was chromatographed on silica gel with gradient elution with methylene chloride/methanol of 99:1 to 91:9 to give it a product as a tan solid (0.661 g, 56% yield). Proton NMR in DMSO-d_6 plus D_2O gave: δ 1.04-1.90 (m, 12H), 2.70-3.00 (m, 2H), 3.40 (m, 1H), 3.66 (s, 3H), 3.69 (s, 3H), 4.05 (s, 2H), 4.11 (s, 1H), 4.35 (m, 1H), 4.60 (m, 1H), 6.48 (s, 1H), 6.77-7.58 (m, 37H). This structure is shown in Figure 6.

Synthesis of PNP Carbonate Derivative of MP-Lys(MMT)-Phe-Lys(MMT)-BHMS. The next step is the synthesis of a p-nitrophenyl (PNP) carbonate derivative for coupling of the doxorubicin moieties to the linker. Simultaneously, the amino-terminus of the peptide is activated by reaction with N-succinimidyl 3-maleimidopropionate. A stirred mixture of H-Lys(MMT)-Phe-Lys(MMT)-BHMS (0.661 g, 0.59 mmol) and N-succinimidyl 3-maleimidopropionate (0.172 g, 1.1 equiv.) in dry dimethylformamide (5 ml) was left stirring at room temperature for 2h. This was diluted with ethyl acetate and washed with water, brine, and then dried over sodium sulfate. The solvents were removed and the residue was dissolved in dry

tetrahydrofuran (10 ml). This was cooled in an ice bath and diisopropylethylamine (0.82 ml, 8 equiv.), p-nitrophenyl chloroformate (0.713 g, 6 equiv.) and pyridine (0.024 ml, 0.5 equiv.) were added. The reaction was left stirring for 1h after which it was quenched by the addition of water and then extracted with methylene chloride. The organic extracts were combined, washed with water, saturated aqueous sodium bicarbonate solution, water, and then dried over sodium sulfate. The solvent was removed and the residue was dissolved in methylene chloride (8 ml). Diethyl ether was added to precipitate the product as a tan solid (0.472 g, 50% yield). Proton NMR in DMSO-d_6 gave: δ 1.04-1.90 (m, 12H), 2.36 (m, 4H), 2.65-3.10 (m, 2H), 3.52 (m, 2H), 3.65 (s, 3H), 3.67 (s, 3H), 4.03 (m, 1H), 4.33 (m, 1H), 4.45 (m, 1H), 5.01 (s, 2H), 5.04 (s, 2H), 6.76-8.30 (m, 52H). This structure is shown in Figure 7.

Synthesis of MP-Lys(MMT)-Phe-Lys(MMT)-BHMS-Dox_2. The next step in the synthesis is the coupling of the two doxorubicin moieties to the activated linker. A stirred mixture of the p-nitrophenyl carbonate derivative of MP-Lys(MMT)-Phe-Lys(MMT)-BHMS (0.472 g, 0.294 mmol) and doxorubicin hydrochloride (0.340 g, 2 equiv.) in dry dimethylformamide (9 ml) at room temperature was treated with diisopropylethylamine (0.153 ml, 3 equiv.). After 7 h, the solution was concentrated to about 3 ml. This was then added dropwise to a stirred solution of methanol (20 ml). The resulting precipitate was collected and washed with methanol and diethyl ether to give the crude product as a red solid (527 mg). This was dissolved in 5 ml of a 2% solution of methanol in methylene chloride and chromatographed on a silica gel column (gradient elution with methylene chloride/methyl alcohol of 99:1 to 93:7) to afford pure material as a red solid (0.308 g, 43% yield). Distinguishing peaks in the proton-NMR spectrum in DMSO-d_6 were: δ 1.09 (brs, 6H, methyl group of Dox), 3.64 (s, 3H, methyl group of monomethoxytrityl), 3.67 (s, 3H, methyl group of monomethoxytrityl), 3.86 (s, 3H, methyl group of Dox), 3.87 (s, 3H, methyl group of Dox). The resulting structure is shown in Figure 7.

Synthesis of MP-Lys-Phe-Lys-BHMS-Dox_2. The next step in the synthesis is the removal of the blocking monomethoxytrityl groups on the ε-amino moieties of the lysine residues. A stirred suspension of MP-Lys(MMT)-Phe-Lys(MMT)-BHMS$(Dox)_2$ (0.300 g, 0.124 mmol) and anisole (2.7 ml, 200 equiv.) in methylene chloride (20 ml) at room temperature was treated with dichloroacetic acid (0.205 ml, 20 equiv.). After 1.5h, the mixture was poured into ethyl acetate (20 ml). The product separated as a fine red solid. This was collected using a centrifuge, washed with ethyl acetate, and dried (0.252 g, 95% yield). Distinguishing peaks in the proton-NMR spectrum in DMSO-d_6 were: δ 1.10 (brs, 6H, methyl group of Dox), 3.88 (s, 3H, methyl group of Dox), 3.89 (s, 3H, methyl group of Dox), 6.99 (s, 2H, maleimide residue). MS-ESI gave a *m/z* of 1872 (MH^+). This resulting structure is shown in Figure 8.

Synthesis of CA-SP-Lys-Phe-Lys-BHMS-Dox_2. The final step in the synthesis is the reaction of cysteamine with the maleimidopropionyl group to yield the amino-terminal cap. MP-Lys-Phe-Lys-BHMS-Dox_2 (0.068 g, 0.032 mmol) was added to a solution of cysteamine hydrochloride (0.012 g, 3.3 equiv.) in methanol (1 ml). After 1 h, the mixture was filtered and concentrated to about 0.5 ml. Diethyl ether was added and the precipitate was collected using a centrifuge. This was washed with diethyl ether and dried to give the product as a red solid (0.063 g, 88% yield). Distinguishing peaks in the proton NMR spectrum in DMSO-d_6 were: δ 1.10 (brs, 6H, methyl group of Dox), 3.88 (s, 3H, methyl group of Dox), 3.89 (s, 3H, methyl group of Dox). MS-ESI yielded *m/z* of 1949 (MH^+) This structure is shown in Figure 8.

Example 7

Cytotoxicity Assays of Hydrolyzable Prodrugs

Materials and Methods

Cell Culture. The BT-20 cell line was maintained in E-MEM-10 (minimal essential medium (Earle's Salts) supplemented with 10% fetal

bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml)) in 5% CO_2 at 37º C. MCF 10A cell line was maintained in DMEM/Ham's F12 supplemented with 5% horse serum, EGF (20 ng/ml), insulin (0.5 µg/ml), hydrocortisone (0.5 µg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml) in 5% CO_2 at 37º C. Corning tissue culture multiwell plates (24 wells/plate) were seeded with 10^5 cells/16 mm well in 2 ml maintenance medium, refed 48 h after seeding and were ready to use in cytotoxicity assays 4 8h later, with the cells just reaching confluency.

