

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 November 2007 (29.11.2007)

PCT

(10) International Publication Number
WO 2007/137170 A2

(51) International Patent Classification:
A61K 39/395 (2006.01)

(21) International Application Number:
PCT/US2007/069262

(22) International Filing Date: 18 May 2007 (18.05.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/802,073 20 May 2006 (20.05.2006) US

(71) Applicant (for all designated States except US): **SEATTLE GENETICS, INC.** [US/US]; 21823 30th Drive SE, Bothell, Washington 98021 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SMITH, Maria Leia** [US/US]; c/o Seattle Genetics, Inc., 21823 30th Drive SE, Bothell, Washington 98021 (US). **DRACHMAN, Jonathan** [US/US]; c/o Seattle Genetics, Inc., 21823 30th Drive SE, Bothell, Washington 98021 (US). **CARTER, Paul** [US/US]; c/o Seattle Genetics, Inc., 21823 30th Drive SE, Bothell, Washington 98021 (US).

(74) Agents: **WOODCOCK WASHBURN LLP** et al.; Cira Centre, 2929 Arch Street, 12th Floor, Philadelphia, Pennsylvania 19104-2891 (US).

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

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

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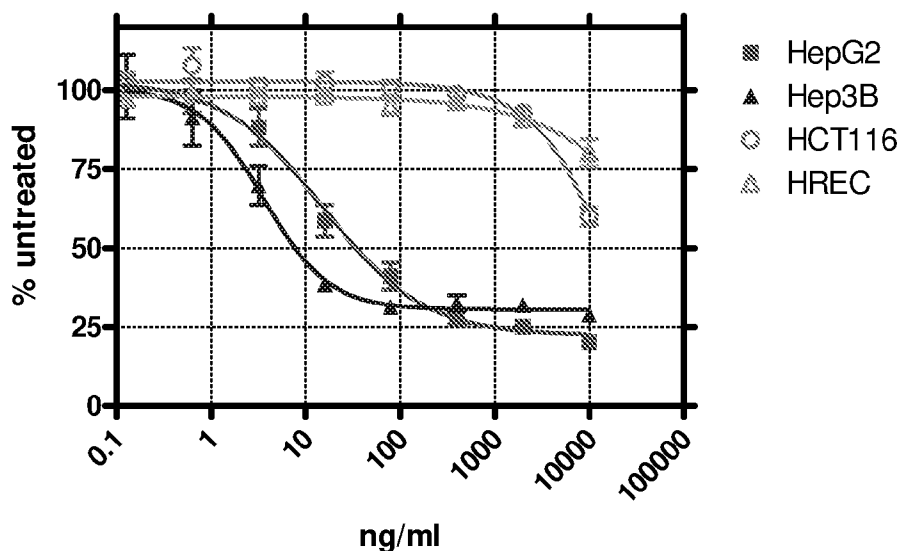
— of inventorship (Rule 4.17(iv))

Published:

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

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

(54) Title: ANTI-GLYPICAN-3 ANTIBODY DRUG CONJUGATES



(57) Abstract: The present invention provides ligand drug conjugates for targeted delivery of drugs to cells expressing GPC3. The ligand drug conjugates have potent cytotoxic activity against antigen-specific targets.

ANTI-GLYPICAN-3 ANTIBODY DRUG CONJUGATES

CONTINUITY

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 60/802,073, filed May 20, 2006, the disclosure of which is incorporated by reference herein in its entirety.

BACKGROUND

[0002] Antibodies are the most rapidly expanding class of therapeutics including for cancer therapy. Indeed, eight antibodies are now approved for oncologic indications in the United States, including RITUXAN (rituximab), HERCEPTIN (trastuzumab) and CAMPATH (alemtuzumab) for non-Hodgkin's lymphoma, metastatic breast cancer and B cell chronic lymphocytic leukemia, respectively. These new drugs provide significant benefits to some patients, but fall well short of the ultimate goal of curing cancer. This significant progress with antibody therapeutics has revitalized interest in strategies to improve the rates and duration of their antitumor responses.

[0003] Antibody conjugation to cytotoxic drugs is a promising way to enhance the antitumor activity of antibodies and reduce the systemic toxicity of drugs (reviewed by Lambert, 2005, *Curr. Opin. Pharmacol.* 5:543-549; and Wu and Senter, 2005, *Nat. Biotechnol.* 23:1137-1146). Antibody drug conjugates (ADCs) can provide target specific delivery of drug to certain target cells, followed by internalization and release of the drug inside the cell. Certain classes of targets, however, are more problematic to target. For example, GPI linked proteins can be more challenging targets for ADCs because the target can be internalized via different pathways, including the lysozomal and caveolar pathways. The efficiency of drug release from the antibody can depend on the internalization pathway.

[0004] There remains a need, therefore, for antibody drug conjugates that deliver cytotoxic amounts of drugs to cells via GPI-linked targets. This and other limitations and problems of the past are solved by the present invention. (The

recitation of any reference in this application is not an admission that the reference is prior art to this application.)

BRIEF SUMMARY

[0005] The present invention provides ligand drug conjugate compounds for targeted delivery of drugs to cells expressing Glypican-3 (GPC-3). The ligand drug conjugates compounds have potent cytotoxic activity against GPC-3-expressing target cells. The ligand drug conjugate compounds comprise at least one Ligand unit that binds to an epitope of GPC-3. One or more Drug units can be covalently linked to the Ligand unit moiety. The Drug units can be covalently linked directly or via a Linker unit (-LU-).

[0006] The invention will best be understood by reference to the following detailed description, taken in conjunction with the accompanying drawings. The discussion below is descriptive, illustrative and exemplary and is not to be taken as limiting the scope defined by any appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] Figure 1. **A**, Hep G2 cells were grown in 96 well plates and anti-Glypican-3 mAbs (1G12, 8H5) or control mouse IgG plus secondary anti-mouse Ab drug conjugate were added for 96 hours. Cell viability was measured using resazurin dye conversion. **B**, Hep3B cells were treated with the anti-Glypican-3 mAbs plus the secondary ADC, as describe above for Figure 1A.

[0008] Figure 2. Differential sensitivity of cells to anti-Glypican-3 drug conjugate (1G12-vcMMAF). HepG2 and Hep3B are GPC-3 positive hepatocellular carcinoma cell lines, HCT116 (colorectal cancer cell line) and HREC (normal human renal epithelial cells) are GPC-3 negative. Cells were incubated with 1G12-vcMMAF for 96 hours and cell viability measured using resazurin dye conversion.

DETAILED DESCRIPTION

[0009] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

Definitions

[0010] Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings. When a trade name is used herein, the trade name also refers to the associated product formulation, the generic drug, and the active pharmaceutical ingredient(s) of the trade name product, unless otherwise indicated by context.

[0011] The term “antibody” herein is used in the broadest sense and refers to intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and to antibody fragments that exhibit the desired biological activity (*e.g.*, antigen-binding). The antibody can be of any type or class (*e.g.*, IgG, IgE, IgM, IgD, and IgA) or sub-class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2).

[0012] An “intact” antibody is one which comprises heavy and light chain antigen-binding variable regions (V_H and V_L , respectively) as well as a light chain constant domain (C_L) and heavy chain constant domains, C_{H1} , C_{H2} , C_{H3} , and/or C_{H4} , as appropriate for the antibody class. The constant domains may be native sequence constant domains (*e.g.*, human native sequence constant domains) or amino acid sequence variants thereof.

[0013] “Single-chain Fv” or “scFv” antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain (*e.g.*, as V_H - V_L or V_L - V_H). The F_v polypeptide typically further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0014] The term “diabody” refers to a small antibody fragment with two antigen-binding sites, which fragments comprise a variable heavy domain (V_H) connected to a variable light domain (V_L) in the same polypeptide chain. By using a linker that is too short to allow pairing between the two domains (V_H and V_L) of the same chain, the domains are forced to pair with the complementary domains of

another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 0 404 097; WO 93/11161; and Hollinger *et al.*, 1993, *Proc. Natl. Acad. Sci. USA* 90:6444-6448. The two antigen-binding sites can be the same or different.

[0015] An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, or to greater than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody’s natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

[0016] An antibody or antibody fragment “which binds” an antigen of interest is one capable of binding that antigen with sufficient affinity such that the antibody is useful in targeting a cell expressing the antigen.

[0017] As used herein, “specific binding” and “specifically binds” refer to antibody binding to a predetermined antigen (i.e., glypican-3). Typically, the antibody binds with an affinity of at least about $1 \times 10^7 \text{ M}^{-1}$, and binds to the predetermined antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen.

[0018] The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which specifically binds to an antigen".

[0019] The term "effective amount" refers to an amount of a drug (*e.g.*, a ligand drug conjugate) effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

[0020] The terms "target polypeptide" or "target antigen" refer to a polypeptide expressed by a target cell.

[0021] The term "compound" refers to and encompasses the chemical compound itself as well as, whether explicitly stated or not, and unless the context makes clear that the following are to be excluded: amorphous and crystalline forms of the compound, including polymorphic forms, where these forms may be part of a mixture or in isolation; free acid and free base forms of the compound, which are typically the forms shown in the structures provided herein; isomers of the compound, which refers to optical isomers, and tautomeric isomers, where optical isomers include enantiomers and diastereomers, chiral isomers and non-chiral isomers, and the optical isomers include isolated optical isomers as well as mixtures of optical isomers including racemic and non-racemic mixtures; where an isomer may be in isolated form or in admixture with one or more other isomers; isotopes of the compound, including deuterium- and tritium-containing compounds, and including compounds containing radioisotopes, including therapeutically- and diagnostically-

effective radioisotopes; multimeric forms of the compound, including dimeric, trimeric, etc. forms; salts of the compound, preferably pharmaceutically acceptable salts, including acid addition salts and base addition salts, including salts having organic counterions and inorganic counterions, and including zwitterionic forms, where if a compound is associated with two or more counterions, the two or more counterions may be the same or different; and solvates of the compound, including hemisolvates, monosolvates, disolvates, etc., including organic solvates and inorganic solvates, said inorganic solvates including hydrates; where if a compound is associated with two or more solvent molecules, the two or more solvent molecules may be the same or different. In some instances, reference made herein to a compound of the invention will include an explicit reference to one or of the above forms, *e.g.*, salts and solvates, however, this reference is for emphasis only, and is not to be construed as excluding other of the above forms as identified above.

[0022] The term "alkyl," whether used alone or as part of another group, refers to a C₁-C₁₈ hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms. Examples are methyl (Me, -CH₃), ethyl (Et, -CH₂CH₃), 1-propyl (n-Pr, n-propyl, -CH₂CH₂CH₃), 2-propyl (i-Pr, i-propyl, -CH(CH₃)CH₂CH₃), 1-butyl (n-Bu, n-butyl, -CH₂CH₂CH₂CH₃), 2-butyl (s-Bu, s-butyl, -CH(CH₃)CH₂CH₃), 2-methyl-1-butyl (i-Bu, i-butyl, -CH₂CH(CH₃)CH₂CH₃), 2-methyl-2-butyl (t-Bu, t-butyl, -C(CH₃)₃), 1-pentyl (n-pentyl, -CH₂CH₂CH₂CH₂CH₃), 2-pentyl (-CH(CH₃)CH₂CH₂CH₂CH₃), 3-pentyl (-CH(CH₃)CH₂CH₂CH₃), 2-methyl-2-butyl (-C(CH₃)₂CH₂CH₃), 3-methyl-2-butyl (-CH(CH₃)CH(CH₃)CH₂CH₃), 3-methyl-1-butyl (-CH₂CH₂CH(CH₃)CH₃), 2-methyl-1-butyl (-CH₂CH(CH₃)CH₂CH₃), 1-hexyl (-CH₂CH₂CH₂CH₂CH₂CH₃), 2-hexyl (-CH(CH₃)CH₂CH₂CH₂CH₃), 3-hexyl (-CH(CH₃)CH₂CH₂CH₂CH₃), 2-methyl-2-pentyl (-C(CH₃)₂CH₂CH₂CH₃), 3-methyl-2-pentyl (-CH(CH₃)CH(CH₃)CH₂CH₃), 4-methyl-2-pentyl (-CH(CH₃)CH₂CH(CH₃)CH₃), 3-methyl-3-pentyl (-C(CH₃)(CH₂CH₃)₂), 2-methyl-3-pentyl (-CH(CH₃)CH(CH₃)CH₂CH₃), 2,3-dimethyl-2-butyl (-C(CH₃)₂CH(CH₃)CH₃), and 3,3-dimethyl-2-butyl (-CH(CH₃)C(CH₃)₃).

