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Activated Prodrug

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Synergism of Selective Tumor Vascular Thrombosis and Protease-Activated Prodrugs

Introduction

Targeted drug activation at selected sites promises reduced toxicity and enhanced efficacy. We propose to investigate and validate a bipartite drug delivery-activation system that employs selective tumor vascular targeted induction of endogenous coagulation protease cascade followed by administration of a protease-activated prodrug¹. The efficacy of this strategy has been demonstrated by a fibronectin motif and Tissue Factor extracellular domain fusion protein that specifically activates coagulation on the tumor vascular endothelial surface when certain integrins, such as $\alpha v \beta 3$ and $\alpha 5 \beta 1$, are expressed and exposed. The activation of the coagulation cascade and tumor vascular thrombosis as well as subsequent activation of thrombolytic pathways leads to the explosive amplification of serine protease cascades and local proteolytic activity within the tumor vasculature. This tumor specific proteolytic activity has also been exploited for targeted local prodrug activation. The combination treatment of selective tumor vascular thrombosis and protease-activated prodrug demonstrated a profound synergism. Indeed, more robust and sustained tumor vascular thrombosis was observed compared to selective tumor vascular thrombosis alone. The activation of local tumor vascular thrombosis substantially increased prodrug activation and retention in tumors. Importantly, the activated prodrug eliminated the remaining tumor cells at the rim of tumors that do not depend on neo-angiogenesis for survival. This coordinated attack on tumors resulted in complete tumor eradication in multiple drug resistant rodent and human tumor models with no apparent toxicity. This synergistic targeted activation of coagulation and prodrug possesses interchangeable targeting potentials with different tumor vascular specific molecules and thus may represent a general therapeutic strategy for breast cancer therapy.

Body

The funding from this synergistic grant provided critical support for us to pursue research in translational medicine and experimental therapeutics in the area of breast cancer. We have made significant progress during the first year of this grant.

Targeted drug activation at selected site promises reduced toxicity and enhanced efficacy. We describe here a bipartite drug delivery-activation system that employs selective tumor vascular targeted induction of endogenous coagulation protease cascade followed by administration of protease-activated prodrug. The efficacy of this strategy is demonstrated by an integrin targeted STVT that specifically activates coagulation on the tumor vascular endothelial surface.

The activation of coagulation cascade and tumor vascular thrombosis as well as the following activation of the thrombolytic pathways led to explosive amplification of serine protease cascades and local proteolytic activity within the tumor vasculature. This tumor specific proteolytic activity is exploited for targeted local prodrug activation. The combination treatment of selective tumor vascular thrombosis and protease-activated prodrug demonstrated profound synergism. More robust and sustained tumor vascular thrombosis are observed compared to selective tumor vascular thrombosis alone. The activation of local tumor vascular thrombosis substantially increased prodrug activation and retention in tumors. Importantly, the activated prodrug eliminated the remaining tumor cells at the rim of tumors that are not dependent on neoangiogenesis for survival. This coordinated attack on tumors resulted in complete tumor eradication in multiple drug resistant rodent and human tumor models with no apparent toxicity. This synergistic targeted activation of coagulation and prodrug has interchangeable targeting potentials with different tumor vascular specific molecules and thus may represent a general therapeutic strategy for cancer therapy.

1. In vivo tumor vascular targeting and protein engineering of selective tumor vascular thrombogen.

a) Production of STVT as a fusion protein of a single chain antibody and TF. (Month 1st to 12th)

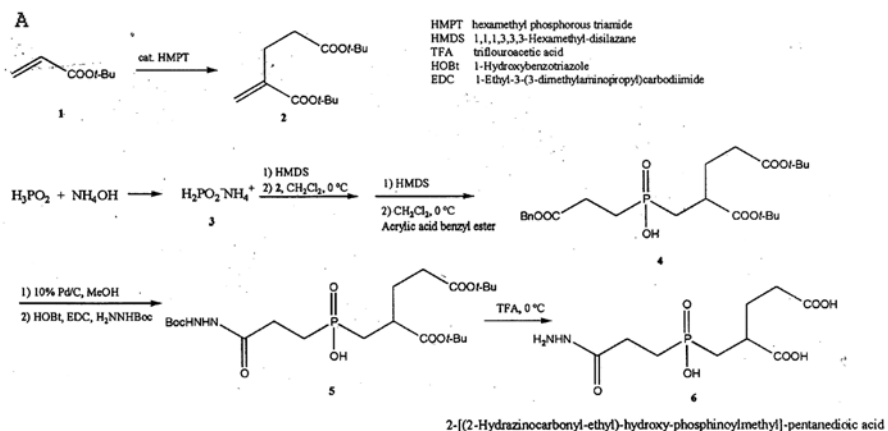
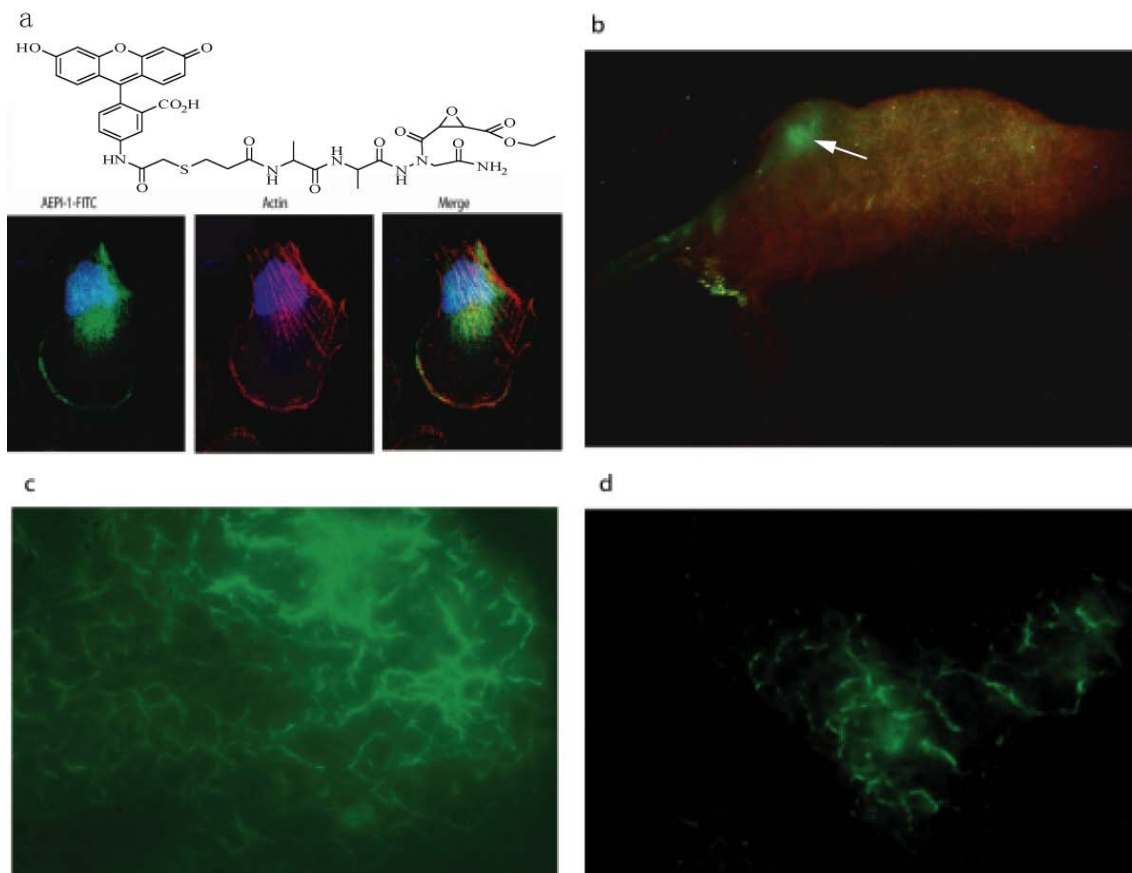
We have completed the design and production of integrin directed STVT and proceeded to the in vivo testing of this STVT. In addition to integrin targeted STVT, proteases associated with angiogenic endothelial surfaces are targeted with legumain targeting STVT and PSMA targeted targeting STVT (there are new reports indicate PSMA is widely expressed in angiogenic tumor vascular endothelial cells including breast cancers) were also constructed and produced. These STVTs are being examined for effective tumor infarction in vivo.

b) Alternating in vitro solid-phase and whole cell panning of a human scFv phage display library against novel and known tumor vascular markers. (Month 1st to 18th)

Panning of phage libraries is ongoing. We have obtained peptides and scFv against tumor vasculatures.

c) Validation of in vivo targeting of antibody by whole mouse imaging. (Month 1st to 18th)

In vivo imaging of antibody and peptides are ongoing. Selected small molecule targeting legumain, PSMA are accomplished.