Solubilization of Hydrolyzable Prodrugs and Anticancer Drugs in Aqueous Medium. Concentrated stocks (200 mM) of the hydrolyzable prodrugs and the anticancer agents doxorubicin, taxol, mitomycin C, and camptothecin were prepared in 100% dimethyl sulfoxide. Dilution with dimethyl sulfoxide to 33.33 mM followed by partial hydration with addition of E-MEM-0% (serum free E-MEM) resulted in 25 mM drug or prodrug in a solvent of 75% dimethyl sulfoxide/25% EMEM-0%. A dilution of 1:1000 in EMEM-0% resulted in 25 µM concentrations with DMSO vehicle concentration of 0.075%. Serial 1:10 dilutions of the 25 µM preparations were made with E-MEM-O. This stepwise hydration of drugs and prodrugs facilitated the delivery of otherwise aqueously insoluble compounds to cells in culture.

Cytotoxicity Assay and Inhibition with E-64 or CA-074. The cytotoxicity assay protocol employing MCF 10A (low cathepsin B secreters) and BT-20 (high cathepsin B secreters) and inhibition of cytotoxicity with L-trans-epoxysuccinyl-leucylamido (4-guanido) butane (cysteine protease inhibitor) or CA-074 (N-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline)(specific cathepsin B inhibitor) was standardized with the only variations occurring with compound, molar concentration, time of exposure, and presence or absence of inhibitors. Maintenance medium was aspirated from the cell walls after feeding, and the cells washed twice with 2 ml/well Hanks balanced salt solution (HBSS). After the second wash, 1.0 ml E-MEM-0 (medium without phenol red indicator) containing chemotherapeutic agents, hydrolyzable prodrug derivatives, medium alone,

or solubilization vehicle controls (0.1% dimethyl sulfoxide). For assays in which inhibitor was employed, E-MEM-0 containing E64, CA-074, or no inhibitor was added 30-60 min prior to addition of anticancer drugs or hydrolyzable prodrugs. Medium was aspirated from the wells and replaced with fresh E-MEM-0 containing anticancer agents, hydrolyzable prodrugs, inhibitors, medium alone, or vehicle controls in the molar concentration and combinations indicated. Incubation was continued in 5% CO_2 at 37°C for the indicated times after drug/prodrug addition. To each well containing 1.0 ml, 50 µl of 0.5% Trypan Blue in HBSS was added, the plate was gently swirled to mix the dye, the plate was allowed to sit for 5 minutes, and the percentage of dead cells (blue stained) was read. In this example, the following compounds were used: Compound (1) was Ac-Phe-Lys-PABC-Dox; Compound (2) was Ac-Phe-Lys-PABC-MMC; Compound (3) was Ac-Phe-Lys-PABC-CPT; Compound (4) was Z-Phe-Lys-PABC-7-Paclitaxel; Compound (5) was CA-SP-Val-Cit-BHMS-Dox_2; Compound (6) was CA-SP-Lys-Phe-Lys-BHMS-Dox_2; Compound (7) was Z-Phe-Lys-Dox; and Compound (8) was 2-Hydroxyethylthio-SP-D-Phe-Lys-PABC-Dox. In these compounds, MMC is mitomycin C, CPT is camptothecin, Z is benzyloxycarbonyl, CA is 2-aminoethylthio, SP is succinimidopropionyl, PABC is p-aminobenzyl carbonyl, and BHMS is p-NH-Ph-CH=C(CH_2OCO-)$_2$.

In the results shown in Figure 9, the prodrugs were used at 100 µM, CA-074 (a cathepsin B-specific inhibitor) was used at 40 µM, BT-20 (high cathepsin B-secreting cells) and MCF-10 (low cathepsin B-secreting cells) were used, and the percent cell kill in 24 hours is shown for Compounds (1), (2), (7) and (8). In a separate experiment at 25 µM drug, Compounds (1) and (2) were 60% and 75% inhibited by 4 µM CA-074, respectively. These results show that high cathepsin B-secreting cells were more readily killed than low cathepsin B-secreting cells. The killing of high cathepsin B-secreting cells was inhibited by CA-074. The killing of low cathepsin B-secreting cells was not inhibited by CA-074. This is attributed to killing, albeit inefficiently, of the low secretors, not as the result of the activity of externally secreted cathepsin B, but rather by non-specific endocytosis of the drug into lysosomes where all cells possess cathepsin B. CA-074 does

not inhibit this killing because it does not enter lysosomes. Compounds (7) and (8) are control compounds, weaker cytotoxic agents than Compounds (1) and (2) because they are less susceptible to cathepsin B-mediated hydrolysis to an active drug because of their particular structures. Compound (7) lacks the PABC-self-immolating linker that facilitates enzymatic cleavage, resulting in probable steric hindrance that places the bulky doxorubicin moiety in the active site of the cathepsin B. Compound (8) has the amino acids in the unnatural D instead of the natural L configuration.

Figure 10 depicts the percent cell kill at various times and prodrug concentrations with BT-20 cells. The results with CA-074 present at 40 μM are given in parentheses. The results indicate that all test compounds show dose- and time-dependent killing of BT-20 (cathepsin B+) cells. The cathepsin B inhibitor CA-074 strongly inhibits cytotoxicity. Compound (8), the compound containing the amino acids in the D-configuration, is very much less active.

The results shown in Figure 11 are the percent cell kill for various times and prodrug concentrations using Compound (6), with both BT-20 (high cathepsin B-secreting) cells and MCF-10A (low cathepsin B-secreting) cells. Results in parentheses are with added E-64 (10 μM), a broad-spectrum cysteine protease inhibitor. (Other experiments showed that E-64 and medium alone caused 10% killing of BT-20 cells after 24 hours.)

These results show that Compound (6), with two doxorubicin moieties linked to the prodrug, causes dose- and time-dependent killing of BT-20 (high cathepsin B-secreting) cells. The killing of MCF-10A (low cathepsin B-secreting) cells is much less efficient. The cysteine protease inhibitor E-64 strongly inhibits cytotoxicity.

In conclusion, tumor cells do indeed secrete enough cathepsin B to release enough cytotoxic drug to kill the cells efficiently. Tumor cells that secrete less cathepsin B are resistant to the hydrolyzable prodrugs. These cells are surrogates for normal cells that secrete no cathepsin B at all.

Cathepsin B inhibitors strongly reduce cytotoxicity of the prodrugs, showing that cathepsin B is the principal means of unmasking them. A prodrug lacking the self-immolating linkers PABC or BHMS has much reduced cytotoxicity, showing that it is an enzyme, presumably cathepsin B, that releases active drug. This is because the absence of the self-immolating linker results in steric hindrance. Additionally, a prodrug with amino acids in the unnatural D configuration has much reduced cytotoxicity, again showing the role of cathepsin B.

Example 8

Stability of Hydrolyzable Prodrugs

Figure 12 shows the stability of a number of hydrolyzable prodrugs according to the present invention linked to doxorubicin using a PABC-self-immolating linker. These hydrolyzable prodrugs release anticancer drug under cathepsin B catalysis at 37°C, pH 7.4, at reasonable rates, and are stable for days or weeks in freshly drawn human plasma under the same conditions.