[0023] The term “C₁-C₈ alkyl,” as used herein, whether used alone or as part of another group, refers to a straight chain or branched, saturated or unsaturated hydrocarbon having from 1 to 8 carbon atoms. Representative “C₁-C₈ alkyl” groups include, but are not limited to, -methyl, -ethyl, -n-propyl, -n-butyl, -n-pentyl, -n-hexyl, -n-heptyl, -n-octyl, -n-nonyl and -n-decyl; while branched C₁-C₈ alkyls include, but are not limited to, -isopropyl, -sec-butyl, -isobutyl, -tert-butyl, -isopentyl, or 2-methylbutyl; unsaturated C₁-C₈ alkyls include, but are not limited to, -vinyl, -allyl, -1-butenyl, -2-butenyl, -isobutylenyl, -1-pentenyl, -2-pentenyl, -3-methyl-1-butenyl, -2-methyl-2-butenyl, -2,3-dimethyl-2-butenyl, 1-hexyl, 2-hexyl, 3-hexyl, -acetylenyl, -propynyl, -1-butyne, -2-butyne, -1-pentyne, -2-pentyne, or -3-methyl-1-butyne; methyl, ethyl, propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, n-hexyl, isohexyl, 2-methylpentyl, 3-methylpentyl, 2,2-dimethylbutyl, 2,3-dimethylbutyl, 2,2-dimethylpentyl, 2,3-dimethylpentyl, 3,3-dimethylpentyl, 2,3,4-trimethylpentyl, 3-methylhexyl, 2,2-dimethylhexyl, 2,4-dimethylhexyl, 2,5-dimethylhexyl, 3,5-dimethylhexyl, 2,4-dimethylpentyl, 2-methylheptyl, 3-methylheptyl, n-heptyl, isoheptyl, n-octyl, and isooctyl. A “C₁-C₈ alkyl” group can be unsubstituted or substituted with one or more groups including, but not limited to, -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH₂, -C(O)NHR', -C(O)N(R')₂, -NHC(O)R', -SO₃R', -S(O)₂R', -S(O)R', -OH, -halogen, -N₃, -NH₂, -NH(R'), -N(R')₂ and -CN; where each R' is independently selected from H, -C₁-C₈ alkyl or aryl. In some embodiments, a “C₁-C₈ alkyl” group is unsubstituted.

[0024] The term “alkenyl,” whether used alone or as part of another group, refers to a C₂-C₁₈ hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms with at least one carbon-carbon, sp² double bond. Examples include, but are not limited to: ethylene or vinyl (-CH=CH₂), allyl (-CH₂CH=CH₂), cyclopentenyl (-C₅H₇), and 5-hexenyl (-CH₂CH₂CH₂CH₂CH=CH₂).

[0025] The term “alkynyl,” whether used alone or as part of another group, refers to a C₂-C₁₈ hydrocarbon containing normal, secondary, tertiary or cyclic carbon

atoms with at least one carbon-carbon, sp triple bond. Examples include, but are not limited to: acetylenic ($-\text{C}\equiv\text{CH}$) and propargyl ($-\text{CH}_2\text{C}\equiv\text{CH}$).

[0026] The term "alkylene," whether used alone or as part of another group, refers to a saturated, branched or straight chain or cyclic hydrocarbon radical of 1-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkane. Typical alkylenes include, but are not limited to: methylene ($-\text{CH}_2-$), 1,2-ethyl ($-\text{CH}_2\text{CH}_2-$), 1,3-propyl ($-\text{CH}_2\text{CH}_2\text{CH}_2-$), 1,4-butyl ($-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$), and the like.

[0027] A " $\text{C}_1\text{-C}_{10}$ alkylene," whether used alone or as part of another group, is a straight chain, saturated hydrocarbon group of the formula $-(\text{CH}_2)_{1-10}-$. Examples of a $\text{C}_1\text{-C}_{10}$ alkylene include methylene, ethylene, propylene, butylene, pentylene, hexylene, heptylene, octylene, nonylene and decalene.

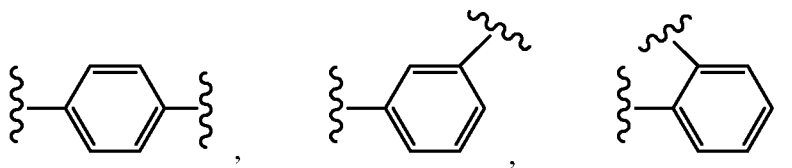
[0028] The term "alkenylene," whether used alone or as part of another group, refers to an unsaturated, branched or straight chain or cyclic hydrocarbon radical of 2-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkene. Typical alkenylene radicals include, but are not limited to: 1,2-ethylene ($-\text{CH}=\text{CH}-$).

[0029] The term "alkynylene," whether used alone or as part of another group, refers to an unsaturated, branched or straight chain or cyclic hydrocarbon radical of 2-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkyne. Typical alkynylene radicals include, but are not limited to: acetylene ($-\text{C}\equiv\text{C}-$), propargyl ($-\text{CH}_2\text{C}\equiv\text{C}-$), and 4-pentynyl ($-\text{CH}_2\text{CH}_2\text{CH}_2\text{C}\equiv\text{CH}-$).

[0030] The term "aryl," whether used alone or as part of another group, refers to a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic

ring system. Some aryl groups are represented in the exemplary structures as "Ar". An aryl group can be unsubstituted or substituted. Typical aryl groups include, but are not limited to, radicals derived from benzene, substituted benzene, phenyl, naphthalene, anthracene, biphenyl, and the like. An aryl can be substituted with one or more groups including, but not limited to, -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH₂, -C(O)NHR', -C(O)N(R')₂, -NHC(O)R', -S(O)₂R', -S(O)R', -OH, -halogen, -N₃, -NH₂, -NH(R'), -N(R')₂ and -CN; wherein each R' is independently selected from H, -C₁-C₈ alkyl or aryl.

[0031] The term "arylene," whether used alone or as part of another group, refers to an aryl group which has two covalent bonds and can be in the ortho, meta, or para configurations as shown in the following structures with phenyl as the exemplary aryl group:



in which the aryl group (*e.g.*, a phenyl group) can be unsubstituted or substituted with up to four groups including, but not limited to, -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH₂, -C(O)NHR', -C(O)N(R')₂, -NHC(O)R', -S(O)₂R', -S(O)R', -OH, -halogen, -N₃, -NH₂, -NH(R'), -N(R')₂ and -CN; wherein each R' is independently selected from H, -C₁-C₈ alkyl or aryl.

[0032] The term "arylalkyl," refers to an acyclic alkyl radical in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or sp³ carbon atom, is replaced with an aryl radical. Typical arylalkyl groups include, but are not limited to, benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl, 2-naphthylethan-1-yl, 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like. The arylalkyl group comprises 6 to 20 carbon atoms, *e.g.*, the alkyl moiety, including alkanyl, alkenyl or alkynyl groups, of the arylalkyl group is 1 to 6 carbon atoms and the aryl moiety is 5 to 14 carbon atoms.

[0033] The terms "substituted alkyl", "substituted aryl", and "substituted arylalkyl" refer to alkyl, aryl, and arylalkyl, respectively, in which one or more hydrogen atoms are each independently replaced with a substituent. Typical substituents include, but are not limited to, -X, -R, -O⁻, -OR, -SR, -S⁻, -NR₂, -NR₃, =NR, -CX₃, -CN, -OCN, -SCN, -N=C=O, -NCS, -NO, -NO₂, =N₂, -N₃, NC(=O)R, -C(=O)R, -C(=O)NR₂, -SO₃⁻, -SO₃H, -S(=O)₂R, -OS(=O)₂OR, -S(=O)₂NR, -S(=O)R, -OP(=O)(OR)₂, -P(=O)(OR)₂, -PO₃⁻, -PO₃H₂, -C(=O)R, -C(=O)X, -C(=S)R, -CO₂R, -CO₂⁻, -C(=S)OR, -C(=O)SR, -C(=S)SR, -C(=O)NR₂, -C(=S)NR₂, or -C(=NR)NR₂, where each X is independently a halogen: F, Cl, Br, or I; and each R is independently -H, C₂-C₁₈ alkyl, C₆-C₂₀ aryl, C₃-C₁₄ heterocycle, a protecting group or a prodrug moiety. Alkylene, alkenylene, and alkynylene groups as described above may also be similarly substituted.

[0034] The term "heterocycle," whether used alone or as part of another group, refers to a ring system in which one or more ring atoms is a heteroatom, *e.g.*, nitrogen, oxygen, and sulfur. The heterocycle radical comprises 1 to 20 carbon atoms and 1 to 4 heteroatoms selected from N, O, P, and S. A heterocycle may be a monocycle, preferably having 3 to 7 ring members (2 to 6 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S) or a bicycle, preferably having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system. Heterocycles are described in Paquette, "Principles of Modern Heterocyclic Chemistry" (W.A. Benjamin, New York, 1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28; and *J. Am. Chem. Soc.* 82:5566 (1960).

[0035] Examples of heterocycles include by way of example and not limitation pyridyl, dihydropyridyl, tetrahydropyridyl (piperidyl), thiazolyl, tetrahydrothiophenyl, sulfur oxidized tetrahydrothiophenyl, pyrimidinyl, furanyl, thienyl, pyrrolyl, pyrazolyl, imidazolyl, tetrazolyl, benzofuranyl, thianaphthalenyl, indolyl, indolenyl, quinolinyl, isoquinolinyl, benzimidazolyl, piperidinyl, 4-piperidonyl,

pyrrolidinyl, 2-pyrrolidonyl, pyrrolinyl, tetrahydrofuranyl, bis-tetrahydrofuranyl, tetrahydropyranyl, bis-tetrahydropyranyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, decahydroquinolinyl, octahydroisoquinolinyl, azocinyl, triazinyl, 6H-1,2,5-thiadiazinyl, 2H,6H-1,5,2-dithiazinyl, thienyl, thianthrenyl, pyranyl, isobenzofuranyl, chromenyl, xanthenyl, phenoxathinyl, 2H-pyrrolyl, isothiazolyl, isoxazolyl, pyrazinyl, pyridazinyl, indolizinyl, isoindolyl, 3H-indolyl, 1H-indazolyl, purinyl, 4H-quinolizinyl, phthalazinyl, naphthyridinyl, quinoxalinyl, quinazolinyl, cinnolinyl, pteridinyl, 4aH-carbazolyl, carbazolyl, β -carbolinyl, phenanthridinyl, acridinyl, pyrimidinyl, phenanthrolinyl, phenazinyl, phenothiazinyl, furazanyl, phenoxazinyl, isochromanyl, chromanyl, imidazolidinyl, imidazolynyl, pyrazolidinyl, pyrazolynyl, piperazinyl, indolynyl, isoindolynyl, quinuclidinyl, morpholynyl, oxazolidinyl, benzotriazolyl, benzisoxazolyl, oxindolyl, benzoxazolynyl, and isatinoyl.