(A) Synthesis pathway of a PSMA targeting inhibitor structure, Compound 6, 2-[2-Hydrazinocarbonyl-ethyl)-hydroxy-phosphinoylmethyl]-pentanedioic acid (PMPA), can be used to conjugate to a peptide.

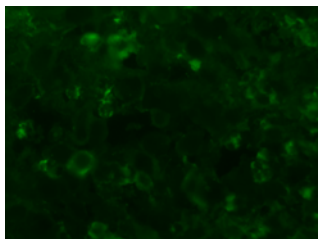
These proteases are expressed on the surface of tumor endothelial cells and will be used to target STVT using small molecule inhibitor as targeting entity.

2. Design and chemical synthesis of a protease-activated prodrug. (Month 1st to 12th)

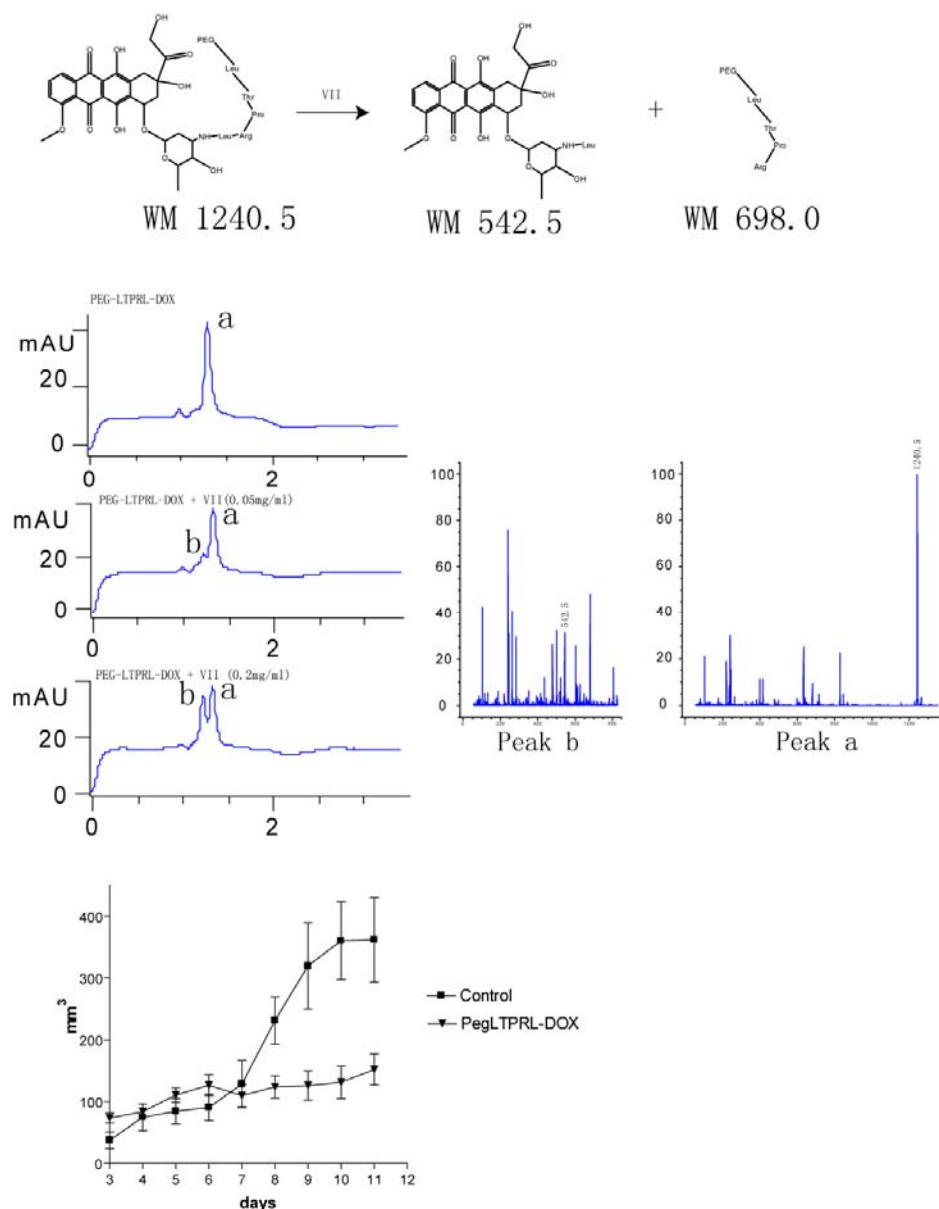
In addition to doxorubicin based prodrug, two paclitaxel based prodrugs that are activated by proteases in the coagulation cascade are prepared. They take advantage of a facile chemistry linking the substrate peptides to the C10 position. These drugs are water soluble compare to paclitaxel parent compound.



One of the key discovery in the course of last year is that factor VII can cross the leaky tumor vasculature and are forming TF:VIIa complex on tumor cell surface.



Factor VIIa is stained with a rat anti-mouse VIIa antibody in 4T1 mammary carcinoma grown in Balb c mice. The green represent VIIa on tumor cell surface. Therefore, TF:VIIa complex is active in the tumor microenvironment outside the blood vessel. This explains the substantial single agent efficacy of TF:VIIa activated prodrug in these models. We are in the process to assess whether Xa and thrombin can exit the tumor vasculature.



The doxorubicin based prodrug with LTPR substrate recognition sequence is properly activated by VIIa and demonstrated significant growth inhibition in 4T1 mouse mammary carcinoma model given at 10 $\mu\text{mol/kg}$ daily. Doxorubicin is ineffective at MTD in this model.

We are in the process to evaluate this compound and the paclitaxel based compound in additional syngeneic and xenograph models of mammary carcinoma.

3. In vivo efficacy of combined selective tumor vascular thrombosis and a protease-activated prodrug.

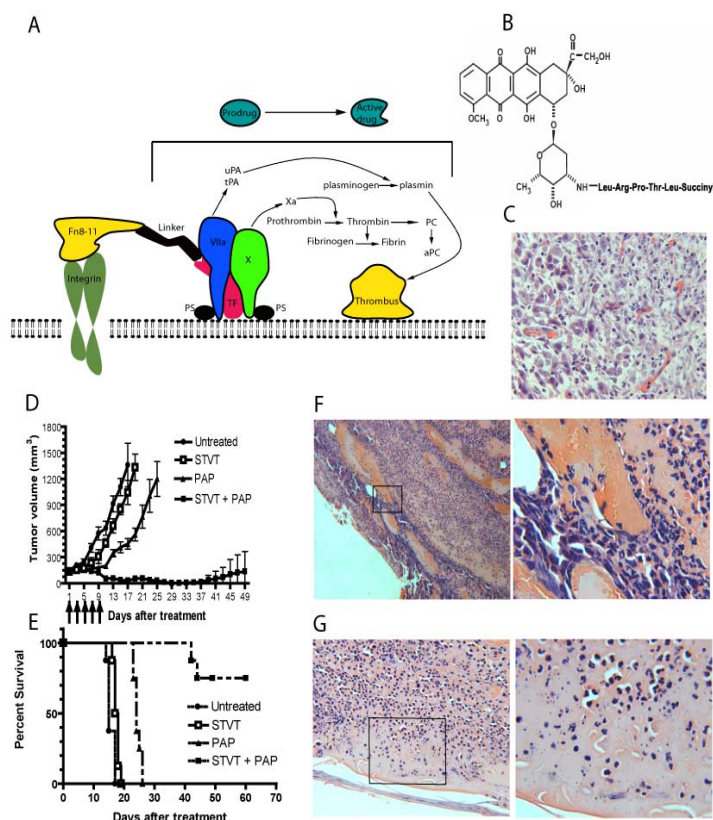
a) The safety and efficacy of STVT and PAP will be assessed in rodent mammary carcinoma models. (Month 3rd to 18th).

Safety and efficacy of integrin targeted STVT have been assessed in animals which demonstrated very local toxicities. Upto 100 ug/mouse are injected i.v. without apparent toxicity and coagulation activation.