ADVANTAGES OF THE PRESENT INVENTION

The present invention provides an efficient way to treat cancer cells secreting peptidohydrolases on their surface, particularly metastatic cells. The hydrolyzable prodrugs of the present invention are usable against many types of metastases and do not depend for their activity on characteristics of the primary tumor cells that might not be shared by the metastases. Hydrolyzable prodrugs of the present invention are readily absorbed and lack toxicity.

Although the present invention has been described with considerable detail, with reference to certain preferred versions thereof, other versions are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of the preferred versions contained herein.

What Is Claimed Is:

1. A hydrolyzable prodrug comprising an amino-terminal capped peptide covalently linked to a therapeutic drug through a self-immolating spacer of sufficient length to prevent the occurrence of steric hindrance, the amino-terminal capped peptide being a substrate for a peptidohydrolase located on the surface of a metastatic cell.

2. The hydrolyzable prodrug of claim 1 wherein the peptidohydrolase is selected from the group consisting of cathepsin B and collagenase IV.

3. The hydrolyzable prodrug of claim 2 wherein the peptidohydrolase is cathepsin B.

4. The hydrolyzable prodrug of claim 3 wherein the amino-terminal capped peptide is benzyloxycarbonylphenylalanyllysine, benzyloxycarbonylvalinyllysine, D-phenylalanylphenylalanyllysine, benzyloxycarbonylvalinylcitrulline, t-butyloxycarbonylphenylalanylysine, benzyloxycarbonylalanyllarginylarginine, benzyloxycarbonylphenylalanyl-N-tosylarginine,2-aminoethylthio-succinimidopropionylvalinylcitrulline, 2-aminoethylthio-succinimidopropionyllysylphenylalanyllysine, acetylphenylalanyllysine, or benzyloxycarbonylphenylalanyl-O-benzoylthreonine.

5. The hydrolyzable prodrug of claim 4 wherein the amino-terminal capped peptide is benzyloxycarbonylphenylalanyllysine.

6. The hydrolyzable prodrug of claim 4 wherein the amino-terminal capped peptide is acetylphenylalanyllysine.

7. The hydrolyzable prodrug of claim 4 wherein the amino-terminal capped peptide is 2-aminoethylthio-succinimidopropionylvalinylcitrulline.

8. The hydrolyzable prodrug of claim 4 wherein the amino-terminal capped peptide is 2-aminoethylthio-succinimidopropionyllysylphenylalanyllysine.

9. The hydrolyzable prodrug of claim 1 wherein the therapeutic drug is an anticancer drug.

10. The hydrolyzable prodrug of claim 9 wherein the anticancer drug is doxorubicin, mitomycin C, taxol, esperamycin, or camptothecin.

11. The hydrolyzable prodrug of claim 10 wherein the anticancer drug is doxorubicin.

12. The hydrolyzable prodrug of claim 1 wherein the self-immolating spacer is selected from the group consisting of p-aminobenzyl carbonyl and p-NH-Ph-CH=(CH_2OCO-)$_2$.

13. The hydrolyzable prodrug of claim 11 wherein the spacer is p-aminobenzyl carbonyl.

14. The hydrolyzable prodrug of claim 1 further comprising a peptide derived from a protein to which metastatic cells adhere in establishing colonies covalently linked to the therapeutic drug.

15. The hydrolyzable prodrug of claim 14 wherein the peptide is a RGD-derived active peptide or a YIGSR-derived active peptide.

16. The hydrolyzable prodrug of claim 14 wherein the peptide is YIGSR (SEQ ID NO:1) or GRGDS (SEQ ID NO:2).

17. Benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin.

18. Acetylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin.

19. Acetylphenylalanyllysyl-p-aminobenzyl carbamoylmitomycin C.

20. Benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbonyl-7-paclitaxel.

21. Acetylphenylalanyllysyl-p-aminobenzyl carbonylcamptothecin.

22. 2-aminoethylthio-succinimidopropionyl-valinylcitrullinyl-bis(hydroxymethyl)styryl-bis-doxorubicin.

23. 2-aminoethylthio-succinimidopropionyl-lysylphenylalanyllysyl-bis(hydroxymethyl)styryl-bis-doxorubicin.

24. Benzyloxycarbonylvalinyllysyl-p-aminobenzyl carbamoyldoxorubicin.

25. D-phenylalanylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin.

26. Benzyloxycarbonylvalinylcitrullinyl-p-aminobenzyl carbamoyldoxorubicin.

27. A method for delivering a therapeutic drug to a metastatic cell comprising the steps of:

(a) contacting a hydrolyzable prodrug comprising an amino-terminal capped peptide covalently linked to a therapeutic drug through a self-immolating spacer of sufficient length to prevent the occurrence of steric hindrance with a metastatic cell, the amino-terminal capped peptide being a substrate for a peptidohydrolase located on the surface of the metastatic cell;

(b) allowing the peptidohydrolase located on the surface of the metastatic cell to hydrolyze the hydrolyzable prodrug and release the therapeutic drug from the prodrug; and

(c) allowing the therapeutic drug to enter the metastatic cell.

28. The method of claim 27 wherein the peptidohydrolase is selected from the group consisting of cathepsin B and collagenase IV.

29. The method of claim 28 wherein the peptidohydrolase is cathepsin B.

30. The method of claim 29 wherein the amino-terminal capped peptide is benzyloxycarbonylphenylalanyllysine, benzyloxycarbonylvalinyllysine, D-phenylalanylphenylalanyllysine, benzyloxycarbonylvalinylcitrulline, t-butyloxycarbonylphenylalanylysine, benzyloxycarbonylalanyllarginylarginine, benzyloxycarbonylphenylalanyl-N-tosylarginine,2-aminoethylthio-succinimidopropionylvalinylcitrulline, 2-aminoethylthio-succinimidopropionyllysylphenylalanyllysine, acetylphenylalanyllysine, or benzyloxycarbonylphenylalanyl-O-benzoylthreonine.

31. The method of claim 30 wherein the amino-terminal capped peptide is benzyloxycarbonylphenylalanyllysine.

32. The method of claim 30 wherein the amino-terminal capped peptide is acetylphenylalanyllysine.

33. The method of claim 30 wherein the amino-terminal capped peptide is 2-aminoethylthio-succinimidopropionylvalinylcitrulline.

34. The method of claim 30 wherein the amino-terminal capped peptide is 2-aminoethylthio-succinimidopropionyllysylphenylalanyllysine.

35. The method of claim 27 wherein the therapeutic drug is an anticancer drug.

36. The method of claim 35 wherein the anticancer drug is doxorubicin, mitomycin C, taxol, esperamycin, or camptothecin.

37. The method of claim 36 wherein the anticancer drug is doxorubicin.

38. The method of claim 27 wherein the spacer is selected from the group consisting of p-aminobenzyl carbonyl and p-NH-Ph-CH=(CH_2OCO-)$_2$.