[0036] By way of example and not limitation, carbon-bonded heterocycles are bonded at the following positions: position 2, 3, 4, 5, or 6 of a pyridine; position 3, 4, 5, or 6 of a pyridazine; position 2, 4, 5, or 6 of a pyrimidine; position 2, 3, 5, or 6 of a pyrazine; position 2, 3, 4, or 5 of a furan, tetrahydrofuran, thiofuran, thiophene, pyrrole or tetrahydropyrrole; position 2, 4, or 5 of an oxazole, imidazole or thiazole; position 3, 4, or 5 of an isoxazole, pyrazole, or isothiazole; position 2 or 3 of an aziridine; position 2, 3, or 4 of an azetidine; position 2, 3, 4, 5, 6, 7, or 8 of a quinoline; or position 1, 3, 4, 5, 6, 7, or 8 of an isoquinoline. Still more typically, carbon bonded heterocycles include 2-pyridyl, 3-pyridyl, 4-pyridyl, 5-pyridyl, 6-pyridyl, 3-pyridazinyl, 4-pyridazinyl, 5-pyridazinyl, 6-pyridazinyl, 2-pyrimidinyl, 4-pyrimidinyl, 5-pyrimidinyl, 6-pyrimidinyl, 2-pyrazinyl, 3-pyrazinyl, 5-pyrazinyl, 6-pyrazinyl, 2-thiazolyl, 4-thiazolyl, or 5-thiazolyl.

[0037] By way of example and not limitation, nitrogen bonded heterocycles are bonded at position 1 of an aziridine, azetidine, pyrrole, pyrrolidine, 2-pyrroline, 3-pyrroline, imidazole, imidazolidine, 2-imidazoline, 3-imidazoline, pyrazole, pyrazoline, 2-pyrazoline, 3-pyrazoline, piperidine, piperazine, indole, indoline, or 1H-indazole; position 2 of a isoindole, or isoindoline; position 4 of a morpholine; and position 9 of a

carbazole, or β -carboline. Still more typically, nitrogen bonded heterocycles include 1-aziridyl, 1-azetetyl, 1-pyrrolyl, 1-imidazolyl, 1-pyrazolyl, and 1-piperidinyl.

[0038] A "C₃-C₈ heterocycle," whether used alone or as part of another group, refers to an aromatic or non-aromatic C₃-C₈ carbocycle in which one to four of the ring carbon atoms are independently replaced with a heteroatom from the group consisting of O, S and N. Representative examples of a C₃-C₈ heterocycle include, but are not limited to, benzofuranyl, benzothiophene, indolyl, benzopyrazolyl, coumarinyl, isoquinolinyl, pyrrolyl, thiophenyl, furanyl, thiazolyl, imidazolyl, pyrazolyl, triazolyl, quinolinyl, pyrimidinyl, pyridinyl, pyridonyl, pyrazinyl, pyridazinyl, isothiazolyl, isoxazolyl and tetrazolyl. A C₃-C₈ heterocycle can be unsubstituted or substituted with up to seven groups including, but not limited to, -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH₂, -C(O)NHR', -C(O)N(R')₂, -NHC(O)R', -S(O)₂R', -S(O)R', -OH, -halogen, -N₃, -NH₂, -NH(R'), -N(R')₂ and -CN; wherein each R' is independently selected from H, -C₁-C₈ alkyl or aryl.

[0039] "C₃-C₈ heterocyclo," whether used alone or as part of another group, refers to a C₃-C₈ heterocycle group defined above wherein one more of the heterocycle group's hydrogen atoms is replaced with a bond. A C₃-C₈ heterocyclo can be unsubstituted or substituted with up to six groups including, but not limited to, -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH₂, -C(O)NHR', -C(O)N(R')₂, -NHC(O)R', -S(O)₂R', -S(O)R', -OH, -halogen, -N₃, -NH₂, -NH(R'), -N(R')₂ and -CN; wherein each R' is independently selected from H, -C₁-C₈ alkyl or aryl.

[0040] The term "carbocycle," whether used alone or as part of another group, refers to a saturated or unsaturated ring having 3 to 7 carbon atoms as a monocycle or 7 to 12 carbon atoms as a bicycle. Monocyclic carbocycles have 3 to 6 ring atoms, still more typically 5 or 6 ring atoms. Bicyclic carbocycles have 7 to 12 ring atoms, e.g., arranged as a bicyclo [4,5], [5,5], [5,6] or [6,6] system, or 9 or 10 ring atoms arranged as a bicyclo [5,6] or [6,6] system. Examples of monocyclic carbocycles include cyclopropyl, cyclobutyl, cyclopentyl, 1-cyclopent-1-enyl, 1-

cyclopent-2-enyl, 1-cyclopent-3-enyl, cyclohexyl, 1-cyclohex-1-enyl, 1-cyclohex-2-enyl, 1-cyclohex-3-enyl, cycloheptyl, and cyclooctyl.

[0041] A “C₃-C₈ carbocycle,” whether used alone or as part of another group, is a 3-, 4-, 5-, 6-, 7- or 8-membered saturated or unsaturated non-aromatic carbocyclic ring. Representative C₃-C₈ carbocycles include, but are not limited to, -cyclopropyl, -cyclobutyl, -cyclopentyl, -cyclopentadienyl, -cyclohexyl, -cyclohexenyl, -1,3-cyclohexadienyl, -1,4-cyclohexadienyl, -cycloheptyl, -1,3-cycloheptadienyl, -1,3,5-cycloheptatrienyl, -cyclooctyl, and -cyclooctadienyl. A C₃-C₈ carbocycle group can be unsubstituted or substituted with one or more groups including, but not limited to, -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH₂, -C(O)NHR', -C(O)N(R')₂, -NHC(O)R', -S(O)₂R', -S(O)R', -OH, -halogen, -N₃, -NH₂, -NH(R'), -N(R')₂ and -CN; where each R' is independently selected from H, -C₁-C₈ alkyl or aryl.

[0042] A “C₃-C₈ carbocyclo,” whether used alone or as part of another group, refers to a C₃-C₈ carbocycle group defined above wherein one more of the carbocycle groups' hydrogen atoms is replaced with a bond.

[0043] The phrase “pharmaceutically acceptable salt” refers to a pharmaceutically acceptable organic or inorganic salt of a ligand drug conjugate or linker drug conjugate. The conjugates may contain at least one amino group, and accordingly acid addition salts can be formed with the amino group. Exemplary salts include, but are not limited to, sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (*i.e.*, 1,1' methylene bis -(2 hydroxy 3 naphthoate)) salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counterion. The counterion may be any organic or inorganic moiety that stabilizes the charge on the parent

compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Instances where multiple charged atoms are part of the pharmaceutically acceptable salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterion.

[0044] The phrases “pharmaceutically acceptable solvate” or “solvate” refer to an association of one or more solvent molecules and a ligand drug conjugate or linker drug conjugate. Examples of solvents that form pharmaceutically acceptable solvates include, but are not limited to, water, isopropanol, ethanol, methanol, DMSO, ethyl acetate, acetic acid, and ethanolamine.

[0045] Examples of a “patient” or “subject” include, but are not limited to, a human, rat, mouse, guinea pig, monkey, pig, goat, cow, horse, dog, cat, bird and fowl. In an exemplary embodiment, the patient or subject is a human.

[0046] The terms “treat” or “treatment,” unless otherwise indicated by context, refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the development or spread of cancer. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

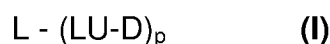
[0047] In the context of cancer, the term “treating” includes any or all of: preventing growth of tumor cells, cancer cells, or of a tumor; preventing replication of tumor cells or cancer cells, lessening of overall tumor burden or decreasing the

number of cancerous cells, and ameliorating one or more symptoms associated with the disease.

Ligand Drug Conjugates

[0048] The present invention provides ligand drug conjugate compounds for targeted delivery of drugs. The inventors have made the surprising discovery that the ligand drug conjugate compounds have potent cytotoxic or cytostatic activity against cells and tissues expressing Glypican-3, a GPI-linked protein. The ligand drug conjugate compounds comprise a Ligand unit covalently linked to at least one Drug unit. The Drug units can be covalently linked directly or via a Linker unit (-LU-).

[0049] In some embodiments, the Ligand Drug conjugate compounds have the following formula:



or a pharmaceutically acceptable salt or solvate thereof; wherein:

L a Ligand unit that specifically binds to an epitope of Glypican-3, and

(LU-D) is a Linker unit-Drug unit moiety, wherein:

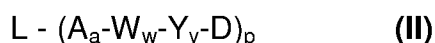
LU- is a Linker unit, and

-D is a Drug unit having cytostatic or cytotoxic activity against a target cell; and

p is an integer from 1 to about 20.

[0050] In some embodiments, p ranges from 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2. In some embodiments, p ranges from 2 to 10, 2 to 9, 2 to 8, 2 to 7, 2 to 6, 2 to 5, 2 to 4 or 2 to 3. In other embodiments, p is 1, 2, 3, 4, 5 or 6. In some embodiments, p is 2 or 4.

[0051] In some embodiments, the Ligand Drug conjugate compounds have the following formula:



or a pharmaceutically acceptable salt or solvate thereof;

wherein:

L a Ligand unit that specifically binds to an epitope of Glypican-3; and

-A_a-W_w-Y_y- is a Linker unit (LU), wherein:

-A- is a Stretcher unit,

a is 0 or 1,

each -W- is independently an Amino Acid unit,

w is an integer ranging from 0 to 12,

-Y- is a self-immolative spacer unit,

y is 0, 1 or 2;

-D is a Drug unit having cytostatic or cytotoxic activity against the target cell; and

p is an integer from 1 to about 20.

[0052] In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0, 1 or 2. In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0 or 1. In some embodiments, p ranges from 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2. In some embodiments, p ranges from 2 to 8, 2 to 7, 2 to 6, 2 to 5, 2 to 4 or 2 to 3. In other embodiments, p is 1, 2, 3, 4, 5 or 6. In some embodiments, p is 2 or 4.

[0053] Each of these units is described in more detail herein.

The Ligand Unit

[0054] A Ligand unit includes within its scope any molecule that binds or reactively associates or complexes with an epitope of Glypican-3. Glypican-3 (GPC-3) has also been referred to as MXR-7. The Glypican-3 is typically a human Glypican-3. The amino acid sequence of Glypican-3 is disclosed as Genbank Accession numbers NP_004475, CAI42277, CAI42761, CAI43110, P51654, AAB58806, AAA98132, AAA93471 and CA86069, and as Unigene Accession No. Hs.567276 (all of the disclosures of which are incorporated by reference herein in their entirety). In some embodiments, the Glypican-3 is a variant, such as those having the amino acid sequence disclosed as Accession numbers BAD96486.1, BAD96481.1, ABC72126.1, AAH35972.1, AAX36128.1, ABC72127.1, ABC72125.1,

Q2L880 (Glypican-3 splice variant B), Q2L882 (Glypican-3 splice variant B), and Q53H15 (Glypican-3 splice variant A); (the disclosures of which are incorporated by reference herein in their entirety).

