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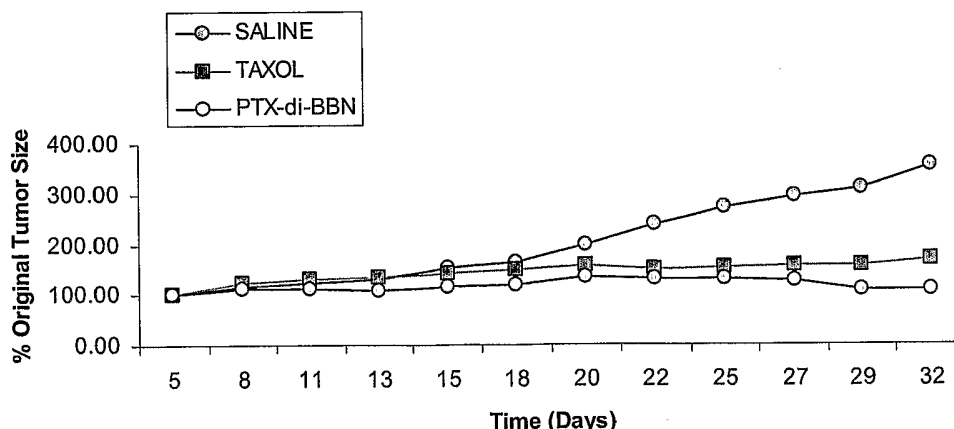
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(54) Title: SINGLE-DRUG MULTI-LIGAND CONJUGATES FOR TARGETED DRUG DELIVERY



(57) Abstract: Described is a single-drug, multi-ligand (SDML) conjugate for targeted drug delivery. The SDML conjugate contains a plurality of targeting elements linked to a treatment molecule, which may be a therapeutic agent or a diagnostic agent. The SDML conjugate may further comprise a solubilizing element and a linker molecule. The plurality of targeting elements contained in the SDML conjugate results in enhanced therapeutic efficiency. Several exemplary SDML conjugates are disclosed as well as methods for their synthesis.

Single-drug Multi-ligand Conjugates for Targeted Drug Delivery

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FIELD OF THE DISCLOSURE

5 The present disclosure relates generally to the field of drug delivery, and specifically to the field of targeted drug delivery.

BACKGROUND

10 Targeted drug delivery is a powerful strategy in the treatment and diagnosis of various human disease states and conditions, such as cancer, bacterial and viral infections and any disease expressing specific and targetable receptors, antigens or other markers. Targeted drug delivery has gained momentum during the past several years based on the specific delivery of therapeutic molecules to target cells comprising the disease state or condition, such as the delivery of oncolytic agents to neoplastic tissue. The ability to specifically target therapeutic molecules to specific cells of interest has advanced considerably during the past several years with the development of monoclonal antibodies and of surface-
15 directed small-molecule peptides (SMPs) capable of binding to target cells. A number of drug, toxin, and radioisotope conjugates of tumor-recognizing molecules have been developed with some currently in clinical use.

Allergic reactions against large-molecule antibodies in humans due to the immunoreactivity of these proteins have hampered the desired development of antibody-drug conjugates for targeted delivery
20 and much attention has been focused on the SMPs which usually show no immunoreactivity due to smaller molecular sizes. SMPs have not been fully utilized as targeting drug delivery systems. Recently, the use of a single SMP in a targeted drug delivery systems with a paclitaxel-bombesin conjugate as a model compound was demonstrated (Safavy, US Patent 6,191,290, 2001; Safavy *et al.*, *J Med Chem*, 1999, 42, 4919-4924). This and similar molecules are referred to as single-drug, single-ligand (SDSL) conjugates. While effective at specifically delivering the therapeutic molecule to the target cell of
25 interest, these SDSL conjugates are limited by having only one targeting site. Multi-drug multi-ligand (MDML) conjugates have also been described (Safavy, US Patent Application No. 10,281,840).

In the present disclosure the design for a single-drug, multi-ligand conjugate (SDML) is disclosed. The disclosure describes the incorporation of two or more targeting elements with a single
30 treatment molecule. The rationale for the design of the SDML conjugate is based on previous results with paclitaxel (PTX) conjugated to monoclonal antibodies (MAbs) [Safavy *et al.*, (2003) *Bioconjug. Chem.* 14 (2): 302; Safavy *et al.* *Bioconjugate Chem*, 2004, 15 (6): 1264]. The high antigen affinity of MAbs may be due in part to the divalency of the MAb molecule resulting in the simultaneous binding of two

antigenic epitopes. While SMPs have a faster circulation clearance, and as a result may cause lower tissue toxicity, the effective dose of antitumor agents delivered by SMPs is still open to improvement due to the inability to bind to more than one site on the target cell. In one embodiment, the present disclosure provides a SDML conjugate comprising one molecule of PTX conjugated to two bombesin (BBN) molecules. By combining the advantage of the multi-ligand binding with the smaller size of SMPs, the present invention provides a targeting vehicle for paclitaxel that has high affinity for its target on the target cell with expected lower toxicity. The SDML conjugate design of the present disclosure may also be extended to other targeting elements and other treatment molecules, limited only by the feasibility of the chemical synthesis. A SDML conjugate as described herein has not been previously described in the art.

Therefore, it would be advantageous to provide such an SDML conjugate capable of increased binding affinity to target cells. It would be further desirable to provide methods for the delivery of such SDML conjugates, either alone or as part of a pharmaceutical composition, for use in a single-step delivery method that is capable of enhanced delivery of a treatment molecules to target cells *via* targeting elements. This approach may result in higher therapeutic indices which may bring about a more significant response from the target cells. The present disclosure provides such SDML conjugates, a strategy for their synthesis and methods of using such SDML conjugates in methods of treatment and methods of diagnosis.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the features, advantages and objects of the disclosure will become clear, are attained and can be understood in detail, reference is made to the appended drawings, which are described briefly below. It is to be noted, however, that the appended drawings illustrate certain embodiments of the disclosure and therefore are not to be considered limiting in their scope.

FIGS. 1A-1E show the synthesis of four embodiments of the SDML conjugates of the present disclosure and a molecular structure for an exemplary SDML conjugate. FIG. 1A shows the synthesis of the PTX-2'-E(YBBN [7-14])₂ conjugate of the present disclosure, utilizing PTX (paclitaxel) as the treatment molecule and two molecules of the bombesin derivative YBBN [7-14] as the targeting ligand. FIG. 1B shows the synthesis of the (PTX-E(YBBN[7-14])₂-7-PEG-R) conjugate of the present disclosure, utilizing PTX as the treatment molecule, two molecules of the bombesin derivative YBBN [7-14] as the targeting ligand and 1 molecule of PEG to increase the solubility of the conjugate. FIG. 1C shows the synthesis of *di*-pegylated conjugate PTX-(PEG)-E(YBBN [7-14])₂, a water-soluble compound. FIG. 1D shows the synthesis of the PTXGL-dPEG24E(dPEG4BBN[7-14])₂ conjugate of the present disclosure, utilizing PTX as the treatment molecule, two molecules of the bombesin derivative YBBN [7-14] as the targeting ligand and multiple molecules of PEG to increase the solubility of the conjugate.