39. The method of claim 38 wherein the spacer is p-aminobenzyl carbonyl.

40. The method of claim 27 wherein the hydrolyzable prodrug further comprises a peptide derived from a protein to which metastatic cells adhere and in establishing colonies covalently linked to the therapeutic drug.

41. The method of claim 40 wherein the peptide is a RGD-derived active peptide or a YIGSR-derived active peptide.

42. The method of claim 41 wherein the peptide is YIGSR (SEQ ID NO:1) or GRGDS (SEQ ID NO:2).

43. The method of claim 27 wherein the hydrolyzable prodrug is benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, acetylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, acetylphenylalanyllysyl-p-aminobenzyl carbamoylmitomycin C, benzyloxycarbonylphenylalanyllysyl-p-aminobenzyl carbonyl-7-paclitaxel, acetylphenylalanyllysyl-p-aminobenzyl carbonylcamptothecin, 2-aminoethylthio-succinimidopropionyl-valinylcitrullinyl-bis(hydroxymethyl)styryl-bis-doxorubicin, 2-aminoethylthio-succinimidopropionyl-lysylphenylalanyllysyl-bis(hydroxymethyl)styryl-bis-doxorubicin, benzyloxycarbonylvalinyllysyl-p-aminobenzyl carbamoyldoxorubicin, D-phenylalanylphenylalanyllysyl-p-aminobenzyl carbamoyldoxorubicin, or benzyloxycarbonylvalinylcitrullinyl-p-aminobenzyl carbamoyldoxorubicin.

44. A pharmaceutical composition comprising:

(a) the hydrolyzable prodrug of claim 1; and

(b) a pharmaceutically acceptable carrier.

45. The pharmaceutical composition of claim 44 wherein the hydrolyzable prodrug further includes a peptide derived from a protein to which metastatic cells adhere in establishing colonies covalently linked to the therapeutic drug.

46. The pharmaceutical composition of claim 45 wherein the peptide is a RGD-derived active peptide or a YIGSR-derived active peptide.

47. The pharmaceutical composition of claim 46 wherein the peptide is

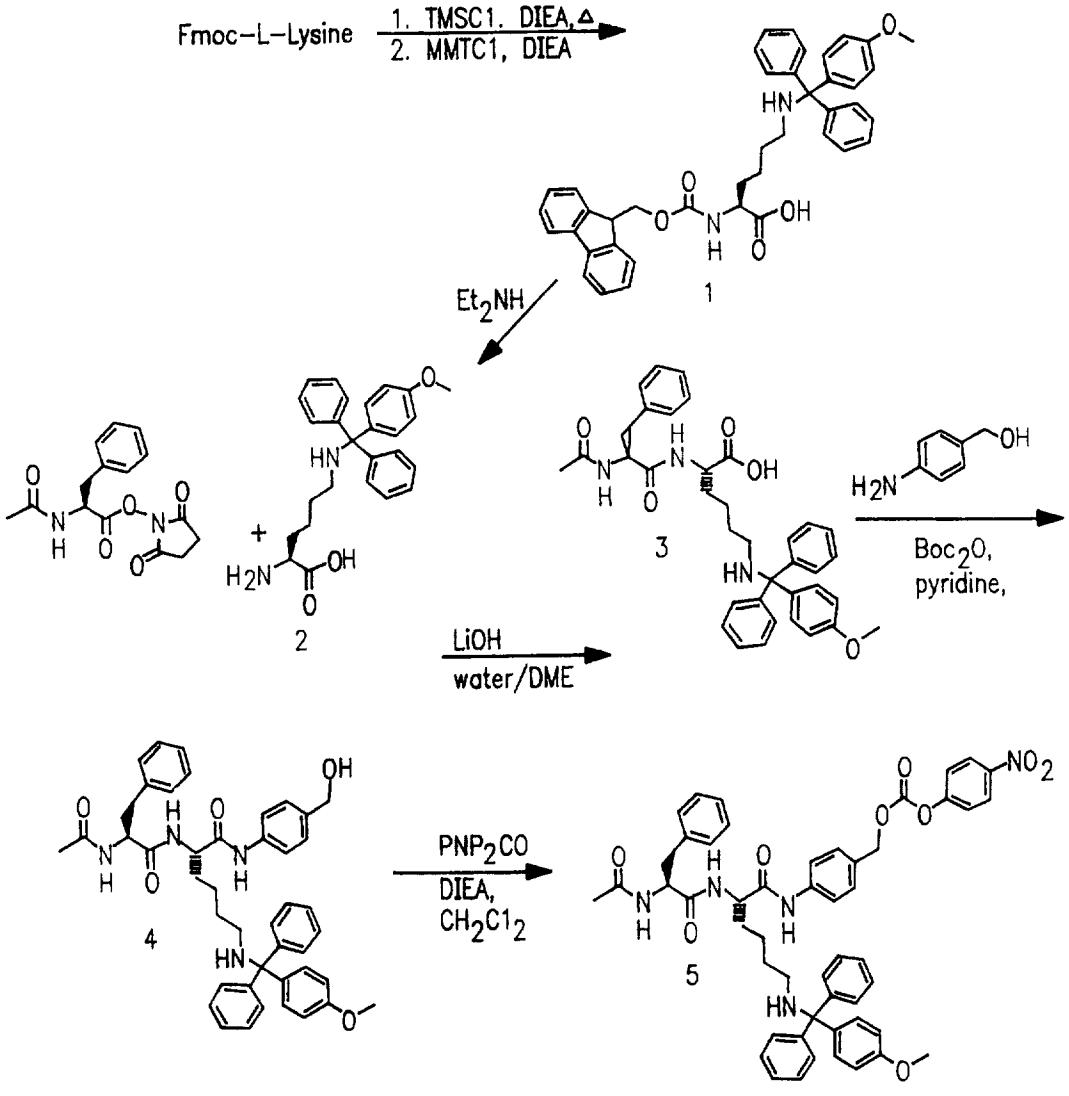
Fmoc-L-Lysine
1. TMSC1. DIEA, Δ
2. MMTC1, DIEA
Et2NH
LiOH
water/DME
Boc2O,
pyridine,
PNP2CO
DIEA,
CH2C12