Two Doxorubicin based and two paclitaxel based prodrugs were prepared and demonstrated efficacy as single agent in breast cancer models since tumor vasculatures are prothrombotic.

b) Synergism of combined treatment of selected highly efficacious candidates will be evaluated in both rodent and human primary as well as metastatic breast cancer models. (Month 6th to 24th)

Early combination evaluation support synergistic effect of STVT and TMEAP in breast cancer models.



Synergism of selective tumor micro-vascular thrombogen (STVT) and protease-activated prodrugs.

A. Model of ternary complex of STVT with VIIa and FX. STVT is a soluble TF and is exploited for selective activation of protease-activated prodrug. B. Schematic structure of a doxorubicin-based cell impermeable prodrug. Cleavage after Arg releases active cell-permeable Leu-dox. C. *In vivo* thrombosis of breast cancer microvasculature occurs immediately after STVT administration. D. Combination therapy of STVT with protease-activated prodrug resulted into synergistic effect (n=12, $P<0.01$), and E. Complete

cancer eradication and tumor free survival (n=12, $P<0.01$). F. Survival of tumor cells at the rim of the tumors received STVT alone and these tumor cells are not dependent on neo-angiogenesis for survival. G. Combination treatment led to complete tumor destruction.

Key Research Accomplishments

1. Demonstrated efficacy of TME activated prodrug against drug resistant prostate cancers.
2. Synergism of integrin targeted STVT with prodrugs.

Reportable Outcomes

1. Characterizing the molecular mechanism of STVT action (reported in Molecular Cancer Therapeutics).
2. Prove of principle of tumor microenvironment activated prodrugs and in vivo demonstration of prodrug efficacy against drug resistant breast cancers (reported in Cancer Research). Finding of TF:VIIa complex formation in tumor microenvironment and activation of prodrug. Demonstration of single agent efficacy of TF:VIIa activated prodrug.
3. Synergism between integrin targeted directed STVT and prodrug.
4. A PCT is filed to protect IP generated from studies supported by this grant (see appendix). This patent covers the prodrug side of the technology.
5. Patent for STVT is awarded (see appendix). Although the initial filing of this patent precedes the award of this grant, the data generated with the support of this grant is instrumental provide additional support to obtain patent. Unfortunately, you can not add new acknowledgement into the patent despite adding new data to support the original invention.
6. The technology developed from this grant is licensed by a commercial entity from TSRI and is undergoing further preclinical development.

Conclusions

The local tumor microenvironment differs greatly from that of other tissues. One key character is that it is enriched in proteolytic activity. Cell surface proteases, such as legumain and PSMA, play important role in cancer progression such as invasion/metastasis and angiogenesis. The coagulation cascade is also activated in tumor vasculature. The over-expression of these cell surface proteases are ideal physical as well as functional targets for cancer therapy. It can serve to target thrombogens to infarct tumors and also to activate prodrugs in the tumor microenvironment as demonstrated by data generated with the support of this grant.

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Appendices

INHIBITING TUMOR CELL INVASION, METASTASIS AND ANGIOGENESIS

This application claims priority to the filing date of U.S. Provisional Application Ser. No. 60/740,575, filed November 29, 2006, which is incorporated herein by reference in its entirety.

This application is related to PCT Application Ser. No. PCT/US2004/017157 filed May 28, 2004, which claims benefit of U.S. Application Ser. No. 60/474,840 filed May 29, 2003, both of which are incorporated by reference herein in their entireties.

Statement of Government Rights

The invention was made with the support of a grant from the Government of the United States of America (CDMRP Grant Numbers W81XWH-05-1-0091 and W81XWH-05-1-0318 from the Department of Defense). The Government may have certain rights to the invention.

Field of the Invention

The present invention relates to methods for treating and/or inhibiting tumor cell invasion, metastasis and/or angiogenesis as well as increasing apoptosis in cancer cells by administering asparaginyl endopeptidase inhibitors. In some embodiments, the invention relates to inhibitors of proteases that are expressed under the hypoxic conditions of the tumor microenvironment. In other embodiments, the invention relates to prodrug agents that become activated by the proteases that are expressed in the tumor microenvironment. The prodrugs become active within the tumor microenvironments of primary and metastatic tumor sites, for example, at the surface of, cancerous cells and tumor stromal cells that express proteases.

Background of the Invention

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is

prior art, or relevant, to the presently described inventions, or that any publication or document that is specifically or implicitly referenced is prior art.

According to the National Cancer Institute, since 1990 over 17 million people have been diagnosed with cancer, and an additional 1,334,100 new cancer cases are expected to be diagnosed in 2003. About 556,500 Americans are expected to die of cancer in 2003, more than 1500 people every day. Cancer is therefore the second leading cause of death in the United States, exceeded only by heart disease. The National Institutes of Health estimate the overall costs of cancer in the year 2002 at \$171.6 billion (Cancer Facts & Figures, 2003). Clearly, cancer is an enormous problem, and more effective cancer treatments are needed.

Two characteristic features of malignant cells are the ability to invade normal tissues and the ability to spread to distant sites. Tumor metastasis and invasion are the main cause of cancer mortality. Malignant cells can spread by several routes including direct local invasion, by the lymphatics or by capillaries. Local invasion is accomplished by an increase of tumor cell mobility and by production of proteases that destroy the normal extracellular matrix and basement membranes. Once the tumor cells escape from their normal boundaries, they are free to enter the circulation through the capillaries and the lymphatic system. The need for methods to prevent tumor invasion and metastasis is critical and constitutes a major goal in the effort to develop effective therapeutic interventions against cancer.

In addition, many cancer cells are capable of inducing angiogenesis. To form blood vessels, angiogenic endothelial cells share some of the same biochemical mechanisms that are used by cancer cells to invade local tissues.

Current cancer treatments generally involve the use of surgery, radiation therapy, and/or chemotherapy. However, these treatments all involve serious side effects. For example, surgery can be complicated by bleeding, damage to internal organs, adverse reactions to anesthesia or other medicines, pain, infection, and slow recovery. Radiation therapy can damage normal cells and can cause fatigue. For many people, chemotherapy is the best option for controlling their cancer. However, chemotherapy can also damage normal cells such as bone marrow and blood cells, cells of the hair follicles, and cells of the reproductive and digestive tracts. Chemotherapy can also cause nausea, vomiting,

constipation, diarrhea, fatigue, changes to the nervous system, cognitive changes, lung damage, reproductive and sexual problems, liver, kidney, and urinary system damage, and, especially with the use of the chemotherapeutic agent doxorubicin, heart damage. Long-term side effects of chemotherapy can include permanent organ damage, delayed development in children, nerve damage, and blood in the urine. Thus, the use of the chemotherapy for cancer treatment is not without serious side effects.

Most agents currently administered to a patient are not targeted to the site where they are needed, resulting in systemic delivery of the agent to cells and tissues of the body where the agent is unnecessary, and often undesirable. Such systemic delivery may result in adverse side effects, and often limits the dose of an agent (*e.g.*, cytotoxic agents and other anti-cancer agents) that can be administered. Accordingly, a need exists for agents and methods that specifically target cancerous cells and tissues.

Thus, it would be desirable to be able to direct various agents to cancer cells and to the tumor microenvironment so as to be able to decrease the dosage of the agents given and to decrease the systemic toxicity and side effects associated with these agents.

Summary of the Invention

According to the invention, the tumor microenvironment creates conditions that induce expression of certain genes, including proteases that are active almost exclusively in the tumor microenvironment. For example, an unexpectedly high level expression of asparaginyl endopeptidases, including legumain, is present in a wide variety of cancer cells, particularly those involved in metastasis. Other proteases that are active in the tumor microenvironment include prostate specific membrane antigen (PSMA)(a carboxypeptidase), fibroblast activation protein (FAP) (a serine peptidase), cathepsin B (a cysteine protease), cathepsin X (a cysteine protease), urokinase-type plasminogen activator (uPA)(a serine protease), tissue factor VIIa (TF VIIa)(a serine protease), matriptase (a membrane-bound serine protease) and Factor XIII. As described herein, tumor-specific protease expression occurs early in the development of cancer cell invasion, just as metastasis begins, and under the hypoxic conditions associated with invasive tumor growth. Moreover, legumain is directly involved in and actually inhibits the cascade of activities

that leads to cellular apoptosis, particularly in cancer cells where legumain is highly expressed.

The tumor specific proteases described herein are cell surface associated proteases. These proteases function in protease networks that play critical roles in modulating extracellular matrix proteins. For instance, certain cancers may employ more cysteine proteases than metalloproteinases or serine protease and vice versa. However the matrix modifying function of each of these proteases is indispensable for tumor metastasis and invasive growth.