FIG. 1E shows a molecular diagram of one SDMLC of the present disclosure.

FIG. 2 shows the antitumor activity of the SDML conjugate in mouse xenograft models. Male nude mice were inoculated with the human prostate carcinoma cell line PC-3. When tumors reached a diameter of approximately 7mm, the mice were divided into 3 groups (N=8 in each group) and injected with either saline, PTX or conjugate 5, (PTX-E(YBBN[7-14])₂). On day 32 post-inoculation, the untreated (saline injected) and the PTX-injected control groups showed, respectively, a 350% and a 68% increase in their tumor sizes, in contrast to an 18% reduction in the tumor size in the mice injected with conjugate 5.

DETAILED DESCRIPTION

The present disclosure illustrates the design and synthesis of a novel class of molecules termed single-drug, multi-ligand (SDML) conjugates. These novel SDML conjugates are shown to provide superior cell killing activity as compared to the free (unconjugated) drug and single drug, single ligand conjugates.

In one embodiment of the present disclosure, there is provided a SDML conjugate comprising a plurality of the targeting elements in combination with a treatment molecule. The synthesis of several embodiments of such SDML conjugates is described in detail.

In another embodiment of the present disclosure, there is provided a SDML conjugate to improve the solubility of the SDML conjugate by the addition of one solubilizing element. Specifically, the synthesis of this embodiment of the SDML conjugate, incorporating PTX as the treatment molecule, two molecules of the bombesin-derived peptide, YBBN[7-14] as the targeting element and PEG as the solubilizing agent is described in detail. In a further embodiment of the present disclosure, there is provided a SDML conjugate to improve the solubility of the SDML conjugate by the addition of two solubilizing elements. Specifically, the synthesis of this embodiment of the SDML conjugate, incorporating PTX as the treatment molecule, two molecules of the bombesin-derived peptide, YBBN[7-14] as the targeting element and two molecules of PEG as the solubilizing element is described in detail.

In yet another embodiment of the present disclosure there is provided a method of treating a disease state or condition comprising administering a SDML conjugate as described herein in a pharmaceutically effective amount to an individual in need of said treatment. Because of the higher therapeutic index achieved by the SDML conjugate, the SDML conjugate may be administered at a lower concentration such that the concentration of the treatment molecule is less than the equivalent concentration of the unconjugated treatment molecule to attain the same or superior treatment results.

In a further embodiment of the present disclosure, there is provided a method of diagnosis of a disease state or condition comprising administering a SDML conjugate as described herein in a diagnostically effective amount to an individual in need of said diagnosis. As discussed above, the

SDML conjugate may be administered at a lower concentration such that the concentration of the treatment molecule is less than the concentration of the unconjugated treatment molecule to attain the same treatment results.

Other and further aspects, features, and advantages of the present disclosure will be apparent from the following description of several embodiments of the invention.

Definitions

The term "targeting element" is meant to include any compound, or a segment of such compound, that can direct the SDML conjugate described to a target cell.

The term "treatment molecule" is meant to include either a therapeutic agent and/or a diagnostic agent as defined in this specification.

The term "solubilizing element" is meant to include any compound, segment of such compound or chemical moiety, associated directly or indirectly with the SDML conjugate, that increases the solubility of the SDML conjugate in a given solution, such as, but not limited to, an aqueous solution.

The term "disease state or condition" is meant to include any disease or condition wherein it is desirable to deliver a treatment molecule to a target cell. Specific diseases and conditions may involve infections by foreign agents, such as, but not limited to, infections caused by bacteria, parasites and viruses, or hyper-proliferation of a subject's own cells, such as, but not limited to, inflammatory diseases and conditions, cardiovascular diseases, hyperplasia, and cancer.

The term "target cell" is meant to include a defined population of cells that may be bound by the targeting element; the defined population of cells may comprise a single cell type or multiple cell types; the defined population of cells may be a cell type endogenous to the subject (such as cancerous cells) or foreign to the subject (such as bacteria, parasites or viruses).

The term "pharmaceutically acceptable salts" is meant to include salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from organic acids like acetic, propionic,

isobutyric, oxalic, maleic, malonic, benzoic, succinic, suberic, fumaric, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (see, for example, Berge, S. M., et al., "Pharmaceutical Salts", Journal of Pharmaceutical
5 Science, 1977, 66, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The terms "treat" and "treating" is meant to include administering a SDML conjugate described, either alone or as part of a pharmaceutical composition, after the onset of clinical symptoms. Such treating need not be absolute to be useful.

10 The term "in need of treatment" is meant to include a judgment made by a caregiver that a patient requires or will benefit from treatment. This judgment is made based on a variety of factors that are in the realm of a caregiver's expertise, but that includes the knowledge that the patient is ill, or will be ill, as the result of a condition that is treatable by a SDML conjugate described, either alone or as part of a pharmaceutical composition.

15 The term "individual", "subject" or "patient" is meant to include any animal, including mammals, preferably mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, or primates, and most preferably humans. The term may specify male or female or both, or exclude male or female.

The term "therapeutically effective amount", in reference to the treating of a disease state or condition, is meant to include an amount of a SDML conjugate described, either alone or as part of a
20 pharmaceutical composition, that is capable of having any detectable, positive effect on any symptom, aspect, or characteristics of the disease state or condition. Such effect need not be absolute to be beneficial.

The term "diagnostically effective amount", in reference to the diagnosing of a disease state or condition, is meant to include an amount of a SDML conjugate described, either alone or as part of a
25 diagnostic composition, that is capable of detecting a disease state or condition. Such detection need not be absolute to be beneficial.

As shown herein, the effect of a given treatment molecule may be enhanced by incorporation of the treatment molecule into a SDML conjugate capable of delivering said treatment molecule to specific target cells of interest by virtue of a plurality of targeting elements incorporated into the SDML
30 conjugate. The present disclosure describes methods for the design and synthesis of said SDML conjugates and delivery of said SDML conjugates for the diagnosis and/or treatment of various human disease states and conditions. Cancer is the exemplary human disease state discussed below and in the Examples, but this disclosure should not be interpreted to be limited only to the diagnosis and treatment of cancer, as one of ordinary skill in the art would be able to adapt the teachings of the disclosure to
35 treatment of alternate disease states and conditions by incorporating the desired targeting element(s)

and/or treatment molecule.

The present disclosure provides embodiments of the SDML conjugate where a bombesin derived peptide is used as the targeting element and a paclitaxel molecule is used as the treatment molecule. However, the present disclosure should not be limited only to the targeting elements and treatment molecule disclosed. Additional targeting elements and treatment molecules may be incorporated into the SDML conjugates described herein. The minimum requirement for targeting elements would be the existence of functionalities suitable for chemical coupling between the targeting element and the treatment molecule, or between the targeting element, the treatment molecule and a linker. Functional groups that may be involved in such chemical coupling include, but are not limited to, organic amines, carboxylic acids, halides, alcohols, sulfides, aldehydes, and ketones. Once present, conjugation may be possible with coupling reagents as is known in the art. Exemplary types of chemical linkages which may be expected to result, include, but are not limited to, amide, amine, ester, ether, thioether, sulfide, disulfide, hemiacetal, acetal, ketal, hydrazide, or hydrazone linkage. The functional groups and chemical bonds discussed above may be useful in coupling reactions described herein. In one embodiment, the targeting elements are linked to a glutamic acid molecule (as exemplified in FIG. 1A and 1B) via the carboxylic acid functionalities on the glutamic acid molecule. amide, amine, ester, ether, thioether, sulfide, disulfide, hemiacetal, acetal, ketal, hydrazide, or hydrazone linkage.