FIG. 1

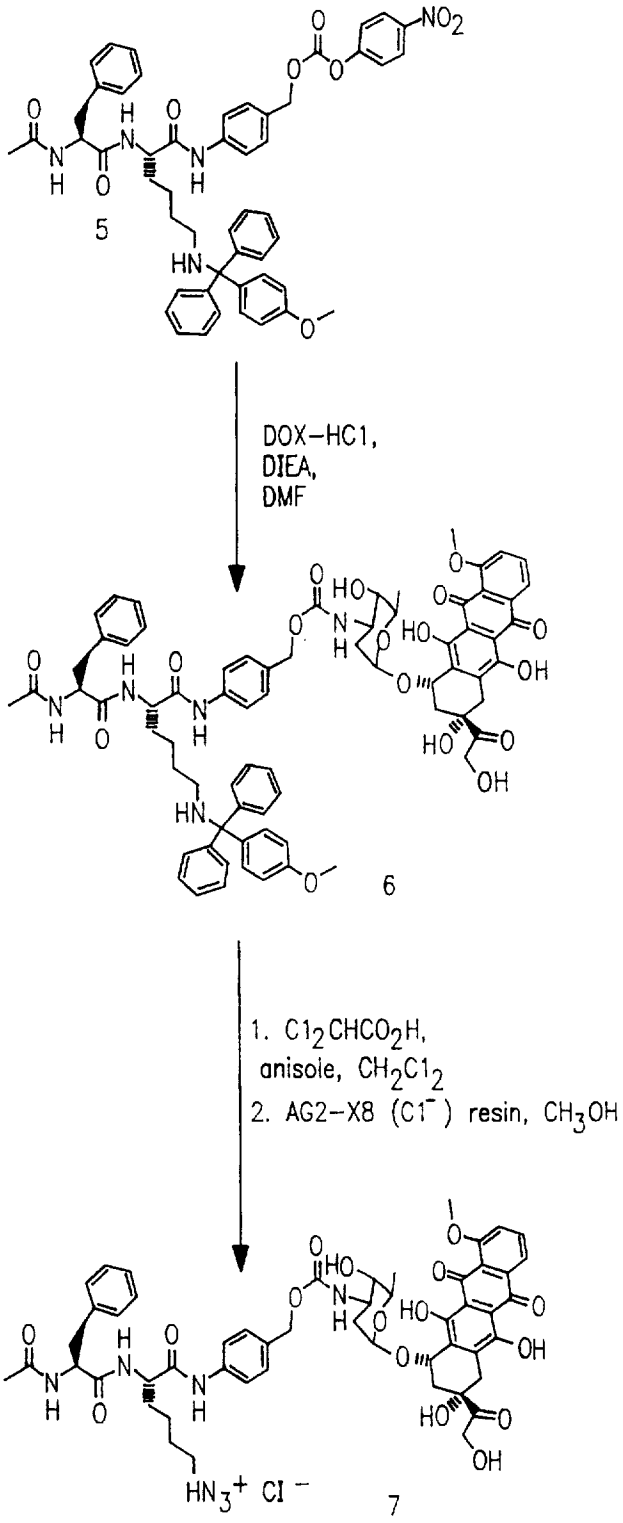
5
DOX–HC1,
DIEA,
DMF
6
1. $C1_2CHCO_2H$,
anisole, CH_2C1_2
2. AG2–X8 ($C1^-$) resin, CH_3OH
HN_3^+ CI^-
7