[0055] In some embodiments, the Ligand unit binds to an epitope of Glypican-3. For example, the Ligand unit can bind to the N-terminal portion of Glypican-3 (*i.e.*, the soluble portion of Glypican-3 released after cleavage, amino acids 1-374 or 1-358 or fragment thereof), or the C-terminal portion of Glypican-3 (*i.e.*, the portion of Glypican-3 remaining bound to the cell membrane after release of the soluble portion, amino acids 375-580 or 359-580). (See, *e.g.*, Capurro et al., 2005, *J. Biol. Chem.* 280: 41201 – 41206.)

[0056] In some embodiments, the Ligand unit is an antibody. Useful polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of immunized animals. Various procedures well known in the art may be used for the production of polyclonal antibodies to GPC-3. For example, for the production of polyclonal antibodies various host animals can be immunized by injection with an antigen of interest or derivative thereof, including but not limited to rabbits, mice, rats, and guinea pigs. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete) adjuvant, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

[0057] In some embodiments, the Ligand unit can be a monoclonal antibody. As used herein, monoclonal antibodies (mAbs) are homogeneous populations of antibodies to a particular antigenic determinant. A monoclonal antibody (mAb) to a target antigen can be prepared by using any technique known in the art. These include, but are not limited to, the hybridoma technique originally described by Köhler and Milstein (1975, *Nature* 256, 495-497), the human B cell

hybridoma technique (Kozbor *et al.*, 1983, *Immunology Today* 4: 72), and the EBV-hybridoma technique (Cole *et al.*, 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and IgD and any subclass thereof. The hybridoma producing the mAbs may be cultivated *in vitro* or *in vivo*.

[0058] Recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques. (See, *e.g.*, Cabilly *et al.*, U.S. Patent No. 4,816,567; and Boss *et al.*, U.S. Patent No. 4,816,397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non-human species having one or more complementarity determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.) Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in International Publication No. WO 87/02671; European Patent Publication No. 0 184 187; European Patent Publication No. 0 171 496; European Patent Publication No. 0 173 494; International Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Publication No. 012 023; Berter *et al.*, 1988, *Science* 240:1041-1043; Liu *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.*, 1987, *J. Immunol.* 139:3521-3526; Sun *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.*, 1987, *Cancer. Res.* 47:999-1005; Wood *et al.*, 1985, *Nature* 314:446-449; Shaw *et al.*, 1988, *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, 1985, *Science* 229:1202-1207; Oi *et al.*, 1986, *BioTechniques* 4:214; U.S. Patent No. 5,225,539; Jones *et al.*, 1986, *Nature* 321:552-525; Verhoeyan *et al.*, 1988, *Science* 239:1534; and Beidler *et al.*, 1988, *J. Immunol.* 141:4053-4060; each of which is incorporated herein by reference in its entirety.

[0059] Human monoclonal antibodies may be made by any of numerous techniques known in the art (*see, e.g.*, Teng *et al.*, 1983, *Proc. Natl. Acad. Sci. USA*.

80:7308-7312; Kozbor *et al.*, 1983, *Immunology Today* 4:72-79; Olsson *et al.*, 1982, *Meth. Enzymol.* 92:3-16; and U.S. Patent Nos. 5,939,598 and 5,770,429).

[0060] Completely human antibodies can be produced using transgenic mice that are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a GPC-3 polypeptide. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, *see, e.g.*, Lonberg and Huszar (1995, *Int. Rev. Immunol.* 13:65-93); and Lonberg (2005, *Nat. Biotechnol.* 23:1117-1125). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see for example*, U.S. Patent Nos. 5,625,126; 5,633,425; 5,569,825; 5,661,016; and 5,545,806; each of which is incorporated herein by reference in its entirety. Other human antibodies can be obtained commercially from, for example, Abgenix, Inc. (now Amgen, Fremont, CA) and Medarex (San Jose, CA).

[0061] Completely human antibodies that recognize a selected epitope can be generated using a technique referred to as “guided selection.” In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers *et al.*, 1994, *Biotechnology* 12:899-903). Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom and Winter, 1991, *J. Mol. Biol.* 227:381; Marks *et al.*, 1991, *J. Mol. Biol.* 222:581; Quan and Carter, 2002, *The rise of monoclonal antibodies as therapeutics*, In *Anti-IgE and Allergic Disease*, Jardieu and Fick Jr., eds., Marcel Dekker, New York, NY, Chapter 20, pp. 427–469).

[0062] In some embodiments, the Ligand Drug conjugate compound is monospecific. In other embodiments, the Ligand Drug conjugate compound is multispecific, such as bi-specific. Multispecific antibodies may be specific for different epitopes of GPC-3 and/or may be specific for both GPC-3 as well as for a heterologous protein. (See, e.g., PCT Publications WO 93/17715, WO 92/08802, WO 91/00360, and WO 92/05793; Tutt *et al.*, 1991, *J. Immunol.* 147:60-69; U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; and 5,601,819; Kostelny *et al.*, 1992, *J. Immunol.* 148:1547-1553.) Multispecific antibodies, including bispecific and trispecific antibodies, useful for practicing the methods described herein are antibodies that immunospecifically bind to both GPC-3 and another target protein.

[0063] Useful Ligand units can include functionally active fragments of human monoclonal antibodies, humanized monoclonal antibodies and chimeric monoclonal antibodies that bind to Glypican-3 (e.g., the extracellular portion). In this regard, "functionally active" means that the fragment is able to elicit anti-anti-idiotypic antibodies that recognize the same antigen that the antibody from which the fragment is derived recognized.

[0064] The Ligand unit also can be a functionally active derivative or analog of an antibody that immunospecifically binds to a desired target antigen. In an exemplary embodiment the antigenicity of the idiotype of the immunoglobulin molecule can be enhanced by deletion of framework and CDR sequences that are C-terminal to the CDR sequence that specifically recognizes the antigen. To determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art (e.g., the BIAcore assay) (see, e.g., Kabat *et al.*, 1991, *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, Md; Kabat *et al.*, 1980, *J. Immunology* 125(3):961-969).

[0065] In some embodiments, the Ligand unit is a single chain antibody. Single chain antibodies can be prepared, for example, as described in U.S. Patent No. 4,946,778; Bird, 1988, *Science* 242:423-42; Huston *et al.*, 1988, *Proc. Natl.*

Acad. Sci. USA 85:5879-5883; Ward *et al.*, 1989, *Nature* 334:544-54; Holliger and Hudson, 2005, *Nat. Biotechnol.* 23:1126-1136; U.S Patent No. 6,545,142; U.S Patent No. 6,492,123; U.S Patent No. 6,248,516; U.S Patent No. 6,010,884; U.S Patent No. 5,837,242; and U.S Patent No. 5,091,513; the disclosures of which are incorporated by reference herein.

[0066] The Ligand unit may also be described or specified in terms of its binding affinity to GPC-3. Typical binding affinities include those with a dissociation constant or K_d less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M. Preferred binding affinities include those with a dissociation constant or K_d less than 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

[0067] The Ligand unit can be modified, *e.g.*, by the covalent attachment of any type of molecule as long as such covalent attachment permits the Ligand unit to retain its binding specificity (*e.g.*, antigen binding immunospecificity). For example, the Ligand unit can be modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to another protein, *etc.* Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis in the presence of tunicamycin, *etc.* Additionally, the Ligand unit can contain one or more unnatural amino acids.

[0068] In specific embodiments, it may be desirable to improve the binding affinity and/or other biological properties of the Ligand unit. Amino acid sequence variants of the Ligand unit (*e.g.*, the V_H and/or V_L) are prepared by introducing appropriate nucleotide changes into the nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or

substitutions of, residues within the amino acid sequences of the Ligand unit (*e.g.*, the V_H and/or V_L). Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the Ligand unit, such as changing the number or position of glycosylation sites.

[0069] A useful method for identification of certain residues or regions of the Ligand unit (*e.g.*, the V_H and/or V_L) that are favored locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989, *Science* 244:1081-1085). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (such as alanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, alanine scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

[0070] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an insertion at the N-terminal methionyl residue or the C terminal residue.

[0071] Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the Ligand unit (*e.g.*, the V_H and/or V_L) replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but framework alterations are also contemplated.

[0072] Substantial modifications in the biological properties of the Ligand unit (*e.g.*, the V_H and/or V_L) are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally-occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0073] A particular type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.*, a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.*, 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.*, binding affinity). In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and the antigen. Such contact residues and

neighboring residues are candidates for substitution. Once such variants are generated, the panel of variants is subjected to screening and antibodies with superior properties in one or more relevant assays may be selected for further development.

[0074] The Ligand unit may be glycosylated at one or conserved positions (see, e.g., Jefferis and Lund, 1997, *Chem. Immunol.* 65:111-128; Wright and Morrison, 1997, *TibTECH* 15:26-32). The oligosaccharide side chains can affect the protein's function (see, e.g., Boyd *et al.*, 1996, *Mol. Immunol.* 32:1311-1318; Wittwe and Howard, 1990, *Biochem.* 29:4175-4180), and the intramolecular interaction between portions of the glycoprotein which can affect the conformation and presented three-dimensional surface of the glycoprotein (see, e.g., Jefferis and Lund, *supra*; Wyss and Wagner, 1996, *Current Opin. Biotech.* 7:409-416). Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. For example, it has been reported that in agalactosylated IgG, the oligosaccharide moiety 'flips' out of the inter-C_H2 space and terminal N-acetylglucosamine residues become available to bind mannose binding protein (Malhotra *et al.*, 1995, *Nature Med.* 1:237-243). Removal by glycopeptidase of the oligosaccharides from CAMPATH-1H (a recombinant humanized murine monoclonal IgG1 antibody which recognizes the CDw52 antigen of human lymphocytes) produced in Chinese Hamster Ovary (CHO) cells resulted in a complete reduction in complement mediated lysis (CMCL) (Boyd *et al.*, 1996, *Mol. Immunol.* 32:1311-1318), while selective removal of sialic acid residues using neuraminidase resulted in no loss of CMCL. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of β (1,4)-N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation of bisecting GlcNAc, was reported to have improved ADCC activity (Umana *et al.*, 1999, *Nature Biotech.* 17:176-180).

[0075] Glycosylation is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine

residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[0076] Glycosylation variants of the Ligand unit are variants in which the glycosylation pattern of an antibody is altered. By altering is meant deleting or adding one or more carbohydrate moieties, changing the composition of glycosylation (glycosylation pattern), the extent of glycosylation, *etc.*

[0077] Addition of glycosylation sites to the Ligand unit is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence (for O-linked glycosylation sites). Similarly, removal of glycosylation sites can be accomplished by amino acid alteration.

[0078] The amino acid sequence is usually altered by altering the underlying nucleic acid sequence. These methods include, but are not limited to, preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

[0079] The glycosylation (including glycosylation pattern) of a Ligand unit may also be altered without altering the amino acid sequence or the underlying nucleotide sequence. Glycosylation largely depends on the host cell used to express the antibody. Since the cell type used for expression of the Ligand unit is rarely the native cell, significant variations in the glycosylation pattern can be expected. See, *e.g.*, Hse *et al.*, 1997, *J. Biol. Chem.* 272:9062-9070. In addition to the choice of host

cells, factors which affect glycosylation during recombinant production of Ligand units include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like. Various methods have been proposed to alter the glycosylation pattern achieved in a particular host organism including introducing or overexpressing certain enzymes involved in oligosaccharide production (*see, e.g.*, U.S. Patent Nos. 5,047,335; 5,510,261; and 5,278,299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example using endoglycosidase H (Endo H). In addition, the recombinant host cell can be genetically engineered, *e.g.*, made defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

[0080] The glycosylation structure can be readily analyzed by conventional techniques of carbohydrate analysis, including lectin chromatography, NMR, mass spectrometry, HPLC, GPC, monosaccharide compositional analysis, sequential enzymatic digestion, and HPAEC-PAD, which uses high pH anion exchange chromatography to separate oligosaccharides based on charge. Methods for releasing oligosaccharides for analytical purposes are also known, and include, without limitation, enzymatic treatment (commonly performed using peptide-N-glycosidase F/endo- β -galactosidase), elimination using harsh alkaline environment to release mainly O-linked structures, and chemical methods using anhydrous hydrazine to release both N- and O-linked oligosaccharides.