The targeting element may be, but is not limited to, a SMP, a peptide of any molecular size, a receptor ligand peptide (meaning a peptide that is designed to specifically bind to one or more designated receptor molecules), a receptor ligand protein, a DNA-directed molecule (meaning a molecule that binds to nucleic acid, such as, but not limited to, DNA and RNA, preferentially over other binding partners; preferentially should not be meant to require a 100% specificity of the binding of such nucleic acid-directed molecule to nucleic acid) or a carbohydrate. All targeting elements may be of natural or synthetic origin, or a combination of both. When the targeting element is a peptide, the peptide may have a length from 2 to 50 amino acids, from 2-25 amino acids, or from 2 to 10 amino acids. The identity of the targeting element is constrained only by the chemistry of linking said targeting element to the remainder of the conjugate molecule. Suitable synthetic schemes and functionalities are presented herein. The targeting element binds to a target on the surface of the target cell. The target may be any molecule on the surface of the target cell, such as but not limited to, a protein moiety, a carbohydrate moiety or a combination thereof. The function of the target need not be known. Suitable cell surface targets include receptor proteins and cell surface antigens. In one embodiment, the targeting element binds to a receptor expressed on the target cell. Exemplary targeting elements include, but are not limited to, bombesin/gastrin-releasing peptide (BBN/GRP) receptor-recognizing peptide, a somatostatin receptor-recognizing peptide, an epidermal growth factor receptor-recognizing peptide, a receptor-recognizing carbohydrate, or any combination of the above. An example of a receptor recognizing

polypeptide that binds to the BBN/GRP receptor and/or the somatostatin receptor is the BBN[7-14] peptide (GlnTrpAlaValGlyHisLeuMet-NH₂) (SEQ ID NO. 1) or the BBN[7-13] peptide (GlnTrpAlaValGlyHisLeu-NH₂) (SEQ ID NO. 2). Either of the BBN[7-14] (SEQ ID NO. 1) or BBN[7-13] peptide (SEQ ID NO. 2) sequences may have a N-terminal tyrosine residue for coupling purposes (SEQ ID NOS. 3 and 4, respectively). The targeting element incorporated into each conjugate may be the same or may be different. The targeting element need not direct the SDML conjugate to only one cell type. The selection of a particular targeting element will depend on the target cell and is within the ordinary skill in the art. The selection of the target cell may depend on the specific disease state or condition to be treated or diagnosed. The targeting elements present on a single SDML conjugate may be different or may be the same.

The treatment molecule can be any diagnostic agent currently known in the art or any therapeutic agent currently known in the art. In certain cases a diagnostic reagent may also be a therapeutic agent, and vice versa. The selection of a particular treatment molecule will depend on the particular disease to be treated or condition to be diagnosed and is within the ordinary skill in the art. The diagnostic agent may be a fluorescent label, a radiolabel, an enzymatic label, a metallic contrast agent, or a quantum dot[®] label. Suitable fluorescent labels include, but are not limited to, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. Suitable radiolabels include, alpha-, beta-, or gamma-emitting radionuclides, such as, but are not limited to, ³H, ¹⁴C, ³²P, ³⁵S, ³⁶Cl, ⁵¹Cr, ⁵⁷Co, ⁵⁸Co, ⁵⁹Fe, ⁸⁸Y, ⁹⁰Y, ^{99m}Tc, ¹²³I, ¹²⁵I, ¹³¹I, ¹⁷⁷Lu, ¹⁸⁶Re, and ¹⁸⁸Re. Suitable enzymatic labels include, but are not limited to, -glucuronidase, -D-glucosidase, -D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. Suitable metallic contrast agents include gadolinium, manganese, iron and derivatives of the foregoing and similar molecules that induce both positive or negative contrast. Diagnosis using the SDML conjugates may be accomplished by administering to the subject a diagnostically effective amount of the SDML conjugate, allowing the SDML conjugate to bind the target cells and detecting and measuring the level of binding to the target cells. An increased binding level as compared to a normal subject free of the disease state or condition to be diagnosed is indicative of the presence of the disease state or condition. When the label is a fluorescent label or a quantum dot[®], the label may be detected by fluorescence microscopy, fluorescence-activated cell sorting or a fluorescence plate reader. When the label is a radiolabel, the radiolabel may be detected by positron emission tomography scanning or similar methods. When the label is an enzymatic label, the enzymatic label may be detected immunohistochemically or by use of a calorimetric assay. When the label is a metallic contrast agent, the label may be detected by MRI or similar technologies.

Therapeutic agents include, but are not limited to, drugs, anti-tumor agents, cytotoxic agents, radionucleotides, and metallic nuclei. An exemplary therapeutic agent described in this specification is a taxane molecule. By "taxane" it is meant to include any taxane derivatives such as paclitaxel (PTX)

(Taxol[®], Bristol-Myers Squibb) and docetaxel (Taxotere[®], Aventis Pharmaceuticals, Inc.) and their analogues and pharmaceutically acceptable salts. In one embodiment, the therapeutic agent PTX is described. Suitable radiolabels include, alpha-, beta-, or gamma-emitting radionuclides, such as, but are not limited to, ³H, ¹⁴C, ³²P, ³⁵S, ³⁶Cl, ⁵¹Cr, ⁵⁷Co, ⁵⁸Co, ⁵⁹Fe, ⁸⁸Y, ⁹⁰Y, ¹²³I, ¹²⁵I, ¹³¹I, ¹⁷⁷Lu, ¹⁸⁶Re, and ¹⁸⁸Re.

5 When injected *via* a systemic route, such as, an intravenous, intramuscular, intraperitoneal or subcutaneous route, the SDML conjugate circulates through the body until the targeting elements detect and bind to their binding target on the target cell, in this case the BBN/GRP receptor. Once bound to the target cell, the therapeutic agents act on the target cell through internalization or proximity to the cell, causing cell damage, decreased proliferation and/or cell death. Alternatively, for treatment of disease
10 states and conditions of the spinal cord or brain, including cancer, the SDML conjugate may be injected directly into the spinal cord of cranium through intrathecal or intracranial (intraventricular) routes allowing a greater dose to be delivered to the these areas for therapeutic or diagnostic purposes while minimizing systemic toxicity.