According to the invention, the combined use of protease activity imaging agents, protease inhibitors, and/or prodrugs described herein represents an integrated precision-guided cancer therapeutic system. The combined use of protease inhibitor and prodrugs are also envisioned, although in some embodiments the prodrug and protease inhibitor targeting same protease are used at different times during the therapeutic regimen.

Also as shown herein, legumain activity is substantially increased on the surface of tumor cells by cell-surface association with integrins, indicating that integrins are co-factors for legumain. Moreover, legumain can activate metalloproteinases (e.g. MMP-2) and cathepsins (e.g., cathepsins B, H and L), which are all proteases involved in promoting tumor cell invasion and metastasis. In addition, asparaginyl endopeptidase expression is also associated with reduced cancer cell apoptosis and increased angiogenesis. Therefore, expression of, and activity by, certain proteases, including legumain, PSMA, FAP, cathepsin B, cathepsin X, uPA, tissue factor VIIa, matriptase (a membrane-bound serine protease) and Factor XIII are cancer and angiogenesis markers and constitute indicia of tumor cell metastasis. The invention therefore provides agents to treat undesirable angiogenesis, tumor cell invasion, tumor cell metastasis and other such cancerous conditions, particularly those conditions involving cells and tissues that express these proteases.

Many tumor cells are largely resistant to chemotherapy, for example, because the chemotherapeutic agents employed are only active against a subset of the tumor cells that comprise a cancerous condition. According to the invention, stromal cells in the tumor microenvironment, such as endothelial cells or tumor associated macrophages (TAMs), can be targeted by the agents of the invention to effectively treat these drug-resistant tumor cell

types. This strategy is also effective for reducing the expression and/or activity of molecules in the tumor microenvironment that attract TAMs and other tumor-associated cells that facilitate tumor growth and invasion. TAMs consist of a polarized M2 (CD206+, F4/80+) macrophage population. TAMs also possess poor antigen presenting capacity and effectively suppress T cell activation. In fact, TAMs actually promote tumor cell proliferation and metastases by producing a wide range of growth factors, pro-angiogenesis factors, metalloproteinases and the like. TAMs also partake in circuits that regulate the function of fibroblasts in the tumor stroma and are particularly abundantly expressed in the tumor stroma.

According to the invention, TAMs express high levels of certain proteases, including legumain, in the tumor microenvironment. In contrast, classical macrophages of the M1 phenotype, that perform key immune-surveillance functions, do not express legumain. Consequently, targeted elimination of TAMs does not interfere with the biological functions of normal (M1) macrophages, including cytotoxicity and antigen presentation. Thus, one aspect of the invention involves targeting legumain-expressing TAMs with prodrugs and/or proteases inhibitors to destroy TAMs and/or inhibit their function.

TAM and endothelial cells are non-transformed cells therefore will not develop drug resistance that is common among malignant cancers. Thus, low dosages of the prodrugs and/or protease inhibitors can be employed when targeting these TAM and endothelial cells. This will down regulate a wide variety of tumor growth factors, pro-angiogenesis factors and enzymes released by these macrophages and lead to inhibition of tumor angiogenesis as well as invasive growth and metastasis.

One aspect of the invention is a method of treating cancer in a mammal by administering to the mammal an effective amount of a prodrug or an inhibitor of a protease that is expressed in the tumor microenvironment. According to the invention, treatment of cancer can involve killing tumor cells, reducing the growth of tumor cells and reducing the growth or function of tumor stromal cells in a mammal. Examples of stromal cells that can be treated by the methods of the invention include tumor-associated macrophages and endothelial cells. Treatment of cancer can also involve promoting apoptosis of cells that express legumain. As shown herein, expression of legumain inhibits apoptosis and cancer

cells that exhibit high levels of legumain expression resist apoptosis. Treatment of cancer can also involve inhibiting angiogenesis of a tumor in a mammal.

Another aspect of the invention is a protease-activated prodrug that is tumoricidal *in vivo*, wherein the protease is a protease that is expressed in the tumor microenvironment (e.g., under the hypoxic conditions of tumor microenvironments). These protease-activated prodrugs have reduced side effects and toxicity relative to currently available chemotherapeutics. While the present prodrugs are useful for treating cancer, they are also useful to treat other conditions and cellular environments that express proteases. For example, certain non-transformed cells support tumor growth and invasion and, as described herein also express proteases when present in the tumor microenvironment. Hence, the present prodrugs can be used to target and kill not only cancer cells but also the cells that support tumor growth and tumor cell metastasis.

A prodrug compound of the invention includes a drug molecule linked to a peptide, wherein the peptide has an amino acid sequence that is specifically recognized by a protease expressed in a tumor microenvironment. Thus, the peptide includes at least two linked amino acids, wherein at least one of the two linked amino acids is an amino acid that is specifically recognized by a tumor-specific protease and forms part of a cleavage site for the protease. For example, legumain is a protease that is specifically expressed in tumor cells and cells that support tumor growth and metastasis (e.g. tumor associated macrophages). Legumain is an asparaginyl protease that specifically recognizes asparagine-containing peptides and cleaves peptides that contain asparagine (Asn). Some of the prodrugs of the invention are therefore designed to be activated by legumain. Legumain cleaves the peptide of the present prodrugs at the site of the Asn to generate an active drug from the prodrug. Prior to cleavage, the prodrug is substantially non-toxic to normal animal cells, whereas after cleavage, the drug is an active drug that can have a beneficial effect upon an animal to which it is administered.

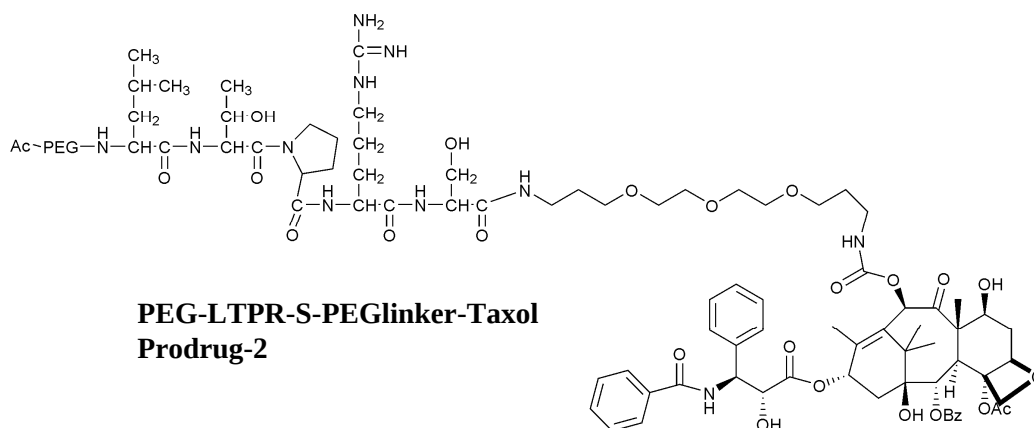
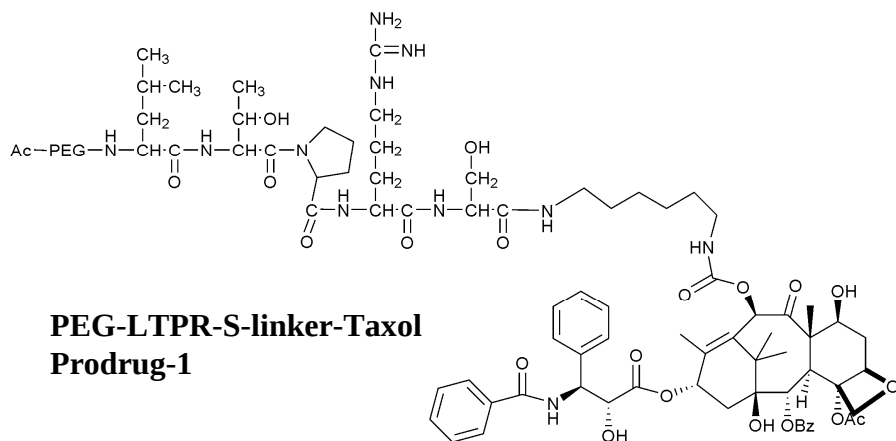
Prodrugs of the invention have the general structure:

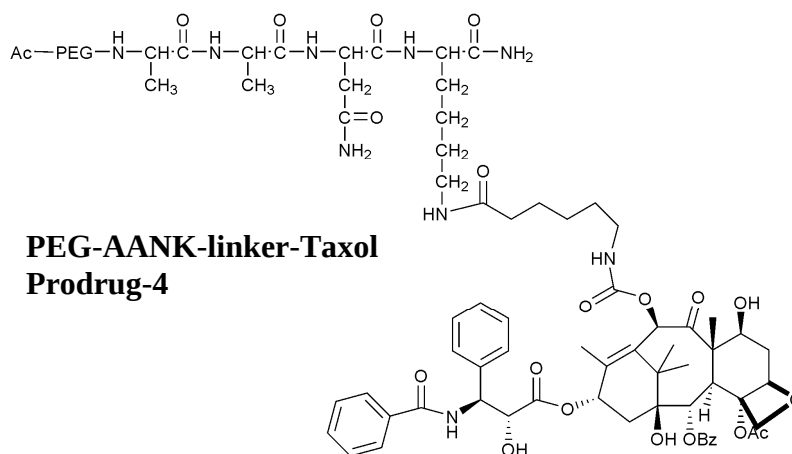
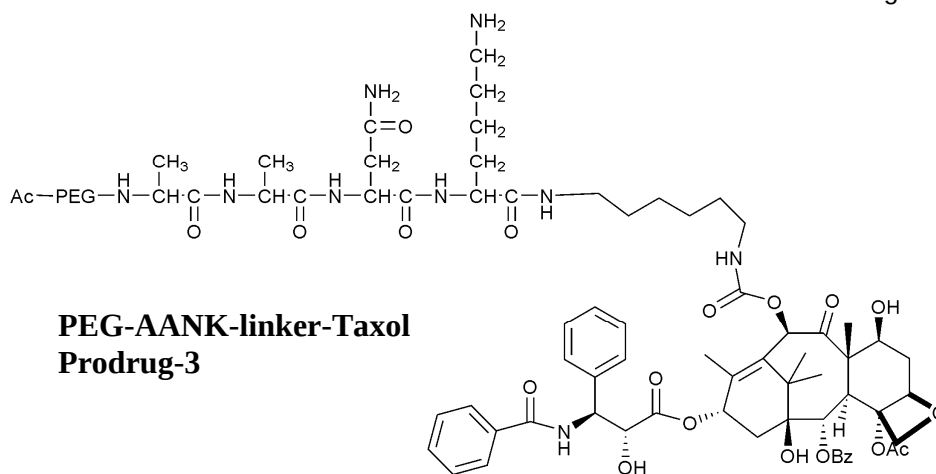


wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; and drug is any therapeutic agent. In some embodiments, the R_1 groups of the present prodrugs are not hydrophobic

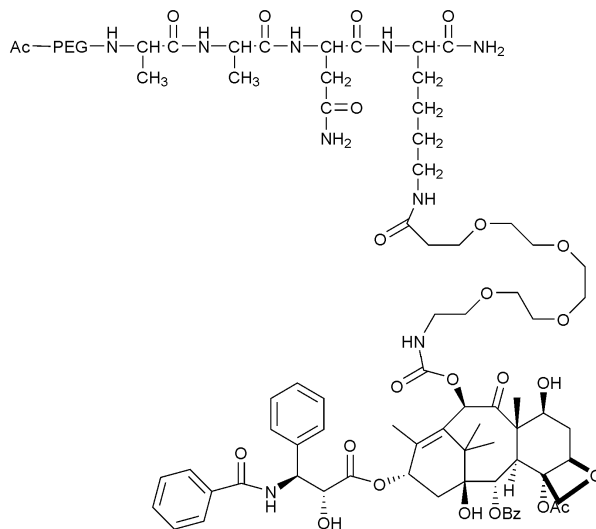
groups or labels. The R₁ and drug moieties can be linked directly to the peptide or they can be linked to the peptide through a linker or spacer molecule. Such a linker or spacer can be an alkylene, a sugar or an oligosaccharide.

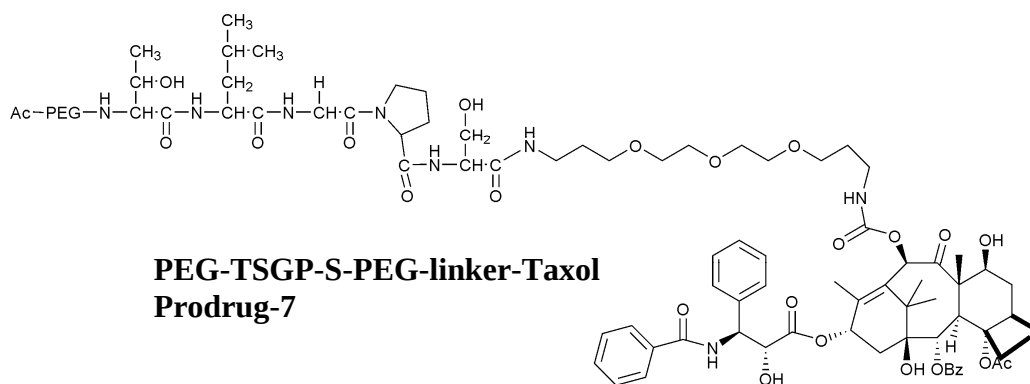
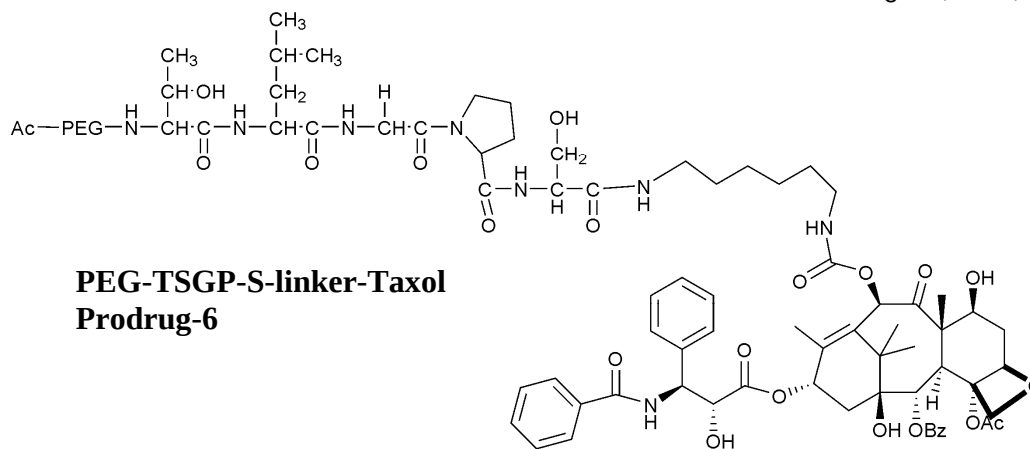
Specific examples of prodrug compounds of the invention include, for example, taxol, paclitaxel, doxorubicin containing prodrugs, including those shown below:





PEG-AANK-PEGlinker-Taxol (Prodrug-5):



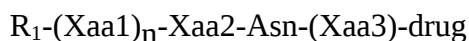


Prodrug-1 and prodrug-2 are activated by serine protease TF VIIa. Prodrug-3, prodrug-4 and prodrug-5 are activated by legumain. Additional legumain prodrugs include LEG-2 (*N*-Succinyl- β -alanyl-L-threoinyl-L-Asparaginyl-L-Leucyl-Doxorubicin) and LEG-3 (*N*-Succinyl- β -alanyl-L-alanyl-L-Asparaginyl-L-Leucyl-Doxorubicin), whose structures are shown in FIG. 9A. In some instances, the prodrugs of the invention do not include LEG-2 or LEG-3, because the inventor has filed a separate application on these compounds. Prodrug-6 and prodrug-7 are activated by fibroblast activation protein (FAP).

The drug employed is any drug whose action is diminished or blocked by attachment of a peptide to the drug. The ability of the drug to enter cells is diminished, inhibited or blocked by attachment of the peptide and hydrophilic groups. Such hydrophilic groups are generally included to facilitate water-solubility and cell impermeability. Hydrophilic groups are generally attached to the peptide so that the function of the drug is not inhibited or blocked by the hydrophilic group once the peptide is cleaved from the prodrug to yield the drug.