[0081] The Ligand units can have modifications (*e.g.*, substitutions, deletions or additions) in amino acid residues that interact with Fc receptors. In particular, the Ligand units include can have modifications in amino acid residues identified as involved in the interaction between the anti-Fc domain and the FcRn receptor (*see, e.g.*, International Publication Nos. WO 97/34631, WO 06/053301, and WO 04/099249, each of which is incorporated herein by reference in its entirety).

[0082] Ligand Units that bind to Glypican-3 can be prepared can be obtained commercially, for example, from commercial companies or produced by any method known to one of skill in the art such as, *e.g.*, chemical synthesis or

recombinant expression techniques. In typical embodiments, the antibody binds to membrane bound GPC-3. In some embodiments, the antibody is mAb 1G12 (BioMosaics) or mAb 8H5 (BioMosaics) (see, *e.g.*, Capurro et al, 2003, *Gastroenterology* 125(1):89-97). In other embodiments, the antibody is not mAb 1G12 or mAb 8H5. In other embodiments, the antibody is an antibody disclosed in U.S. Patent Application Publication Nos. 2004-0236080 or 2005-0233392, European Patent Application Publication No. 1 541 680 or PCT Published Application WO 2006/006693 (the disclosures of which are incorporated by reference herein).

Linker units

[0083] A “Linker unit” (LU) is a bifunctional compound that can be used to link a Drug unit and a Ligand unit to form a Ligand Drug conjugate compound. In some embodiments, the Linker unit has the formula:



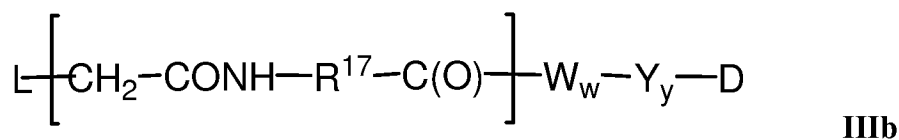
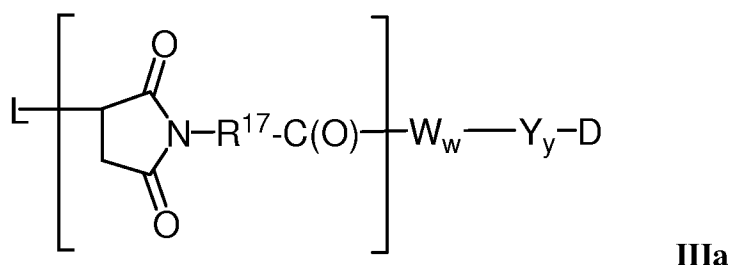
wherein: -A- is a Stretcher unit,
 a is 0 or 1,
 each -W- is independently an Amino Acid unit,
 w is an integer ranging from 0 to 12,
 -Y- is a self-immolative Spacer unit, and
 y is 0, 1 or 2.

[0084] In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0, 1 or 2. In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0 or 1.

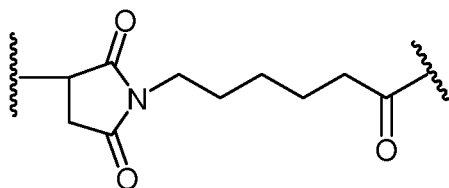
The Stretcher Unit

[0085] The Stretcher unit (A), when present, is capable of linking a Ligand unit to an Amino Acid unit (-W-), if present, to a Spacer unit (-Y-), if present; or to a Drug unit (-D). In one embodiment, the Stretcher unit forms a bond with a sulfur atom of the Ligand unit. The sulfur atom can be derived from a sulfhydryl group of a

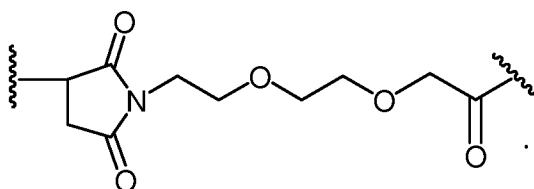
Ligand. Representative Stretcher units of this embodiment are depicted within the square brackets of Formulas IIIa and IIIb, wherein L-, -W-, -Y-, -D, w and y are as defined above, and R¹⁷ is selected from -C₁-C₁₀ alkylene-, -C₃-C₈ carbocyclo-, -O-(C₁-C₈ alkyl)-, -arylene-, -C₁-C₁₀ alkylene-arylene-, -arylene-C₁-C₁₀ alkylene-, -C₁-C₁₀ alkylene-(C₃-C₈ carbocyclo)-, -(C₃-C₈ carbocyclo)-C₁-C₁₀ alkylene-, -C₃-C₈ heterocyclo-, -C₁-C₁₀ alkylene-(C₃-C₈ heterocyclo)-, -(C₃-C₈ heterocyclo)-C₁-C₁₀ alkylene-, -(CH₂CH₂O)_r-, or -(CH₂CH₂O)_r-CH₂-; and r is an integer ranging from 1-10. It is to be understood from all the exemplary embodiments that even where not denoted expressly, from 1 to 20 drug moieties can be linked to a Ligand (p = 1-20).



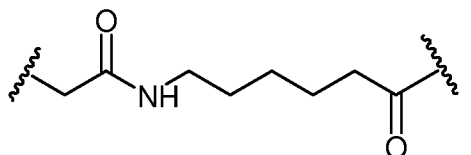
[0086] An illustrative Stretcher unit is that of Formula IIIa wherein R¹⁷ is -(CH₂)₅-:



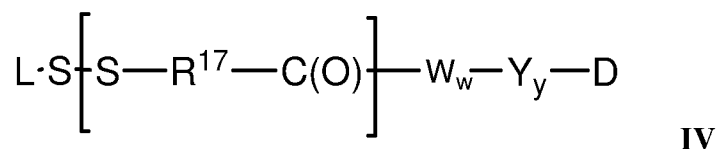
[0087] Another illustrative Stretcher unit is that of Formula IIIa wherein R¹⁷ is -(CH₂CH₂O)_r-CH₂-; and r is 2:



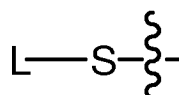
[0088] Still another illustrative Stretcher unit is that of Formula IIIb wherein R^{17} is $-(CH_2)_5-$:



[0089] In another embodiment, the Stretcher unit is linked to the Ligand unit via a disulfide bond between a sulfur atom of the Ligand unit and a sulfur atom of the Stretcher unit. A representative Stretcher unit of this embodiment is depicted within the square brackets of Formula IV, wherein R^{17} , L-, -W-, -Y-, -D, w and y are as defined above.

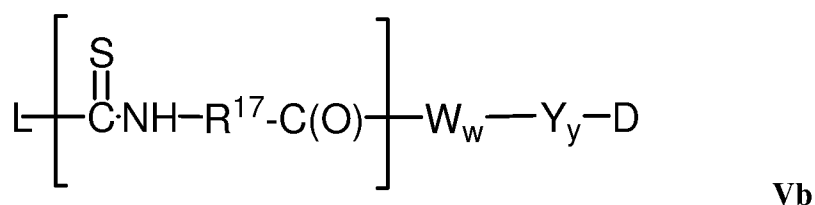
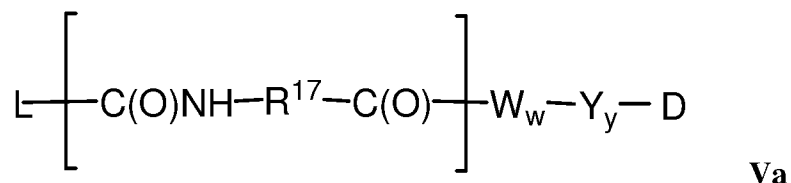


[0090] It should be noted that throughout this application, the S moiety in the formula below refers to a sulfur atom of the Ligand unit, unless otherwise indicated by context.

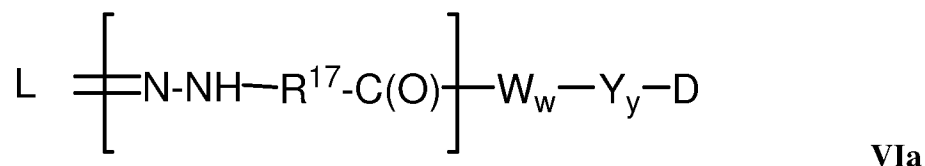


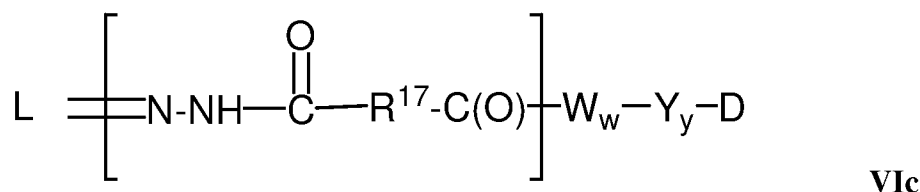
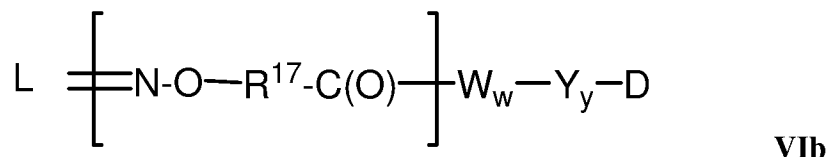
[0091] In yet another embodiment, the Stretcher contains a reactive site that can form a bond with a primary or secondary amino group of a Ligand. Examples of these reactive sites include, but are not limited to, activated esters such as succinimide esters, 4 nitrophenyl esters, pentafluorophenyl esters,

tetrafluorophenyl esters, anhydrides, acid chlorides, sulfonyl chlorides, isocyanates and isothiocyanates. Representative Stretcher units of this embodiment are depicted within the square brackets of Formulas Va and Vb, wherein $-R^{17}$ -, L-, -W-, -Y-, -D, w and y are as defined above;



[0092] In some embodiments, the Stretcher contains a reactive site that is reactive to a modified carbohydrate's (-CHO) group that can be present on a Ligand. For example, a carbohydrate can be mildly oxidized using a reagent such as sodium periodate and the resulting (-CHO) unit of the oxidized carbohydrate can be condensed with a Stretcher that contains a functionality such as a hydrazide, an oxime, a primary or secondary amine, a hydrazine, a thiosemicarbazone, a hydrazine carboxylate, and an arylhydrazide such as those described by Kaneko *et al.*, 1991, *Bioconjugate Chem.* 2:133-41. Representative Stretcher units of this embodiment are depicted within the square brackets of Formulas VIa, VIb, and VIc, wherein $-R^{17}$ -, L-, -W-, -Y-, -D, w and y are as defined above.