Solubility of the SDML conjugate can be improved by addition of at least one solubilizing
15 element. More than one solubilizing agent may be added if desired. If more than one solubilizing agent is added, each solubilizing agent may be the same or different. Suitable solubilizing elements include, but are not limited to, polyethylene glycol (PEG), a carbohydrate, a salt in conjunction with a component of the SDML conjugate (such as a pharmaceutically acceptable salt), a peptide, a charged molecule, or a water-soluble natural or synthetic polymer, or any other molecule of high water solubility, to the SDML
20 conjugate. In one embodiment, PEG is used as the solubilizing element. The chemical and biological properties of PEG molecules have been extensively studied and the pharmaceutically useful characteristics of this polymer have been noted. These include aqueous as well as organic solubilities, lack of immunogenicity, and favorable blood clearance patterns and *in vivo* behavior. Furthermore, PEG molecules are available in a wide range of chemistries, facilitating the formation of SDML conjugates
25 with less steric hindrance, allowing favorable antigen or receptor binding ability. Any form of PEG may be used as desired, including, but not limited to, mono-dispersed discrete PEG (dPEG). The solubilizing molecules may be positioned at any place in the conjugate desired provided that the chemistry of the functional groups present on the conjugate and/or the solubilizing agent allow for such placement. A solubilizing element may be placed at more than one location on a given conjugate. Exemplary
30 placements of the solubilizing agent are provided in Example 2.

A linking molecule may be used to link the targeting element and the therapeutic molecule and the solubilizing element (if used). The use of a linking molecule is not required in every embodiment, as the targeting element, the therapeutic molecule and the solubilizing molecule (if used) may be linked together directly. Suitable linking molecules include, but are not limited to, amino acids. In one
35 embodiment glutaric acid or succinic acid are used as the linking molecule. The choice glutaric acid or

succinic acid as a linking molecule may be based, at least in part, on the stability of the linking molecule to degradation by components present in the bloodstream of a subject. The Applicant has shown that the use of glutaric acid increases the stability of the SDML molecule as compared to a corresponding SDML conjugate where succinic acid is the linking molecule. The synthetic chemistry is essentially identical for both glutaric acid and succinic acid. In Examples 1 and 2 below, succinic acid is used as the linking molecule and provides a carboxylic acid group useful in the synthesis. In these examples, succinic acid was coupled to the 2' position of paclitaxel to form paclitaxel-2'-hemisuccinate which was coupled to the targeting elements. Other compounds with suitable groups for the coupling reactions may also be used as the linking molecules. Such linking molecules are well known in the art and may be any molecule capable of linking the targeting molecule, the therapeutic agent and the solubilizing element (if used). The linker molecules are designed to reduce the effects of steric hindrance and increase the overall yield of the synthetic reaction. The linking molecule may be cleavable once the SDML conjugate is internalized into the cell. However, the use of a linker molecule is optional.

Exemplary synthetic schemes for the preparation of the SDML conjugates of the present disclosure are shown in FIGS. 1A-1D and are discussed in Examples 1 and 2 below. FIG. 1A illustrates the synthesis of one embodiment of the SDML conjugate comprising a therapeutic agent as the treatment agent (in this example PTX) and two targeting elements (in this case the BBN[7-14] peptide). FIG. 1B illustrates the addition on one solubilizing element, in this embodiment one molecule of PEG, to the SDML conjugate described in FIG. 1A. FIG. 1C illustrates the synthesis of a SDML conjugate comprising two solubilizing elements, in this embodiment two molecules of PEG. FIG. 1E shows the molecular structure of the embodiment of the SDML conjugate whose synthesis is illustrated in FIG. 1A.

The incorporation of two targeting elements allows improved binding of the SDML conjugate. This improved binding affinity allows increased delivery of the treatment molecule to the target, enhancing the delivery of the treatment molecule to the intended target cells. This increased delivery of the treatment molecule will allow a lower dose of the treatment molecule to be administered, which in turn, is expected to reduce systemic toxicity. The selection of the treatment molecule to be used will depend upon the target cell selected and the choice between a therapeutic administration or a diagnostic administration. Such selection is within the ordinary skill in the art of those in the field. In the same manner, the selection of the specific targeting element to be used will also be determined by the target cell selected which may depend on the disease state or condition to be treated or diagnosed. Again, selection of the appropriate targeting element is within the ordinary skill in the art of those in the field.

The SDML conjugates of the present disclosure may be administered alone or in combination with pharmaceutically acceptable carriers as known in the art, including, but not limited to, vehicles, adjuvants, excipients, or diluents. Typically, the pharmaceutically acceptable carrier is chemically inert to the active compounds and has no detrimental side effects or toxicity under the conditions of use. The

pharmaceutically acceptable carriers can include polymers and polymer matrices. The conjugates described may also be used in combination with other therapeutic agents as a part of a multi-agent treatment regimen.

The total amount of the conjugate administered will also be determined by the route, timing and frequency of administration as well as the existence, nature, and extent of any adverse side effects that might accompany the administration of the conjugate and the desired physiological effect. It will be appreciated by one skilled in the art that various conditions or disease states, in particular chronic conditions or disease states, may require prolonged treatment involving multiple administrations.

EXAMPLES

Example 1: Exemplary Synthesis of the One Embodiment of the SDML Conjugate

The synthesis of one embodiment of the SDML conjugate is described below. The SDML conjugate comprises two targeting elements linked to the treatment molecule. In this example, the targeting element is the YBBN[7-14] peptide (SEQ ID NO. 1) and the treatment agent is a therapeutic agent, in this example one molecule of PTX. This SDML conjugate is referred to as PTX-2'-E(YBBN[7-14])₂.

The PTX-2'-E(YBBN[7-14])₂ SDML conjugate was synthesized according to FIG. 1A. YBBN[7-14], **2**, was prepared by standard solid-phase methods and fluorenyl methoxycarbonyl (Fmoc) chemistry (Synpep, CA). SEQ. ID NO. 1 was condensed with Boc-glutamic acid (Boc-E(OH)-OH, **1**) by HOBT/DCC reaction and in dry DMF as solvent to compound **3**. The Boc protecting group was removed by trifluoroacetic acid (TFA) and the resulting intermediate, **4**, was purified by reversed-phase (RP) HPLC. The latter compound was then coupled to paclitaxel-2'-hemisuccinate in the presence of *N*-hydroxy succinimide (NHS) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as the coupling reagents to afford the target product, **5** (PTX-2'-E(YBBN[7-14])₂) which was purified by RP-HPLC. All intermediate compounds and final products were analyzed and purified by chromatographic methods and identified by mass spectroscopic methods.

Example 2: Exemplary Synthesis of Three Embodiments of a Water-soluble SDML Conjugate

Due to a low water solubility of the paclitaxel molecule, and also to the lipophilic nature of the YBBN[7-14] peptide, conjugate **5** is not as water soluble as may be desired. In therapy protocols and clinical applications, this low aqueous solubility would necessitate the use of solubilizing excipients which may be immunogenic and/or allergenic in human patients. A well known and related example is

the parenteral formulation of paclitaxel with reported side-effects and toxicities in humans. To overcome this problem, two exemplary model water-soluble SDML conjugates incorporating PEG as the solubilizing element were designed and synthesized as shown in FIGS. 1B and 1C.