In some embodiments, the drug can be a cytotoxin or a photosensitizing agent. Such a cytotoxin can be aldesleukin, 5-aminolevulinic acid, bleomycin sulfate, camptothecin, carboplatin, carmustine, cisplatin, cladribine, lyophilized cyclophosphamide, non-lyophilized cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, diethyistilbestrol, epoetin alfa, esperamycin, etidronate, etoposide, filgrastim, floxuridine, fludarabine phosphate, fluorouracil, goserelin, granisetron hydrochloride, idarubicin, ifosfamide, immune globulin, interferon alpha-2a, interferon alpha-2b, leucovorin calcium, leuprolide, levamisole, mechlorethamine, medroxyprogesterone, melphalan, methotrexate, mitomycin, mitoxantrone, octreotide, ondansetron hydrochloride, paclitaxel, pamidronate, pegaspargase, plicamycin, protoporphyrin IX, sargramostim, streptozocin, taxol, thiotepa, teniposide, vinblastine, or vincristine. In some embodiments, the drug is doxorubicin, 5-aminolevulinic acid, protoporphyrin IX, taxol or paclitaxel.

In one embodiment, the prodrug is activated by asparaginyl proteases (e.g., legumain) and has a peptide amino acid sequence comprising SEQ ID NO:3:



wherein:

R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group;

n is an integer of about 0 to about 50;

Xaa1 and Xaa2 are separately any amino acid;

Xaa3 is either nothing or an amino acid that has no substantial effect on the activity of the drug; and

the drug employed is a drug whose action is diminished or blocked by attachment of a peptide to the drug.

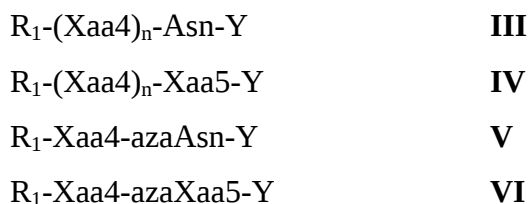
In some embodiments, the R_1 groups of the present prodrugs are not hydrophobic groups or labels. For example, when cytotoxic drugs are part of the prodrug, a hydrophilic group is preferably used for R_1 to limit cell uptake by non-target cells.

Examples of peptide sequences that may be used in the prodrugs of the invention include amino acid sequence Asn-Leu, Ala-Asn-Leu, Thr-Asn-Leu, Ala-Ala-Asn-Leu (SEQ ID NO:5), Ala-Thr-Asn-Leu (SEQ ID NO:6), and Boc-Ala-Ala-Asn-Leu (SEQ ID NO:4). Examples of prodrugs provided by the invention include Boc-Ala-Ala-Asn-Leu-

doxorubicin (SEQ ID NO:7), succinyl-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:8), N-(-*t*-Butoxycarbonyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:9), N-(Succinyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:10), N-(-*t*-Butoxycarbonyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:11), N-(Succinyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:12), N-(-*t*-Butoxycarbonyl-Thr-Leu)doxorubicin (SEQ ID NO:13), N-(Succinyl-Thr-Leu)doxorubicin (SEQ ID NO:14),

As described herein a hydrophilic R₁ group (sometimes abbreviated herein as “Hyd”) facilitates prodrug and inhibitor water solubility and inhibits cell uptake and tissue retention of the prodrug before activation and of the inhibitor before binding to a protease (e.g. the legumain: integrin complex). A variety of hydrophilic protecting groups can be utilized. Hydrophilic R₁ groups of the invention can be sugars (monosaccharides and disaccharides), dicarboxylic acids (e.g., succinate, malate, fumarate, oxaloacetate, citrate, isocitrate), glycans, polyalkylene oxides, lower alkyl carboxylates, carboxyalkyls, carboxyalkylene carboxylates, charged amino acids (e.g., any of the hydrophilic, acidic, basic and polar amino acids described herein) and the like. In some embodiments the protecting group is a hydrophilic amino protecting group. Specific examples of R₁ groups that can be used include glucuronide, succinyl, polyethylene glycol (PEG) or glutathione. Hydrophobic groups can be used if cellular uptake of the drug is desired. Hydrophobic groups that can be used include those listed herein.

The invention also provides a protease inhibitor having including formulae **III**, **IV**, **V** or **VI**:



wherein:

wherein R₁ is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group;

n is an integer of about 2 to about 5;

each Xaa4 and Xaa5 is an amino acid or an amino acid mimetic;

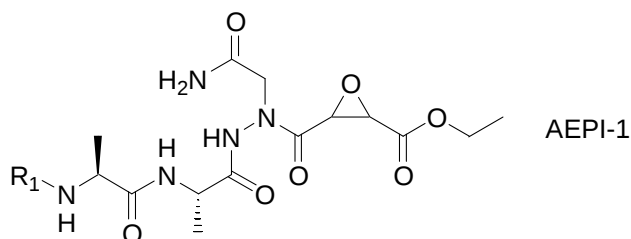
Y is alkyl, alkenyl epoxide, fluoromethylketone or a Michael acceptor, optionally substituted with 1-3 halo or hydroxy, alkylamino, dialkylamino, alkyldialkylamino, or cycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, aryl; (C₅ - C₁₂)arylalkyl or (C₅ -C₁₂)arylalkenyl,

wherein the aryl groups of the arylalkyl or arylalkenyl can be 0-4 heteroatoms selected from N, O and S, and are optionally substituted with halo, cyano, nitro, haloalkyl, amino, aminoalkyl, dialkylamino, alkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, carboxyl, carboalkoxy, alkylcarboxamide, (C₅ -C₆)aryl, --O-(C₅ -C₆)aryl, arylcarboxamide, alkylthio or haloalkylthio; and

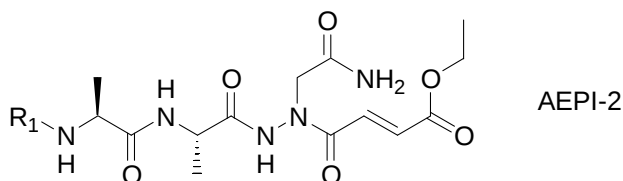
wherein each of the inhibitors of formulae **III**, **IV**, **V** and **VI** bind to a protease expressed in a tumor microenvironment.

Examples of asparaginyl endopeptidase inhibitors (AEPIs) that may be used in the methods of the invention include the following:

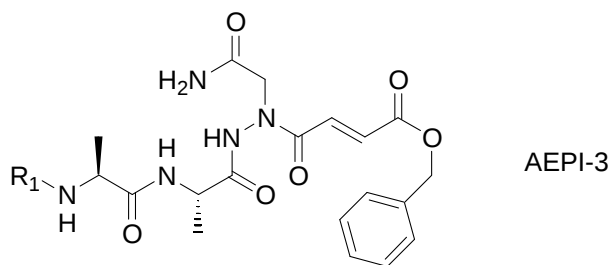
AEPI-1 is R₁-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



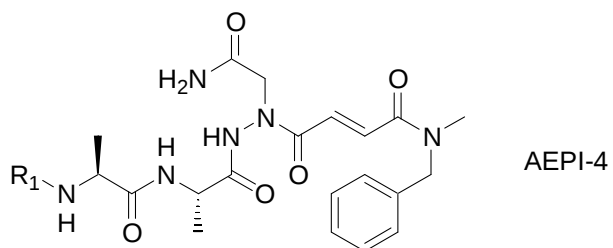
AEPI-2 is R₁-Ala-Ala-AzaAsn-CH=CH-COOEt, for example, a compound of the structure:



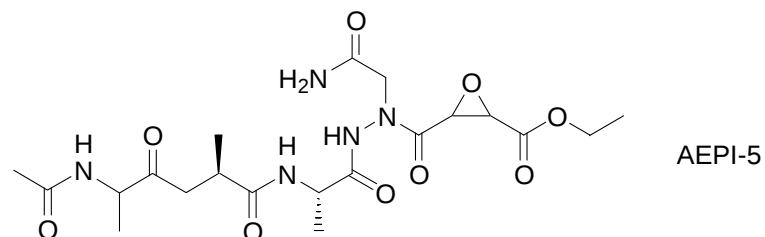
AEPI-3 is R₁-Ala-Ala-AzaAsn-CH=CH-COOBzl, for example, a compound of the structure:



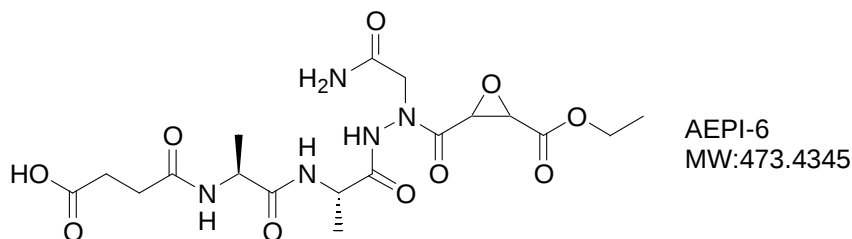
AEPI-4 is R_1 -Ala-Ala-AzaAsn-CH=CH-CON(CH₃)Bzl, for example, a compound of the structure:



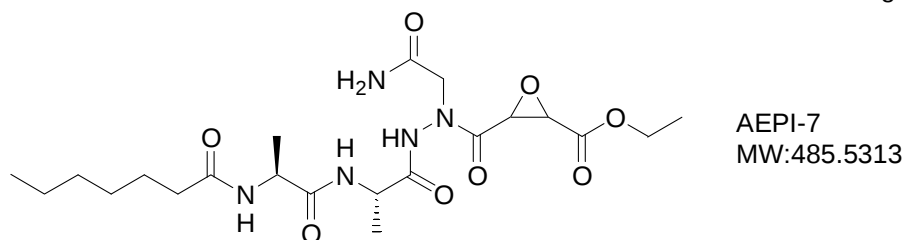
AEPI-5 is N-acetyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



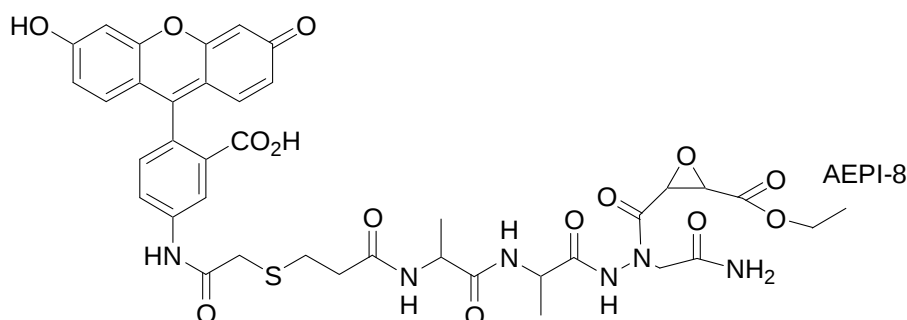
AEPI-6 is N-succinyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



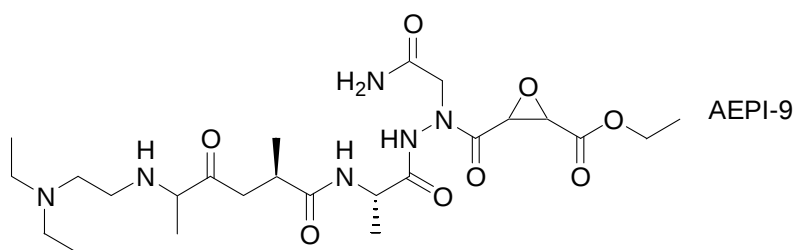
AEPI-7 is N-heptanoyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



AEPI-8 is 3-(N-carbamoylmethyl-N'-{fluorescein}-methylsulfanyl)-N-propionyl)-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:

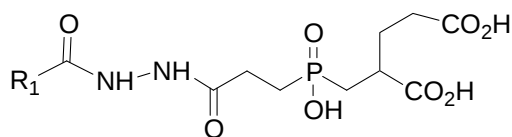


AEPI-9 is N-triethylamino-Ala-(N-isopropanoyl-Ala-AzaAsn-(S,S)-EPCOOEt), a compound of the structure:



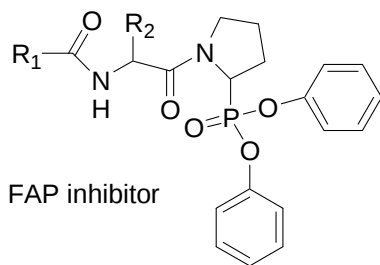
wherein R₁ is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; Ep is epoxy; Et is ethyl; and Bzl is benzyl.

Inhibitors of PSMA that can be used in the invention include the following:



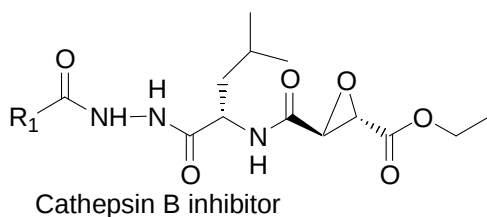
wherein R₁ is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Inhibitors of fibroblast activation protein (FAP) that can be used in the invention include the following:



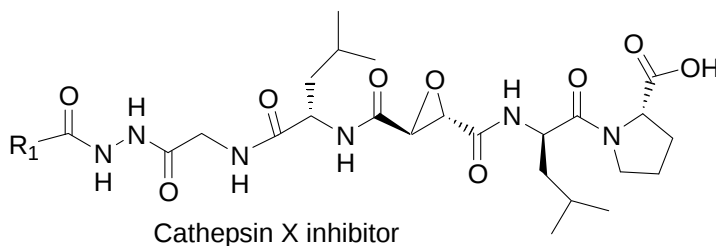
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; and R_2 is hydrogen, hydroxymethylene (CH_2OH), lower alkyl (e.g., methyl, ethyl, propyl, isopropyl ($\text{CH}(\text{CH}_3)_2$), butyl, isobutyl), or benzyl.

Cathepsin B inhibitors useful in the invention include the following:



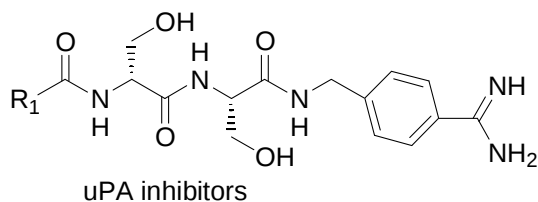
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Cathepsin X inhibitors useful in the invention include the following:



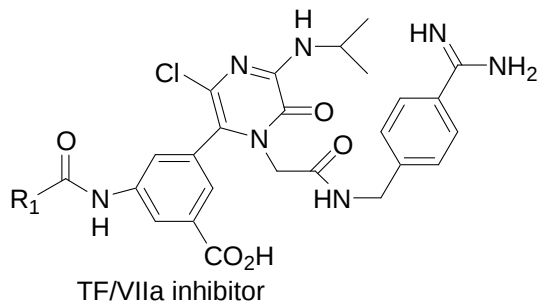
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Urokinase-type plasminogen activator (uPA) inhibitors useful in the invention include the following:



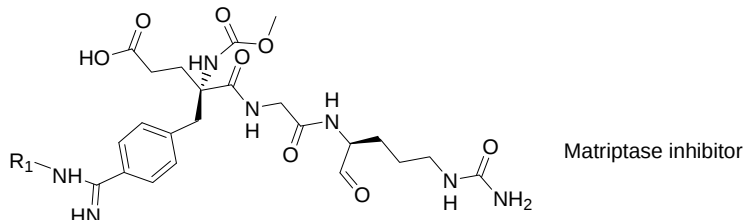
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Tissue factor VIIa (TF VIIa) inhibitors useful in the invention include the following:



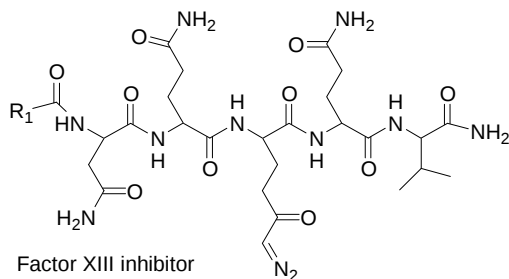
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Matriptase inhibitors useful in the invention include the following.