FIG. 2

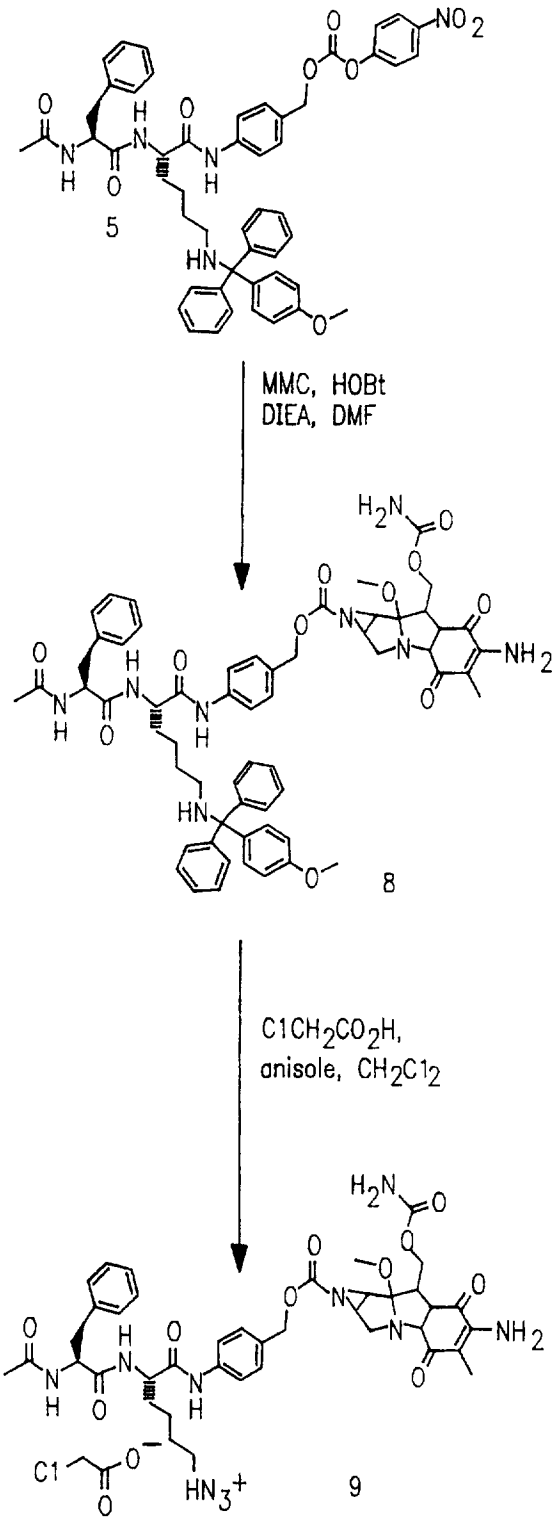

FIG. 3

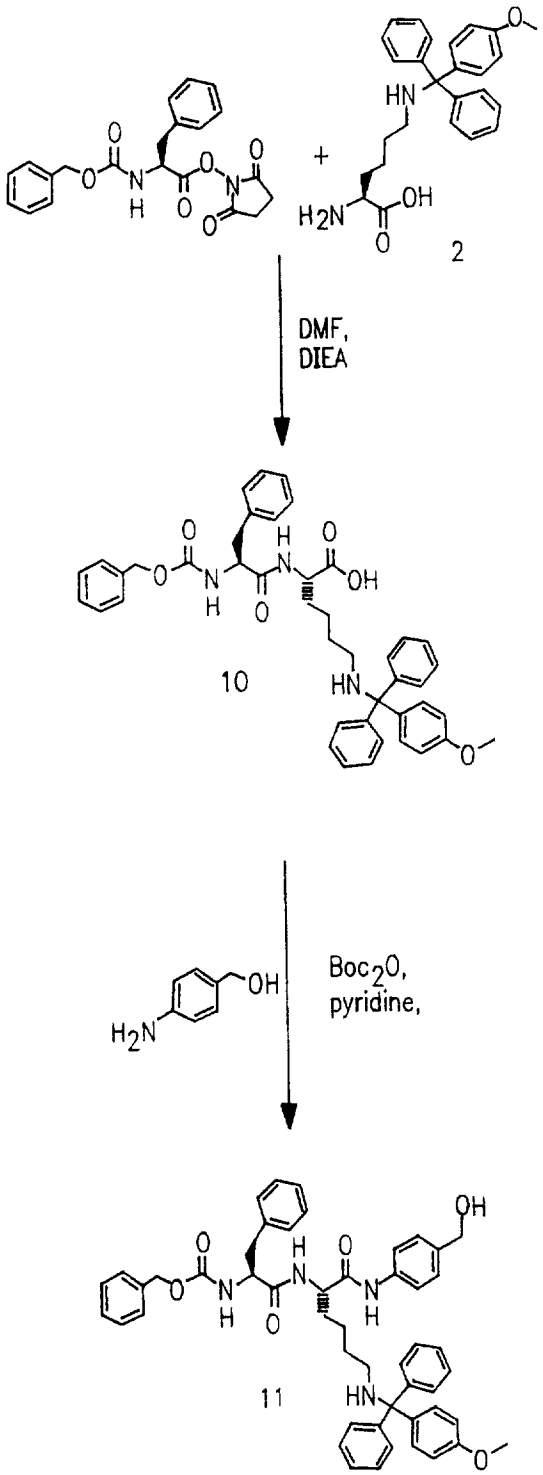

FIG. 4

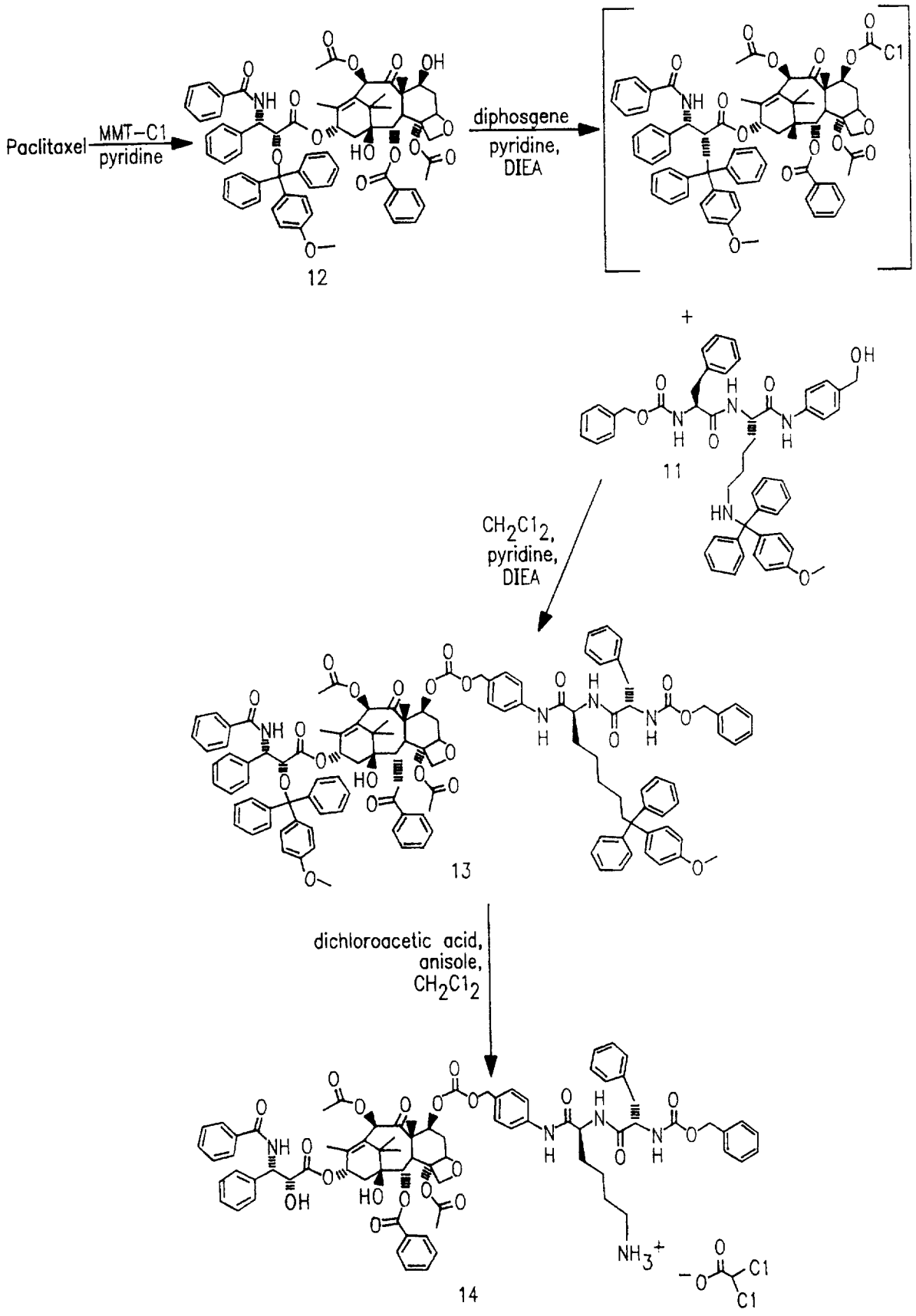
Paclitaxel
MMT-C1
pyridine
12
diphosgene
pyridine,
DIEA
+
11
CH_2C1_2,
pyridine,
DIEA
13
dichloroacetic acid,
anisole,
CH_2C1_2
14