The chemical and biological properties of PEG have been extensively studied and some useful characteristics of this polymer have been noted. These include aqueous as well as organic solubilities, lack of immunogenicity, and favorable blood clearance patterns and *in vivo* behavior. The chemistry and properties of PEG are described in J.M. Harris, Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications, Plenum, New York, N.Y., 1992; and J.M. Harris and S. Zalipsky, Chemistry and Biological Applications of Polyethylene Glycol, ACS Books, Washington, D.C., 1997 and in US Patent No. 5,643,575.

As shown in FIG. 1B, conjugate **5** was reacted with *N*-trifluoroacetyl-amino-PEG carboxylic acid, **6**, in the presence of EEDQ and in dry dichloromethane (DCM) and at 0°C for 12 hours. The product **7** (PTX-2'-E(YBBN[7-14])₂-7-PEG-R) was purified by RP-HPLC. All intermediate compounds and final products were analyzed and purified by chromatographic methods and identified by mass spectroscopic methods.

As shown in FIG. 1C, conjugate **13** was synthesized by reacting compound **4** with the Fmoc-protected PEG amino carboxylic acid **8** in dry DMF followed by the removal of the Fmoc protecting group by piperidine. Repeat of the same step with a second molecule of **8** afforded the intermediate conjugate **11** which was coupled to PTX by the same procedure as described in the previous section. The highly water-soluble final conjugate **13** (PTX-(PEG)₂-E(YBBN[7-14])₂) was purified by HPLC.

As shown in FIG. 1D, conjugate **20** was synthesized to introduce a multiple solubilizing agents into the structure. In this embodiment, the synthesis utilized mono-dispersed *discrete* PEGs (dPEGs). Four-unit and 24-unit dPEGs (dPEG4 and dPEG24, respectively) were used in this synthesis. Peptide **2** (prepared as described above in Example 1) was coupled to amine-protected dPEG4 carboxylic acid **14** by a DCC coupling protocol, followed by the removal of the Boc protecting group by TFA to afford compound **15**. Coupling of **15** to the Boc protected glutamic acid **1** at a 2:1, conjugate **15**: conjugate **1** molar ratio, and removal of the Boc protecting group afforded product **16**. The water-soluble PTXGL segment was synthesized by condensation of paclitaxel hemi-glutarate (PTXGL, **17**) with an equivalent of dPEG24 amino acid **18** to yield the soluble drug moiety **19**. Compound **19** was then conjugated to the dipeptide segment **16** to afford the highly water-soluble target conjugate **20**. All the intermediate and the final product were identified by mass spectroscopic analysis.

Example 3: Cytotoxicity Screening

The cytotoxic efficacy of the SDML conjugate **5**, (PTX-2'-E(YBBN[7-14])₂) was tested in a

number of different human cancer cell lines that express the bombesin receptor (the target for the YBBN[7-14] targeting elements. The cell lines included FADU, SKNAS, JNPSRLT, and HUTU-80, human carcinoma cell lines (obtained from the commercial sources indicated). These cell lines were maintained in culture conditions as known in the art. Cytotoxicity was determined as a measure of cell survival through the following protocol.

In these experiments, PTX was used as a reference compound. The cells were incubated in 96-well plates, and in separate groups, with the SDML conjugate **5** or PTX alone for 24 hours, at which time the treatments were removed by washing and aspirating. The surviving cell populations were counted on the fourth day after treatment (96 hour post-treatment). All counts were normalized against an untreated control which represented 100% survival. The results of these assays are shown in Table 1. As can be seen, these experiments determined a superior performance by the SDML conjugate **5** as compared to unconjugated PTX in all treated cell lines. Results are expressed as the percentage of viable cells on the fourth day after treatment.

Furthermore, a comparison of the cytotoxic activity of the SDML conjugate **5** with the cytotoxic activity of the PTX-*mono*-BBN[7-14] conjugates (in other words, a SDSL conjugate containing only 1 copy of the YBBN[7-14] peptide as the targeting element) indicated higher cell-killing activity by the SDML conjugate **5** (PTX-2'-E(YBBN[7-14])₂) in all human cancer cell lines tested.

Example 4: Antitumor Activity of PTX-2'-E(YBBN[7-14])₂

To assess the antitumor efficacy of the SDML conjugate in animal models, three groups of male nude mice (N=8 per group) were subcutaneously inoculated with the human prostate carcinoma cell line PC-3, used as a model cell line. When tumors reached a diameter of approximately 7 mm, the mice were injected with saline (mice 1-8), PTX (mice 9-17) and the SDML conjugate **5** (PTX-2'-E(YBBN[7-14])₂) (mice 18-24). Due to the insolubility of PTX and conjugate **5**, dry dimethyl sulfoxide (DMSO) was used as the delivery vehicle for conjugate **5** and PTX. A total of five intraperitoneal injections were given at three day intervals. The product of two diameters of each tumor, measured by calipers every third day, was used as a measure of tumor growth.

As can be seen in FIG. 2, the SDML conjugate **5** (PTX-2'-E(YBBN[7-14])₂) reduced tumor size in a mouse xenograft model in which nude mice were inoculated with tumor cells from a human prostate carcinoma cell line and provides an improved approach to drug delivery and treatment of a disease state and conditions in comparison to that obtained from treatment with a therapeutic agent alone. These results show that specific delivery of a therapeutic agent by a plurality of targeting elements enhances the effects of the therapeutic agent and serves as an improved method for drug delivery.

FIG. 2 shows the tumor growth in the control and the treated groups. On day 32 post-inoculation, the untreated (saline injected) and the PTX- injected control groups showed, respectively, a

350% and a 68% increase in their tumor sizes. In contrast, the mice injected with conjugate 5 had an 18% reduction in tumor size. This result indicates a surprising increase in antitumor activity using the SDML conjugates of the present disclosure.

As indicated, the SDML conjugate used in mouse xenograft study, was the water-insoluble conjugate 5. Even higher antitumor activities may be expected with the SDML conjugates containing one or more solubilizing elements (e.g., PEG or carbohydrates). This is based on the assumption that the water-insoluble compounds may partially precipitate out of the circulation, and therefore, may not reach the tumor at the originally delivered concentration. This means that FIG 4 may show the activity of only a fraction of the administered dose of the SDML conjugate 5. The PTX, although also insoluble, may have a higher relative retained concentration due to its significantly smaller molecular size, and a lower lipophilicity, as compared to conjugate 5.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. The appended claim is attached solely for the purposes of foreign priority, if required.

One skilled in the art will appreciate readily that the present disclosure is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are representative of certain embodiments, are exemplary, and are not intended as limitations on the scope of the disclosure.