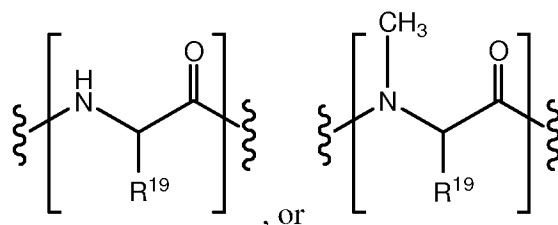




The Amino Acid Unit

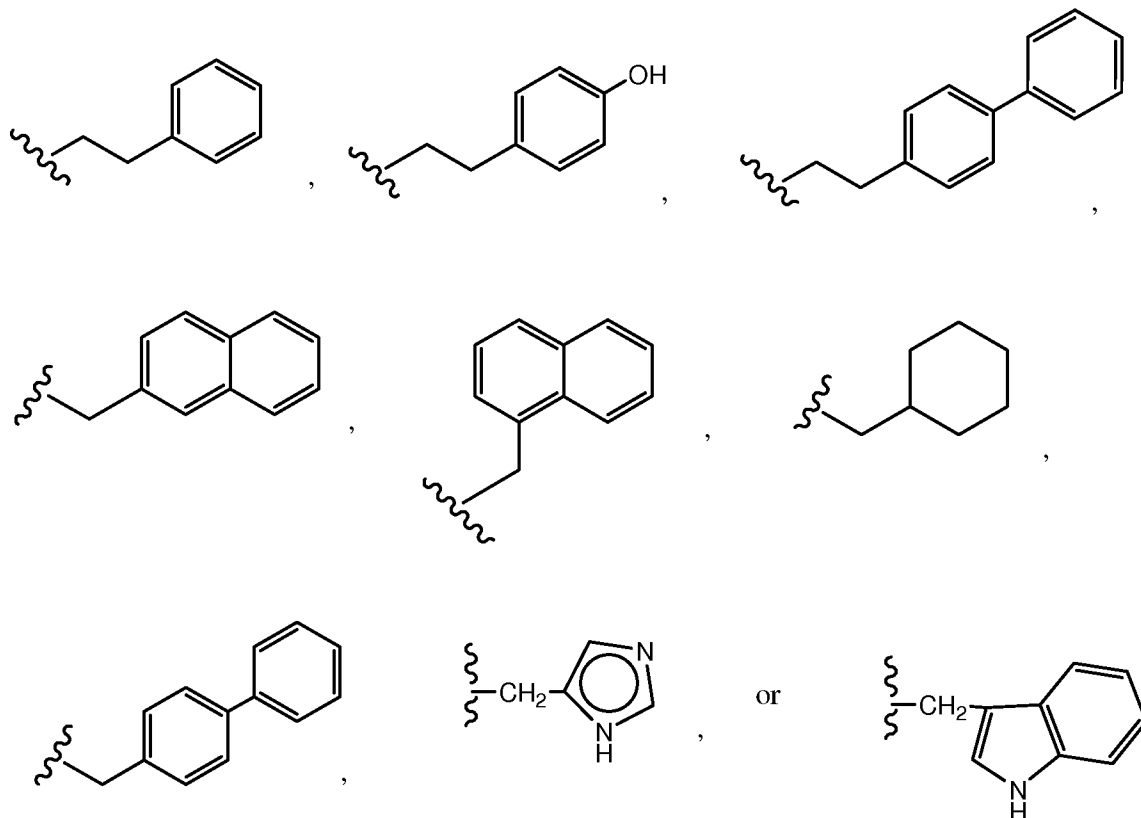
[0093] The Amino Acid unit (-W-), when present, links the Stretcher unit to the Spacer unit if the Spacer unit is present, links the Stretcher unit to the Drug moiety if the Spacer unit is absent, and links the Ligand unit to the Drug unit if the Stretcher unit and Spacer unit are absent.

[0094] W_w- is a dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide, decapeptide, undecapeptide or dodecapeptide unit. Each -W- unit independently has the formula denoted below in the square brackets, and w is an integer ranging from 0 to 12:



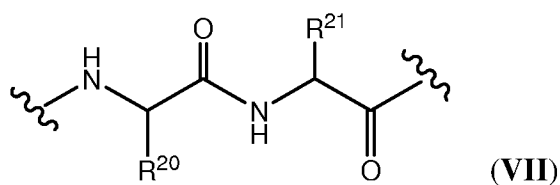
wherein R¹⁹ is hydrogen, methyl, isopropyl, isobutyl, *sec*-butyl, benzyl, *p*-hydroxybenzyl, -CH₂OH, -CH(OH)CH₃, -CH₂CH₂SCH₃, -CH₂CONH₂, -CH₂COOH, -CH₂CH₂CONH₂, -CH₂CH₂COOH, -(CH₂)₃NHC(=NH)NH₂, -(CH₂)₃NH₂, -(CH₂)₃NHCOCH₃, -(CH₂)₃NHCHO, -(CH₂)₄NHC(=NH)NH₂, -(CH₂)₄NH₂, -(CH₂)₄NHCOCH₃, -(CH₂)₄NHCHO, -(CH₂)₃NHCONH₂, -(CH₂)₄NHCONH₂,

-CH₂CH₂CH(OH)CH₂NH₂, 2-pyridylmethyl-, 3-pyridylmethyl-, 4-pyridylmethyl-, phenyl, cyclohexyl,

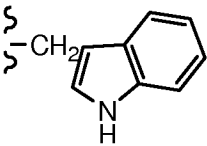


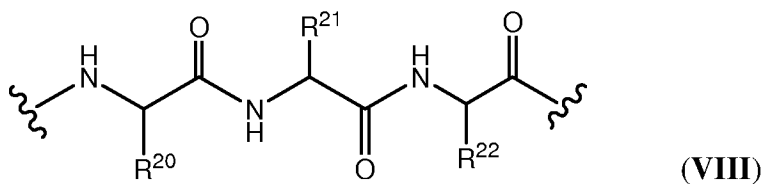
[0095] In some embodiments, the Amino Acid unit can be enzymatically cleaved by one or more enzymes, including a cancer or tumor-associated protease, to liberate the Drug unit (-D), which in one embodiment is protonated *in vivo* upon release to provide a Drug (D).

[0096] In certain embodiments, the Amino Acid unit can comprise natural amino acids. In other embodiments, the Amino Acid unit can comprise non-natural amino acids. Illustrative Ww units are represented by formulas (VII)-(IX):



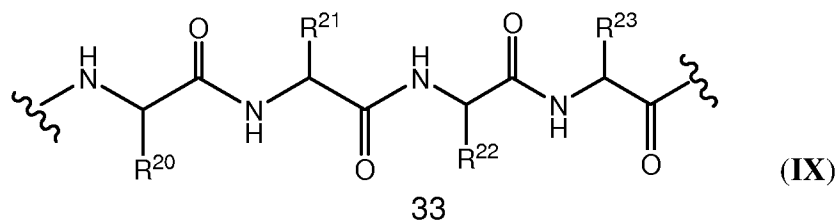
wherein R^{20} and R^{21} are as follows:

R^{20}	R^{21}
Benzyl	$(CH_2)_4NH_2$;
methyl	$(CH_2)_4NH_2$;
isopropyl	$(CH_2)_4NH_2$;
isopropyl	$(CH_2)_3NHCONH_2$;
benzyl	$(CH_2)_3NHCONH_2$;
isobutyl	$(CH_2)_3NHCONH_2$;
sec-butyl	$(CH_2)_3NHCONH_2$;
	$(CH_2)_3NHCONH_2$;
benzyl	methyl;
benzyl	$(CH_2)_3NHC(=NH)NH_2$;



wherein R^{20} , R^{21} and R^{22} are as follows:

R^{20}	R^{21}	R^{22}
benzyl	benzyl	$(CH_2)_4NH_2$;
isopropyl	benzyl	$(CH_2)_4NH_2$; and
H	benzyl	$(CH_2)_4NH_2$;



wherein R^{20} , R^{21} , R^{22} and R^{23} are as follows:

$\underline{R^{20}}$	$\underline{R^{21}}$	$\underline{R^{22}}$	$\underline{R^{23}}$
H	benzyl	isobutyl	H; and
methyl	isobutyl	methyl	isobutyl.

[0097] Exemplary Amino Acid units include, but are not limited to, units of formula VII where: R^{20} is benzyl and R^{21} is $-(CH_2)_4NH_2$; R^{20} is isopropyl and R^{21} is $-(CH_2)_4NH_2$; or R^{20} is isopropyl and R^{21} is $-(CH_2)_3NHCONH_2$. Another exemplary Amino Acid unit is a unit of formula VIII wherein R^{20} is benzyl, R^{21} is benzyl, and R^{22} is $-(CH_2)_4NH_2$.

[0098] Useful $-W_w-$ units can be designed and optimized in their selectivity for enzymatic cleavage by a particular enzyme, for example, a tumor-associated protease. In one embodiment, a $-W_w-$ unit is that whose cleavage is catalyzed by cathepsin B, C and D, or a plasmin protease.

[0099] In one embodiment, $-W_w-$ is a dipeptide, tripeptide, tetrapeptide or pentapeptide. When R^{19} , R^{20} , R^{21} , R^{22} or R^{23} is other than hydrogen, the carbon atom to which R^{19} , R^{20} , R^{21} , R^{22} or R^{23} is attached is chiral.

[0100] Each carbon atom to which R^{19} , R^{20} , R^{21} , R^{22} or R^{23} is attached is independently in the (S) or (R) configuration.

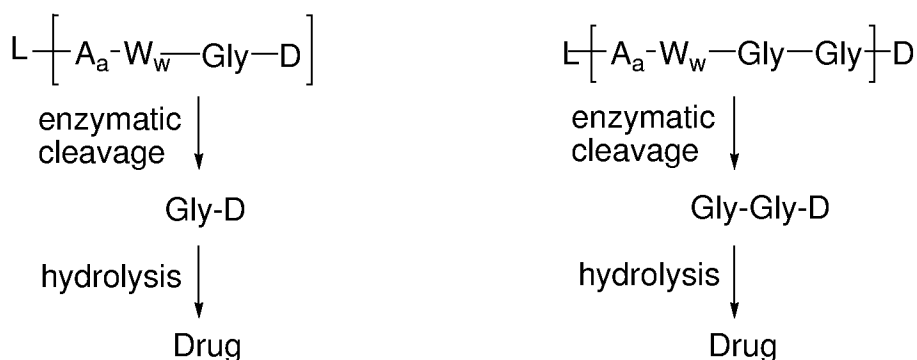
[0101] In one aspect of the Amino Acid unit, the Amino Acid unit is valine-citrulline (vc or val-cit). In another aspect, the Amino Acid unit is phenylalanine-lysine (i.e., fk). In yet another aspect of the Amino Acid unit, the Amino Acid unit is N-methylvaline-citrulline. In yet another aspect, the Amino Acid unit is 5-aminovaleric acid, homo phenylalanine lysine, tetraisoquinolinecarboxylate lysine, cyclohexylalanine lysine, isonepecotic acid lysine, beta-alanine lysine, glycine serine valine glutamine and isonepecotic acid.

The Spacer Unit

[0102] The Spacer unit (-Y-), when present, links an Amino Acid unit to the Drug unit when an Amino Acid unit is present. Alternately, the Spacer unit links the Stretcher unit to the Drug unit when the Amino Acid unit is absent. The Spacer unit also links the Drug unit to the Ligand unit when both the Amino Acid unit and Stretcher unit are absent.

[0103] Spacer units are of two general types: non self-immolative or self-immolative. A non self-immolative Spacer unit is one in which part or all of the Spacer unit remains bound to the Drug moiety after cleavage, particularly enzymatic, of an Amino Acid unit from the Ligand Drug conjugate compound. Examples of a non self-immolative Spacer unit include, but are not limited to a (glycine-glycine) Spacer unit and a glycine Spacer unit (both depicted in Scheme 1) (*infra*). When a conjugate containing a glycine-glycine Spacer unit or a glycine Spacer unit undergoes enzymatic cleavage via an enzyme (*e.g.*, a tumor-cell associated-protease, a cancer-cell-associated protease or a lymphocyte-associated protease), a glycine-glycine-Drug moiety or a glycine-Drug moiety is cleaved from L-Aa-Ww-. In one embodiment, an independent hydrolysis reaction takes place within the target cell, cleaving the glycine-Drug moiety bond and liberating the Drug.