FIG. 5

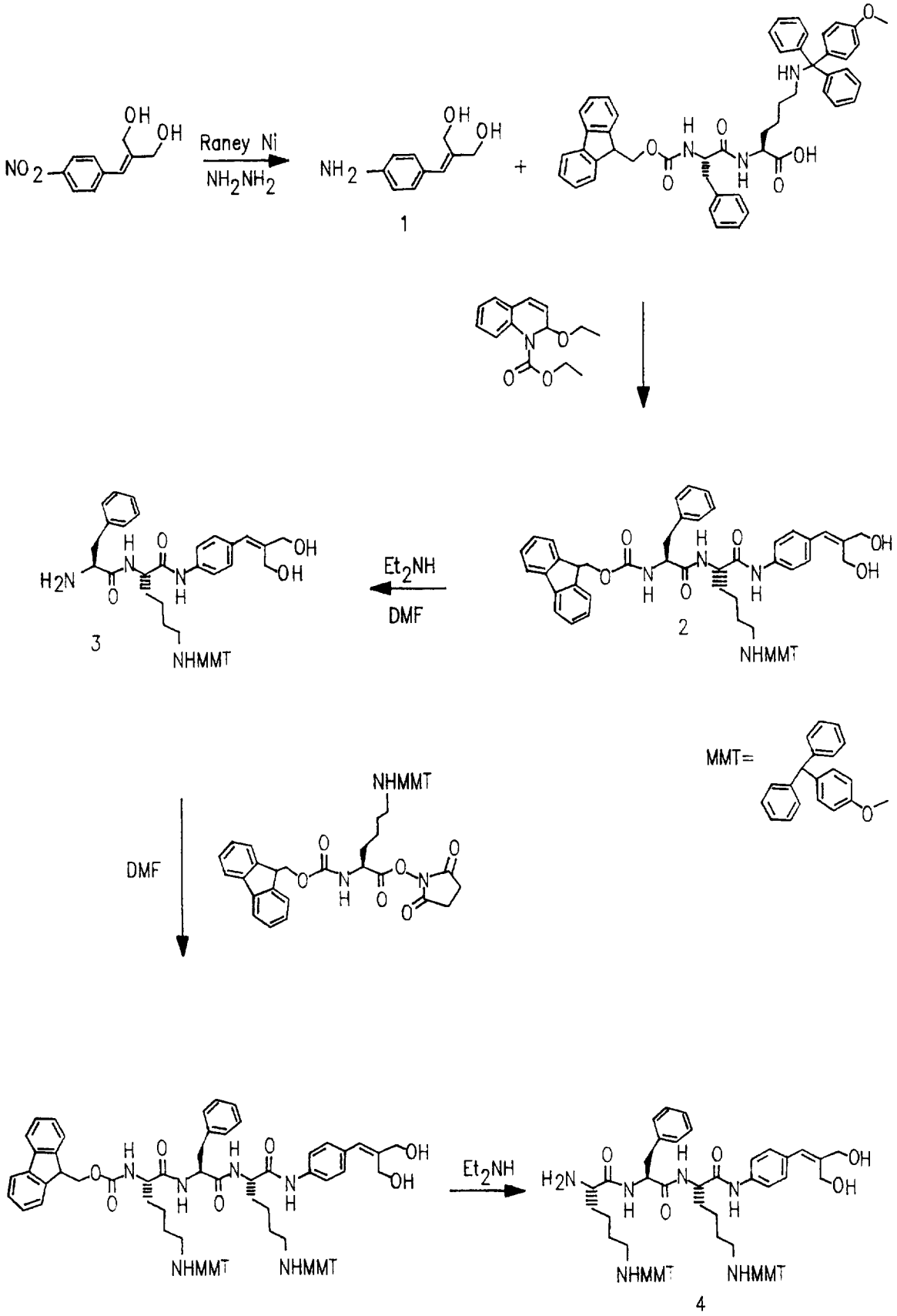

FIG. 6

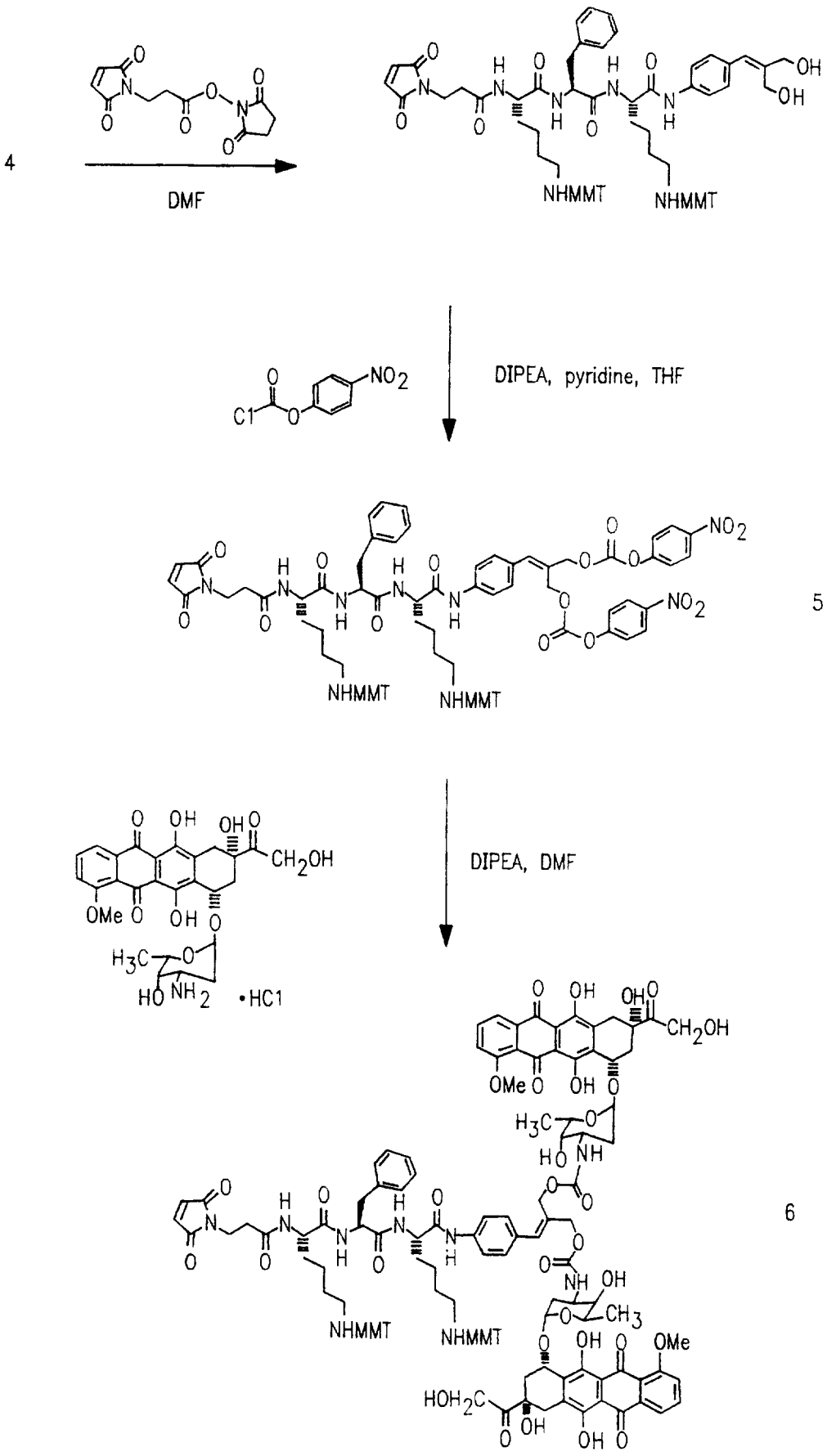

FIG. 7

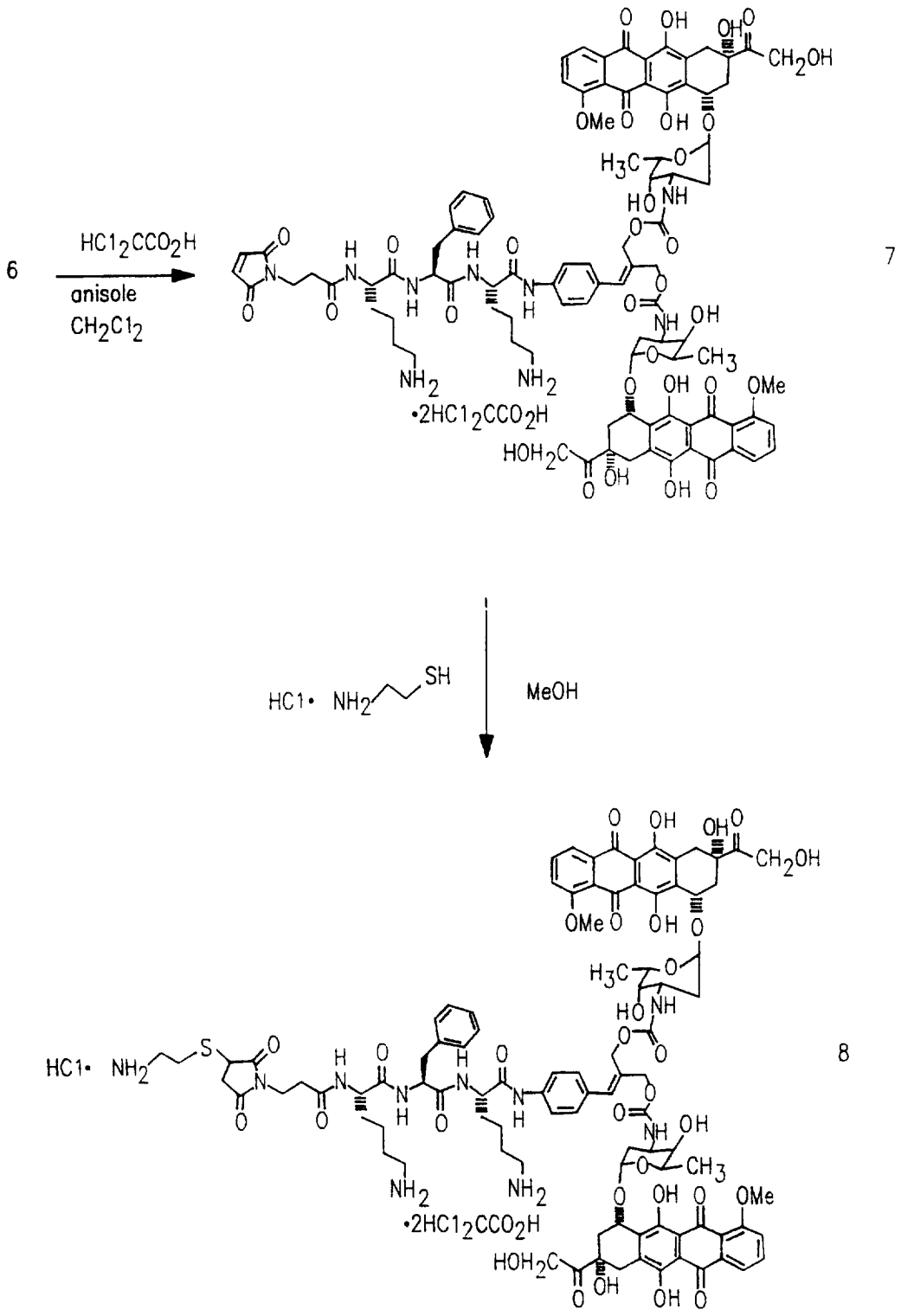
6
HCl2CCO2H
anisole
CH2Cl2
7
•2HCl2CCO2H
HCl• NH2 SH
MeOH
8
•2HCl2CCO2H

FIG. 8

Comparison of Killing of High- and Low-Secreting Cells

Compound	CA-074	BT-20 Cells	MCF-10 Cells
1	-	50	25
	+	5	25
2	-	20	10
7	-	10	5
8	-	10	5

FIG. 9

Cell Killing of BT-20 at Various Times and Prodrug Concentrations

Compound	Concentration μM	2h	4h	8h	24h
1	25	5(1)	10(5)	20(5)	25(5)
	2.5	1(1)	5(1)	5(1)	10(1)
	0.25	1(1)	5(1)	5(1)	5(1)
2	25	5(1)	10(5)	15(5)	20(5)
	2.5	1(1)	5(1)	10(5)	10(5)
	0.25	1(1)	5(1)	5(1)	5(1)
3	25	10(1)	15(1)	15(1))	40(20)
	2.5	1(1)	5(1)	10(1)	20(5)
	0.25	1(1)	1(1)	5(1)	10(5)
4	25	10(5)	15(5)	20(5)	90(60)
	2.5	5(1)	5(1)	10(1)	60(30)
	0.25	1(1)	1(1)	5(1)	40(15)
5	25	20(10)	25(10)	25(10)	25(10)
	2.5	10(5)	15(5)	15(5)	15(5)
	0.25	5(1)	10(1)	10(1)	10(1)
8	25	1(1)	1(1)	1(1)	5(5)
	2.5	1(1)	1(1)	1(1)	1(1)
	0.25	1(1)	1(1)	1(1)	1(1)

Results with 40 μM CA-074 are in parenthesis.

FIG. 10

Cell Kill Results with Compound 6

BT-20 Cells

Concentration, μM	2h	4h	8h	24h
25	5(5)	10(10)	20(15)	50(30)
2.5	1(1)	5(5)	10(5)	20(15)
0.25	1(1)	5(1)	5(1)	20(10)
0.025	1(1)	1(1)	5(1)	10(10)
0.0025	1(1)	1(1)	1(1)	5(10)

MCF-10A Cells

25	1(1)	1(1)	1(1)	5(5)
2.5	1(1)	1(1)	1(1)	1(1)

FIG. 11

Stability of Hydrolyzable Prodrugs

Prodrug	t½, pH 7.4, 37°C with Cathepsin B	Plasma, No Cathepsin B