CLAIMS

What is claimed:

1. A single-drug, multi-ligand conjugate comprising one treatment molecule and two or more targeting elements functionally linked together, said targeting elements directing said conjugate to a target cell.
2. The conjugate of claim 1 where chemically reactive groups on said treatment molecule or said targeting elements are used to functionally link the treatment molecule and the targeting elements.
3. The conjugate of claim 1 further comprising a linking molecule disposed between said treatment molecule and said targeting elements, said linking molecule functionally linking the treatment molecule and the targeting elements.
4. The conjugate of claim 3 where said linking molecule is an amino acid.
5. The conjugate of claim 3 where said linking molecule is glutaric acid or succinic acid.
6. The conjugate of claim 1 further comprising a solubilizing element.
7. The conjugate of claim 3 where said linking is accomplished by an amide, amine, ester, ether, thioether, sulfide, disulfide, hemiacetal, acetal, ketal, hydrazide, or hydrazone linkage.
8. The conjugate of claim 6 where said solubilizing element is polyethylene glycol or a carbohydrate moiety.
9. The conjugate of claim 6 where said solubilizing element is a charged molecule such as a salt.
10. The conjugate of claim 6 where said solubilizing element is an amino acid, a peptide, a natural polymer, or a synthetic polymer.
11. The conjugate of claim 6 where said solubilizing element is water soluble.
12. The conjugate of claim 3 further comprising at least one solubilizing element.
13. The conjugate of claim 12 where said solubilizing element is polyethylene glycol or a carbohydrate moiety.
14. The conjugate of claim 12 where said solubilizing element is a charged molecule such as a salt.
15. The conjugate of claim 12 where said solubilizing element is an amino acid, a peptide, a natural polymer, or a synthetic polymer.
16. The conjugate of claim 12 where said solubilizing element is water soluble.
17. The conjugate of claim 1 where the targeting elements are independently selected from the group consisting of: a SMP, a peptide, a receptor ligand peptide, a receptor ligand protein, a DNA-directed molecule and a carbohydrate.
18. The conjugate of claim 17 where the targeting element is a SMP.
19. The conjugate of claim 17 where said SMP is selected from the group consisting of: a bombesin/gastrin-releasing peptide receptor-recognizing peptide, a

somatostatin receptor recognizing peptide, and an epidermal growth factor receptor recognizing peptide.

20. The conjugate of claim 17 where said SMP has the sequence shown in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 and SEQ ID NO. 4.

5 21. The conjugate of claim 1 wherein the treatment molecule is a diagnostic agent or a therapeutic agent.

22. The conjugate of claim 21 where the therapeutic agent is selected from the group consisting of: a drug, an anti-tumor agent, a cytotoxic agent, a radionucleotide and a metallic nucleus.

23. The conjugate of claim 21 where the therapeutic agent is a taxane.

10 24. The conjugate of claim 21 where the therapeutic agent is paclitaxel or docetaxel.

25. The conjugate of claim 20 where the therapeutic agent is radionucleotide selected from the group consisting of: ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{88}Y , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{123}I , ^{125}I , ^{131}I , ^{177}Lu , ^{186}Re , and ^{188}Re .

15 26. The conjugate of claim 21 where the diagnostic agent is a fluorescence label, a radiolabel, an enzymatic label, a metallic contrast agent, or a quantum dot label.

27. The conjugate of claim 21 where the diagnostic agent is a fluorescence label selected from the group consisting of: fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow.

20 28. The conjugate of claim 21 where the diagnostic agent is a radiolabel selected from the group consisting of: ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{88}Y , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{123}I , ^{125}I , ^{131}I , ^{177}Lu , ^{186}Re , and ^{188}Re .

29. The conjugate of claim 21 where the diagnostic agent is an enzymatic label selected from the group consisting of: β -glucuronidase, β -D-glucosidase, β -D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase.

25 30. The conjugate of claim 21 where the diagnostic agent is a metallic contrast label selected from the group consisting of: gadolinium, manganese, iron and a derivative of any of the foregoing.

31. The conjugate of claim 3 where the targeting elements are independently selected from the group consisting of: a SMP, a peptide, a receptor ligand peptide, a receptor ligand protein, a DNA-directed molecule and a carbohydrate.

30 32. The conjugate of claim 31 where the targeting element is a SMP.

33. The conjugate of claim 31 where said SMP is selected from the group consisting of: a bombesin/gastrin-releasing peptide receptor-recognizing peptide, a somatostatin receptor recognizing peptide, and an epidermal growth factor receptor recognizing peptide.

35 34. The conjugate of claim 31 where said SMP has the sequence shown in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 and SEQ ID NO. 4.

35. The conjugate of claim 3 wherein the treatment molecule is a diagnostic agent or a therapeutic agent.
36. The conjugate of claim 35 where the therapeutic agent is selected from the group consisting of: a drug, an anti-tumor agent, a cytotoxic agent, a radionucleotide and a metallic nucleus.
- 5 37. The conjugate of claim 35 where the therapeutic agent is a taxane.
38. The conjugate of claim 35 where the therapeutic agent is paclitaxel or docetaxel.
39. The conjugate of claim 35 where the therapeutic agent is radionucleotide selected from the group consisting of: ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{88}Y , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{123}I , ^{125}I , ^{131}I , ^{177}Lu , ^{186}Re , and ^{188}Re .
- 10 40. The conjugate of claim 35 where the diagnostic agent is a fluorescence label, a radiolabel, an enzymatic label, a metallic contrast agent, or a quantum dot label.
41. The conjugate of claim 35 where the diagnostic agent is a fluorescence label selected from the group consisting of: fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow.
- 15 42. The conjugate of claim 35 where the diagnostic agent is a radiolabel selected from the group consisting of: ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{88}Y , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{123}I , ^{125}I , ^{131}I , ^{177}Lu , ^{186}Re , and ^{188}Re .
43. The conjugate of claim 35 where the diagnostic agent is an enzymatic label selected from the group consisting of: β -glucuronidase, β -D-glucosidase, β -D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase.
- 20 44. The conjugate of claim 35 where the diagnostic agent is a metallic contrast label selected from the group consisting of: gadolinium, manganese, iron and a derivative of any of the foregoing.
45. The conjugate of claim 1 where each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide and the treatment molecule is paclitaxel or docetaxel.
- 25 46. The conjugate of claim 12 where each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide, the treatment molecule is paclitaxel or docetaxel, the linking molecule is glutaric acid or succinic acid and the solubilizing element is one molecule of PEG.
- 30 47. The conjugate of claim 46 where the solubilizing element is attached to any functional group of the linker, the treatment molecule or the targeting elements.
48. The conjugate of claim 12 where each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide, the treatment molecule is paclitaxel or docetaxel and the solubilizing elements are two or more molecules of PEG.
- 35 49. The conjugate of claim 48 where the solubilizing element is attached to any

functional group of the linker, the treatment molecule or the targeting elements.

50. A method of treating an individual having a disease state or condition comprising the step of administering to a subject in need of such treatment a therapeutically effective amount of the conjugate of any one of claims 1-48 in an amount sufficient to treat the disease state or condition.

5

51. The method of claim 50 where the targeting element and the treatment molecule are selected based on the disease state or condition to be treated.

52. The method of claim 50 where the disease is an infection or a condition involving the hyper-proliferation of cells.

- 10 53. The method of claim 52 where said infection is caused by a bacterium, a parasite or a virus.

54. The method of claim 52 where said condition involving the hyper-proliferation of cells is an inflammatory condition, an autoimmune condition, restonosis, atherosclerosis or cancer.

55. The method of claim 50 where the conjugate has the following composition: each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide; the treatment molecule is paclitaxel or docetaxel; and the linking molecule is glutaric acid or succinic acid.