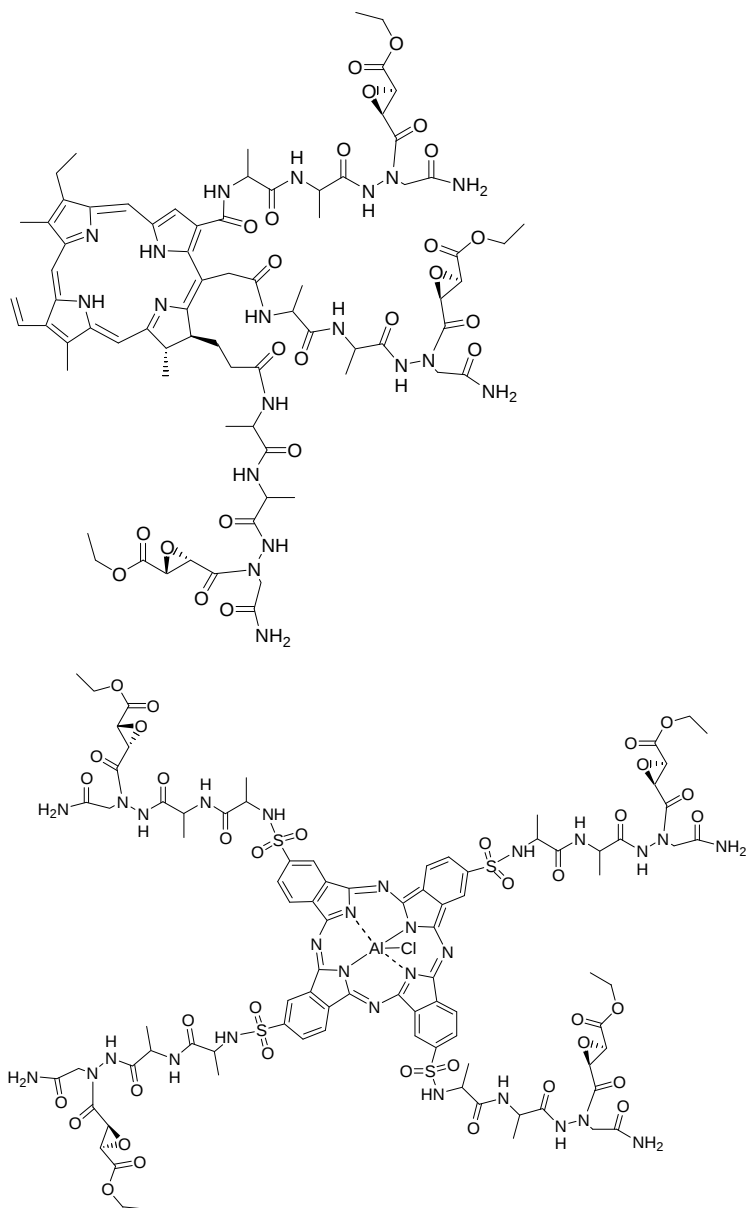
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Factor XIII inhibitors useful in the invention include the following.



wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

In some embodiments, the inhibitor is linked to a photosensitizing agent. While any available photosensitizing agent can be used (e.g., any of the photosensitizing agents contemplated for use with the present prodrugs), specific examples of photosensitizing agents include chlorin e6 and aluminum phthalocyanine tetrasulfonate (AlPcS4). Examples of inhibitors with chlorin e6 and AlPcS4 are shown below.

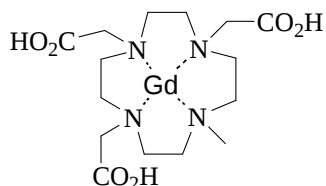


Hydrophilic R₁ groups of the invention can be sugars (monosaccharides and disaccharides), dicarboxylic acids (e.g., succinate, malate, fumarate, oxaloacetate, citrate, isocitrate), glycans, polyalkylene oxides, lower alkyl carboxylates, carboxyalkyls, carboxyalkylene carboxylates, and the like.

Hydrophobic R₁ groups of the invention can be alkyl, aryl, alkylene aryl, arylalkyl, hydrophobic amino protecting agents, beta-alanyl and related hydrophobic groups.

Any convenient amino protecting group available in the art can be used in the invention including, for example, carbobenzyloxy (Cbz), *tert*-butyloxycarbonyl (BOC), 9-fluorenylmethyloxycarbonyl (Fmoc) and benzyl groups.

A variety of labels can be used with the inhibitors of the invention to generate imaging agents or reagents for detection of cancer. Such labels can be fluorophores, radioisotopes, metals, enzymes, enzyme substrates, luminescent moieties, and the like. One example of a label that may be used is gadolinium or a gadolinium complex. For example, the following gadolinium complex can be used as a label:



In other embodiments, the legumain inhibitor can be cystatin, stefin, a peptide including the sequence Ala-Leu-β-Asn-Ala-Ala (SEQ ID NO:15) or an antibody that inhibits legumain activity.

Another aspect of the invention is a pharmaceutical composition that includes at least one of the prodrug compounds of the invention or at least one of the protease inhibitors of the invention and a pharmaceutically acceptable carrier. In some embodiments, the carrier is a liposome. Combinations of the present prodrug compounds and/or protease inhibitors can also be included in the compositions of the invention.

Another aspect of the invention is a method of detecting and treating cancer in a mammal, comprising administering to the mammal an inhibitor of the invention, wherein R₁ is a label, to detect whether the mammal has cancer and to detect which type of tumor specific protease is associated with the cancer, and administering a prodrug of the invention to treat the cancer, wherein the prodrug has a cleavage site for the tumor specific protease associated with the cancer.

Another aspect of the invention is a method for treating a mammal having, or suspected of having cancer. The method includes administering to the mammal a prodrug compound and/or a protease inhibitor of the invention in amounts and at intervals effective

to prevent, reduce, or eliminate one or more of the symptoms associated with cancer. The cancer can be an invasive or metastatic cancer. The cancer can also be a tumor that is prone to angiogenesis. Cancers that can be treated by the invention include solid tumors and cancers as well as cancers associated with particular tissues, including breast cancer, colon cancer, lung cancer, prostate cancer, ovarian cancer, cancer of the central nervous system, carcinomas, leukemias, lymphomas, melanomas, fibrosarcomas, neuroblastoma, and the like. The cancer can, for example, be autoimmune deficiency syndrome-associated Kaposi's sarcoma, cancer of the adrenal cortex, cancer of the cervix, cancer of the endometrium, cancer of the esophagus, cancer of the head and neck, cancer of the liver, cancer of the pancreas, cancer of the prostate, cancer of the thymus, carcinoid tumors, chronic lymphocytic leukemia, Ewing's sarcoma, gestational trophoblastic tumors, hepatoblastoma, multiple myeloma, non-small cell lung cancer, retinoblastoma, or tumors in the ovaries.

The invention also provides a method for inhibiting cancer metastasis and/or tumor cell invasion in an animal, including administering a protease inhibitor compound and/or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis and/or tumor cell invasion.

The invention also provides a method for inhibiting cell migration in an animal that includes administering a protease inhibitor compound and/or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method of killing a cell in a tissue, including contacting the cell with a prodrug of the invention in amounts and at intervals effective to kill the cell, wherein the tissue includes cells that express legumain.

The invention also provides a method for treating cancer in animal that includes administering to the animal a protease inhibitor compound or a prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms of cancer in the animal.

The invention also provides a method for inhibiting cancer metastasis in a tissue that includes contacting the tissue with a protease inhibitor compound or prodrug of the

invention in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis.

The invention also provides a method for inhibiting cancer cell migration in a tissue that includes contacting the tissue with a protease inhibitor compound or prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method for treating inflammation in an animal, which includes administering to the mammal a protease inhibitor compound or prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms associated with inflammation.

The invention also provides a method for delivering a drug to a cell in a tumor microenvironment of a mammal, which includes administering to the mammal an effective amount of a prodrug of the invention.

The invention also provides a method for diagnosing cancer in a tissue that includes contacting the tissue with of an agent that specifically binds to a protease that is expressed in a tumor microenvironment, and detecting whether the agent binds to the tissue. The invention also provides a method for diagnosing cancer in an animal that includes administering to the animal an agent that specifically binds to a protease that is expressed in a tumor microenvironment, and detecting whether the agent accumulates in a tissue. For example, the protease can be selected from the group legumain, PSMA, FAP, Cathepsin B, Cathepsin X, uPA, TF VIIa, matriptase and Factor XIII. These methods can further include diagnosing the patient as having or not having cancer and monitoring the progression of a cancer.

The invention also provides a method for imaging a tissue that expresses a protease. For example, the protease can be selected from the group legumain, PSMA, FAP, Cathepsin B, Cathepsin X, uPA, TF VIIa, matriptase and Factor XII. The method includes contacting a test tissue suspected of expressing the protease with of an agent that specifically binds to the protease, and detecting whether the agent binds to the test tissue. The method can further include quantifying and comparing amounts of the agent bound to the test tissue with amounts of the agent bound to a control tissue that does not express the

protease. The agent can be protease inhibitor-imaging agent or a labeled antibody that specifically binds to the protease.

In another embodiment, the prodrug and inhibitor compounds of the invention can be used for the manufacture of a medicament useful for treating diseases such as cancer.