Z-Phe-Lys-PABC-Dox	32 min	>20d
Z-Val-Lys-PABC-Dox	36 min	>20d
D-Phe-Phe-Lys-PABC-Dox	54 min	>16d
Z-Val-Cit-PABC-Dox	16 h	>16d
Boc-Phe-Lys-PABC-MMC	32 min	6d

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17410

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 38/00; C07K 5/00
US CL :530/330, 331; 514/17, 18, 19
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. : 530/330, 331; 514/17, 18, 19

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)

CAS, EMBASE, WPIDS, FILE REG, USPAT, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	EP 0 624 377 A2 (BRISTOL-MYERS SQUIBB COMPANY) 17 November 1994, see entire document.	1-3, 9-13, 27-29 and 35-39 --- 1-47
X --- Y	US 4,703,107 A (MONSIGNY et al.) 27 October 1987, see entire document.	1-3, 9-11, 27-29 and 35-37 --- 1-47

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

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 12 NOVEMBER 1997	Date of mailing of the international search report 12 DEC 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BENNETT CELSA Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17410

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CASTRONOVO, V. et al. Laminin and Fibronectin Increase The Steady State Level Of The 67 kD High Affinity Metastasis-Associated Laminin Receptor mRNA In Human Cancer Cells. Biochem. Biophys. Res. Commun. 16 May 1990, Vol. 168, No. 3, pages 1110-1117, see especially pages 1112-1116.	1-47