56. The method of claim 50 where the conjugate has the following composition: each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide; the treatment molecule is paclitaxel or docetaxel; the linking molecule is glutaric acid or succinic acid; and the solubilizing element is one molecule of PEG.

57. The method of claim 50 where the conjugate has the following composition: each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide; the treatment molecule is paclitaxel or docetaxel; the linking molecule is glutaric acid or succinic acid; and the solubilizing elements are two or more molecules of PEG.

- 25 58. A method of diagnosing a subject suspected of having a disease state or condition comprising the step of administering to a subject in need of such diagnosis a therapeutically effective amount of the conjugate of any one of claims 1-48 in an amount sufficient to treat the disease state or condition.

59. The method of claim 58 where the targeting element and the treatment molecule are selected based on the disease state or condition to be diagnosed.

60. The method of claim 58 where the disease is an infection or a condition involving the hyper-proliferation of cells.

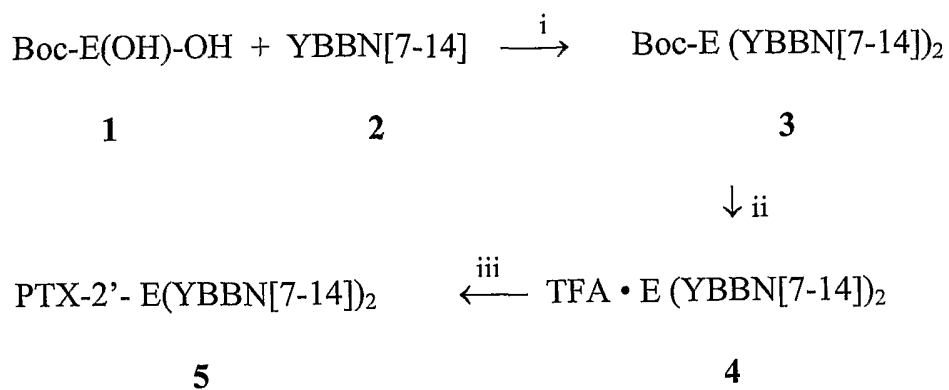
61. The method of claim 60 where said infection is caused by a bacterium, a parasite or a virus.

62. The method of claim 60 where said condition involving the hyper-proliferation of cells is an

inflammatory condition, an autoimmune condition, restenosis, atherosclerosis or cancer.

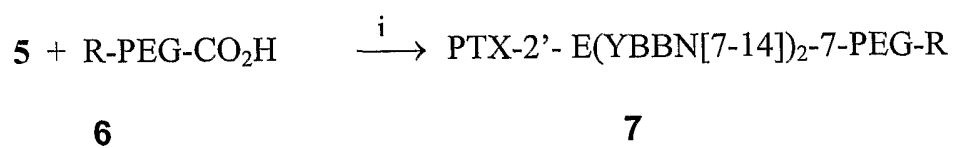
63. The method of claim 58 where the conjugate has the following composition: each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide; the treatment molecule is paclitaxel or docetaxel; and the linking molecule is glutaric acid or succinic acid.
64. The method of claim 58 where the conjugate has the following composition: each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide; the treatment molecule is paclitaxel or docetaxel; the linking molecule is glutaric acid or succinic acid; and the solubilizing element is one molecule of PEG.
65. The method of claim 58 where the conjugate has the following composition: each targeting element is independently selected from the group consisting of the YBBN[7-14] peptide and the YBBN [7-13] peptide; the treatment molecule is paclitaxel or toxotere; the linking molecule is glutaric acid or succinic acid; and the solubilizing elements are two or more molecules of PEG.

FIG. 1A



(i) HOBT, DCC, DMF, RT; (ii) TFA, DCM; (iii) NHS, EEDQ, DMF.

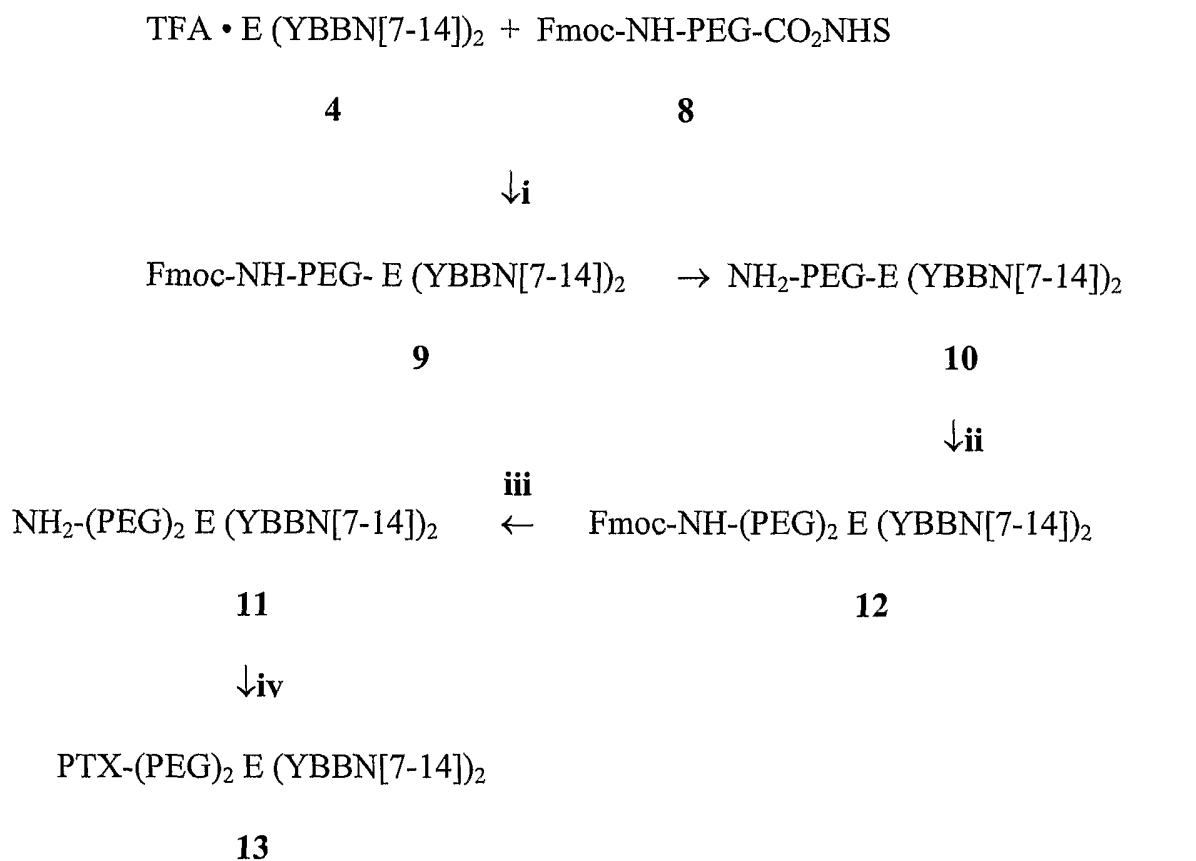
FIG. 1B



R = Trifluoroacetyl

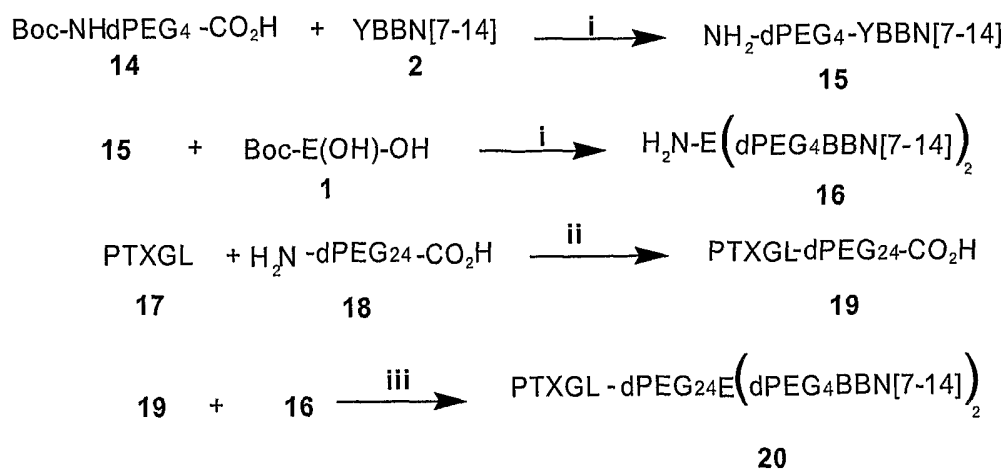
(i) EDC, DCM, 0 °C

FIG. 1C



(i) DIEA, DMF, then piperidine/DMF to **10**; (ii) **8**, DMF, DIEA; (iii) piperidien/DMF; PTX, NHS, EEDQ, DMF.

FIG. 1D



(i) 1: HOBT, DCC, DMF ; 2: TFA ; (ii) 1: NHS, EEDQ + 18, 2: 19 ; (iii) HOBT, EDC, THF, CHCl₃.

FIG. 1E

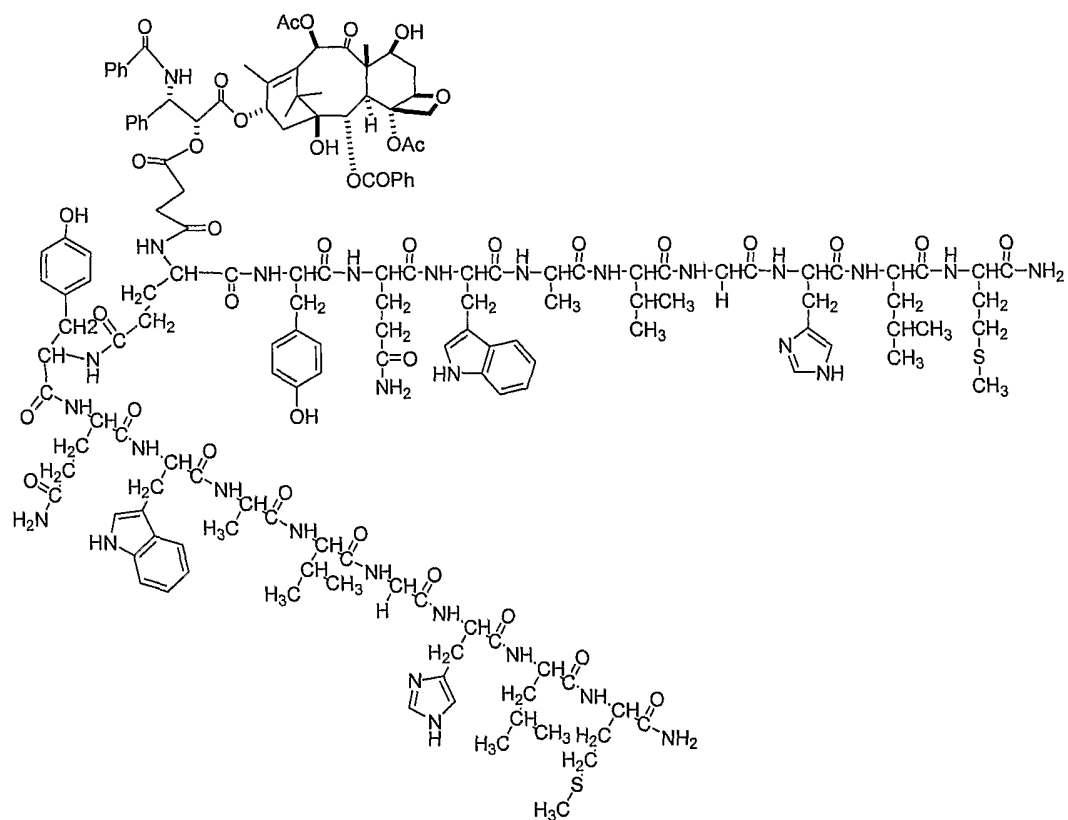


FIG. 2

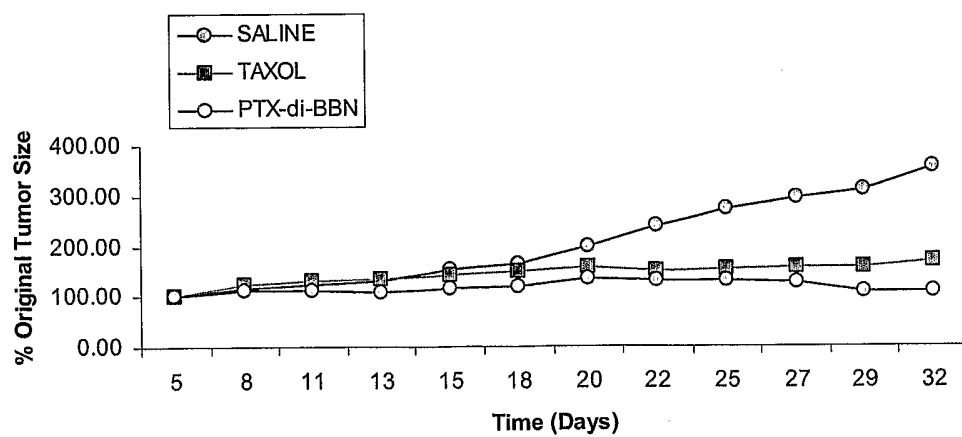


Table 1. Cytotoxicity of PTX-2'-E(YBBN[7-14])₂ SDML conjugate, PTX-BBN[7-14] SDSL conjugate and PTX in selected human carcinoma cell lines. Results are expressed as a percentage of viable cells after treatment as described. Untreated controls represented 100% survival.

Cell Line	Dose (ng)	PTX	PTXYBBN[7-14]	PTXE(YBBN[7-14]) ₂
FADU	100	15-36%	73%	11%
	200			
SKNAS	100	42-49%	78% 46%	20%
	200			
JNPSRLT	100	72% 12%	0%	18% 6%
	200			
HUTU-80	100	15-26%	84%	19% 11%
	200			

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