Scheme 1



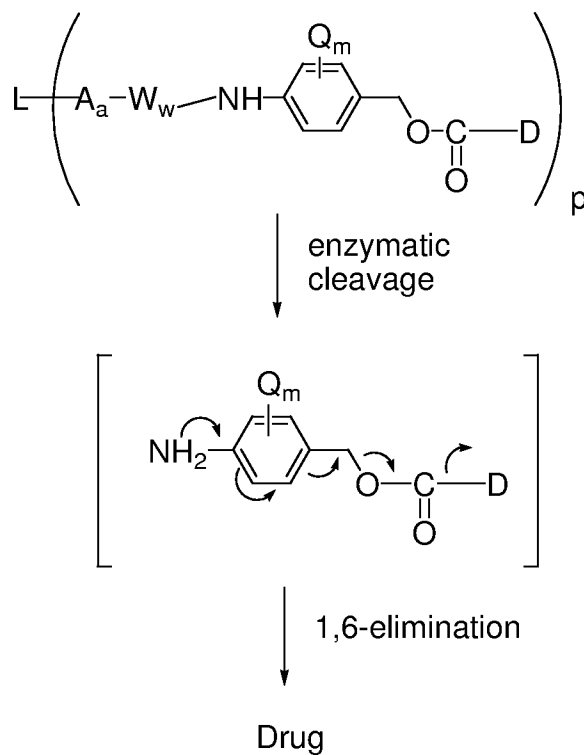
[0104] In some embodiments, a non self-immolative Spacer unit (-Y-) is -Gly-. In some embodiments, a non self-immolative Spacer unit (-Y-) is -Gly-Gly-.

[0105] In one embodiment, a Drug-Linker conjugate compound is provided in which the Spacer unit is absent ($y=0$), or a pharmaceutically acceptable salt or solvate thereof.

[0106] Alternatively, a conjugate containing a self-immolative Spacer unit can release -D. As used herein, the term “self-immolative Spacer” refers to a bifunctional chemical moiety that is capable of covalently linking together two spaced chemical moieties into a stable tripartite molecule. It will spontaneously separate from the second chemical moiety if its bond to the first moiety is cleaved.

[0107] In some embodiments, -Y_y- is a p-aminobenzyl alcohol (PAB) unit (see Schemes 2 and 3) whose phenylene portion is substituted with Q_m wherein Q is -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -halogen, - nitro or -cyano; and m is an integer ranging from 0-4.

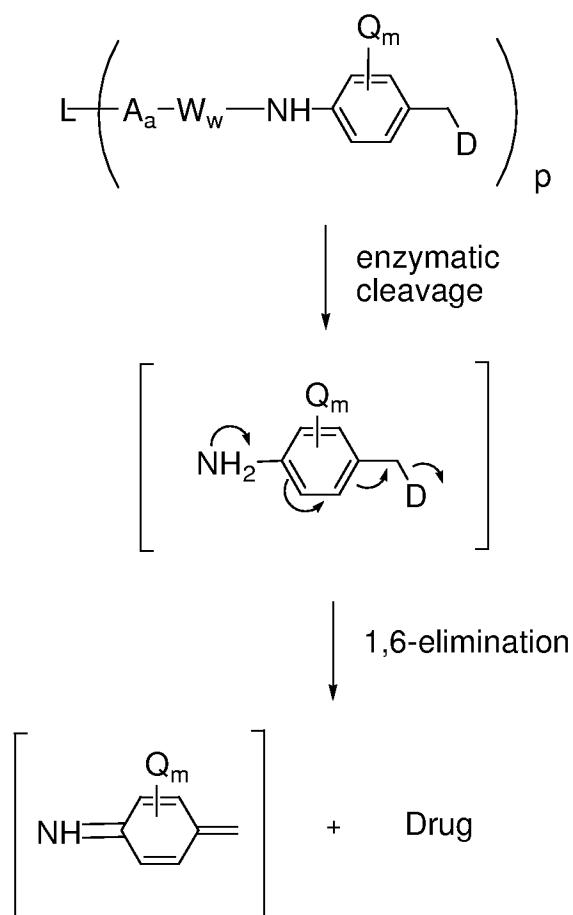
[0108] In some embodiments, -Y- is a PAB group that is linked to -W_w- via the amino nitrogen atom of the PAB group, and connected directly to -D via a carbonate, carbamate or ether group. Without being bound by any particular theory or mechanism, Scheme 2 depicts a possible mechanism of Drug release of a PAB group which is attached directly to -D via a carbamate or carbonate group as described by Toki *et al.*, 2002, *J. Org. Chem.* 67:1866-1872.

Scheme 2

In Scheme 2, Q is -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -halogen, -nitro or -cyano; m is an integer ranging from 0-4; and p ranges from 1 to about 20.

[0109] Without being bound by any particular theory or mechanism, Scheme 3 depicts a possible mechanism of Drug release of a PAB group which is attached directly to -D via an ether or amine linkage, wherein D includes the oxygen or nitrogen group that is part of the Drug unit.

Scheme 3



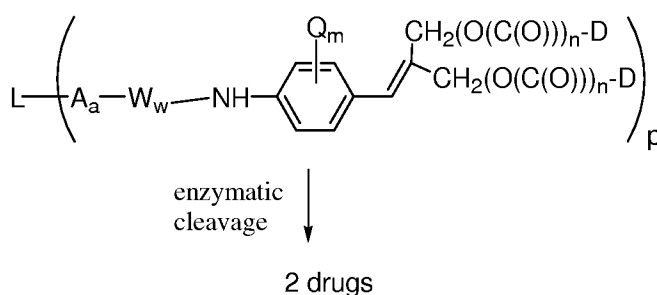
In Scheme 3, Q is -C₁-C₈ alkyl, -O-(C₁-C₈ alkyl), -halogen, -nitro or -cyano; m is an integer ranging from 0-4; and p ranges from 1 to about 20.

[0110] Other examples of self-immolative spacers include, but are not limited to, aromatic compounds that are electronically similar to the PAB group such as 2-aminoimidazol-5-methanol derivatives (Hay *et al.*, 1999, *Bioorg. Med. Chem. Lett.* 9:2237) and ortho or para-aminobenzylacetals. Spacers can be used that undergo cyclization upon amide bond hydrolysis, such as substituted and unsubstituted 4-aminobutyric acid amides (Rodrigues *et al.*, 1995, *Chemistry Biology* 2:223), appropriately substituted bicyclo[2.2.1] and bicyclo[2.2.2] ring systems (Storm *et al.*, 1972, *J. Amer. Chem. Soc.* 94:5815) and 2-aminophenylpropionic acid amides (Amsberry *et al.*, 1990, *J. Org. Chem.* 55:5867). Elimination of amine-containing

drugs that are substituted at the α -position of glycine (Kingsbury *et al.*, 1984, *J. Med. Chem.* 27:1447) are also examples of self-immolative spacers.

[0111] In one embodiment, the Spacer unit is a branched bis(hydroxymethyl)-styrene (BHMS) unit as depicted in Scheme 4, which can be used to incorporate and release multiple drugs.

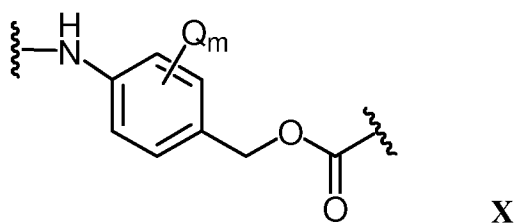
Scheme 4



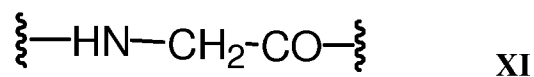
In Scheme 4, Q is $-C_1-C_8$ alkyl, $-O-(C_1-C_8$ alkyl), -halogen, -nitro or -cyano; m is an integer ranging from 0-4; n is 0 or 1; and p ranges raging from 1 to about 20.

In some embodiments, the -D moieties are the same. In yet another embodiment, the -D moieties are different.

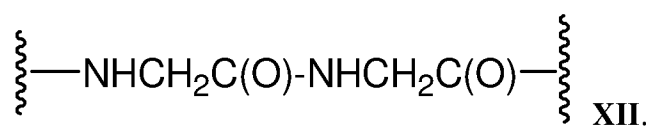
[0112] In one aspect, Spacer units ($-Y_y-$) are represented by Formulas (X)-(XII):



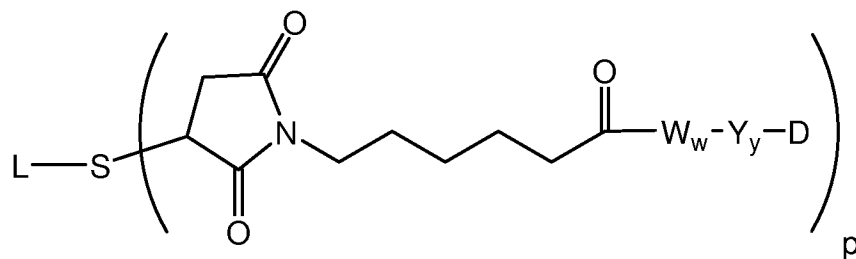
wherein Q is $-C_1-C_8$ alkyl, $-O-(C_1-C_8$ alkyl), -halogen, -nitro or -cyano; and m is an integer ranging from 0-4;



and

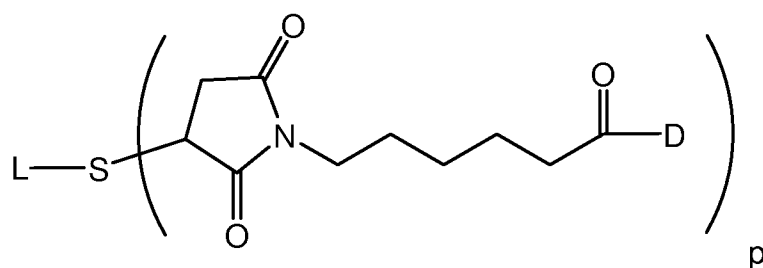


[0113] Embodiments of the Formula I and II comprising Ligand-drug conjugate compounds can include:

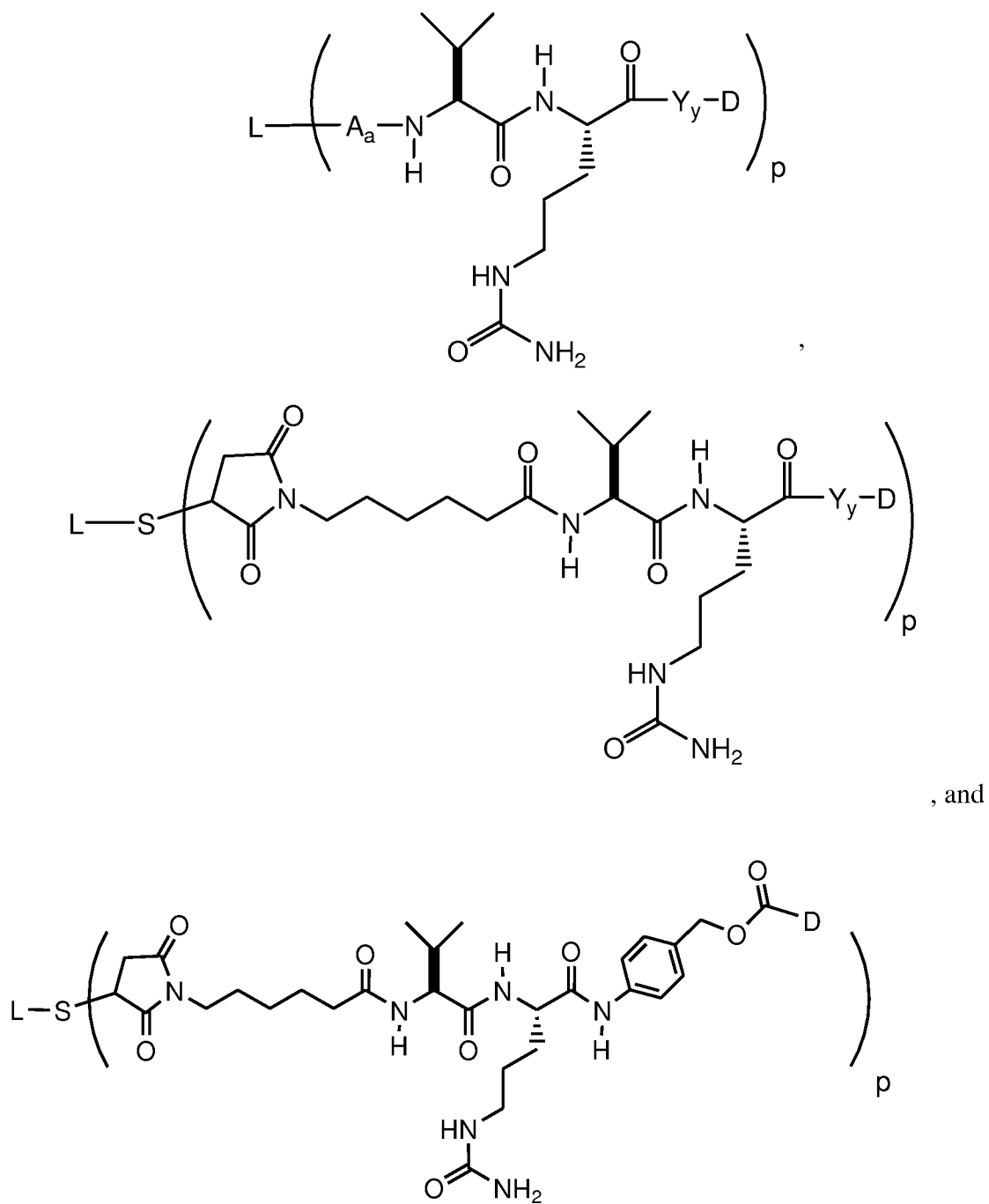


wherein w and y are each 0, 1 or 2,

and,



wherein w and y are each 0,



The Drug Unit

[0114] The Drug moiety (D) can be any cytotoxic, cytostatic or immunomodulatory drug. D is a Drug unit (moiety) having an atom that can form a bond with the Spacer unit, with the Amino Acid unit, with the Stretcher unit or with the Ligand unit. In some embodiments, the Drug unit D has a nitrogen atom that can form a bond with the Spacer unit. As used herein, the terms “Drug unit” and “Drug moiety” are synonymous and used interchangeably.

[0115] Useful classes of cytotoxic or immunomodulatory agents include, for example, antitubulin agents, auristatins, DNA minor groove binders, DNA replication inhibitors, alkylating agents (*e.g.*, platinum complexes such as cis-platin, mono(platinum), bis(platinum) and tri-nuclear platinum complexes and carboplatin), anthracyclines, antibiotics, antifolates, antimetabolites, chemotherapy sensitizers, duocarmycins, etoposides, fluorinated pyrimidines, ionophores, lexitropsins, nitrosoureas, platinols, pre-forming compounds, purine antimetabolites, puromycins, radiation sensitizers, steroids, taxanes, topoisomerase inhibitors, vinca alkaloids, or the like.

[0116] Individual cytotoxic or immunomodulatory agents include, for example, an androgen, anthramycin (AMC), asparaginase, 5-azacytidine, azathioprine, bleomycin, busulfan, buthionine sulfoximine, calicheamicin, camptothecin, carboplatin, carmustine (BSNU), CC-1065, chlorambucil, cisplatin, colchicine, cyclophosphamide, cytarabine (cytidine arabinoside), cytochalasin B, dacarbazine, dactinomycin (formerly actinomycin), daunorubicin, decarbazine, docetaxel, doxorubicin, etoposide, an estrogen, 5-fluorodeoxyuridine, 5-fluorouracil, gemcitabine, gramicidin D, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine (CCNU), maytansine, mechlorethamine, melphalan, 6-mercaptopurine, methotrexate, mithramycin, mitomycin C, mitoxantrone, nitroimidazole, paclitaxel, palytoxin, plicamycin, procarbazine, rhizoxin, streptozotocin, tenoposide, 6-thioguanine, thioTEPA, topotecan, vinblastine, vincristine, vinorelbine, VP-16 and VM-26.

[0117] In some typical embodiments, suitable cytotoxic agents include, for example, DNA minor groove binders (*e.g.*, enediynes and lexitropsins, a CBI

compound; see also U.S. Patent No. 6,130,237), duocarmycins, taxanes (*e.g.*, paclitaxel and docetaxel), puromycins, vinca alkaloids, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, echinomycin, combretastatin, netropsin, epothilone A and B, estramustine, cryptophysins, cemadotin, maytansinoids, discodermolide, eleutherobin, and mitoxantrone.

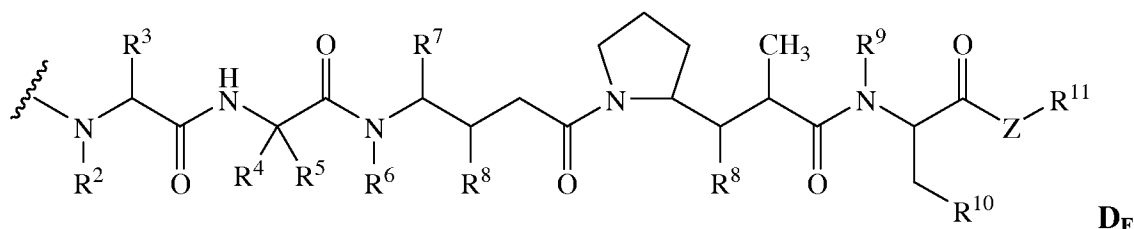
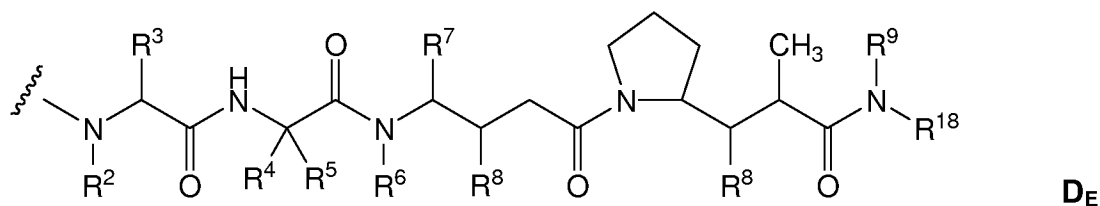
[0118] In some embodiments, the Drug is an anti-tubulin agent. Examples of anti-tubulin agents include, but are not limited to, taxanes (*e.g.*, Taxol® (paclitaxel), Taxotere® (docetaxel)), T67 (Tularik; now Amgen South San Francisco) and vinca alkyls (*e.g.*, vincristine, vinblastine, vindesine, and vinorelbine). Other antitubulin agents include, for example, baccatin derivatives, taxane analogs (*e.g.*, epothilone A and B), nocodazole, colchicine and colcimid, estramustine, cryptophysins, cemadotin, maytansinoids, combretastatins, discodermolide, and eleutherobin.

[0119] In certain embodiments, the cytotoxic agent is a maytansinoid, another group of anti-tubulin agents. For example, in specific embodiments, the maytansinoid is maytansine or DM-1 (ImmunoGen, Inc.; see also Chari *et al.*, 1992, Cancer Res. 52:127-131).

[0120] In some embodiments, the Drug is an auristatin, such as auristatin E or a derivative thereof. Typically, the auristatin E derivative is, *e.g.*, an ester formed between auristatin E and a keto acid. For example, auristatin E can be reacted with paraacetyl benzoic acid or benzoylvaleric acid to produce AEB and AEVB, respectively. Other typical auristatin derivatives include AFP, MMAF, and MMAE. The synthesis and structure of auristatin derivatives are described in U.S. Patent Application Publication Nos. 2003-0083263, 2005-0238649 and 2005-0009751; International Patent Publication No. WO 04/010957, International Patent Publication No. WO 02/088172, and U.S. Patent Nos. 6,323,315; 6,239,104; 6,034,065; 5,780,588; 5,665,860; 5,663,149; 5,635,483; 5,599,902; 5,554,725; 5,530,097; 5,521,284; 5,504,191; 5,410,024; 5,138,036; 5,076,973; 4,986,988;

4,978,744; 4,879,278; 4,816,444; and 4,486,414, each of which is incorporated by reference herein in its entirety.

[0121] In some embodiments, -D is an auristatin of the formula D_E or D_F:



wherein, independently at each location:

R² is H or C₁-C₈ alkyl;

R³ is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

R⁴ is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

R⁵ is H or methyl;

or R⁴ and R⁵ jointly form a carbocyclic ring and have the formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from H, C₁-C₈ alkyl or C₃-C₈ carbocycle and n is selected from 2, 3, 4, 5 or 6;

R⁶ is H or C₁-C₈ alkyl;

R⁷ is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

each R⁸ is independently selected from H, OH, C₁-C₈ alkyl, C₃-C₈ carbocycle or O-(C₁-C₈ alkyl);

R⁹ is H or C₁-C₈ alkyl;

R^{10} is aryl or C₃-C₈ heterocycle;
 Z is O, S, NH, or NR¹², wherein R¹² is C₁-C₈ alkyl;
 R^{11} is H, C₁-C₂₀ alkyl, aryl, C₃-C₈ heterocycle, $-(R^{13}O)_m-R^{14}$, or $-(R^{13}O)_m-CH(R^{15})_2$;
 m is an integer ranging from 1-1000;
 R^{13} is C₂-C₈ alkyl;
 R^{14} is H or C₁-C₈ alkyl;
each occurrence of R^{15} is independently H, COOH, $-(CH_2)_n-N(R^{16})_2$, $-(CH_2)_n-SO_3H$, or $-(CH_2)_n-SO_3-C_1-C_8$ alkyl;
each occurrence of R^{16} is independently H, C₁-C₈ alkyl, or $-(CH_2)_n-COOH$;
 R^{18} is $-C(R^8)_2-C(R^8)_2$ -aryl, $-C(R^8)_2-C(R^8)_2$ -(C₃-C₈ heterocycle), or $-C(R^8)_2-C(R^8)_2$ -(C₃-C₈ carbocycle); and
 n is an integer ranging from 0 to 6.

[0122] In one embodiment, R³, R⁴ and R⁷ are independently isopropyl or sec-butyl and R⁵ is -H. In an exemplary embodiment, R³ and R⁴ are each isopropyl, R⁵ is H, and R⁷ is sec-butyl.

[0123] In another embodiment, R² and R⁶ are each methyl, and R⁹ is H.

[0124] In still another embodiment, each occurrence of R⁸ is -OCH₃.

[0125] In an exemplary embodiment, R³ and R⁴ are each isopropyl, R² and R⁶ are each methyl, R⁵ is H, R⁷ is sec-butyl, each occurrence of R⁸ is -OCH₃, and R⁹ is H.

[0126] In one embodiment, Z is -O- or -NH-.

[0127] In one embodiment, R¹⁰ is aryl.

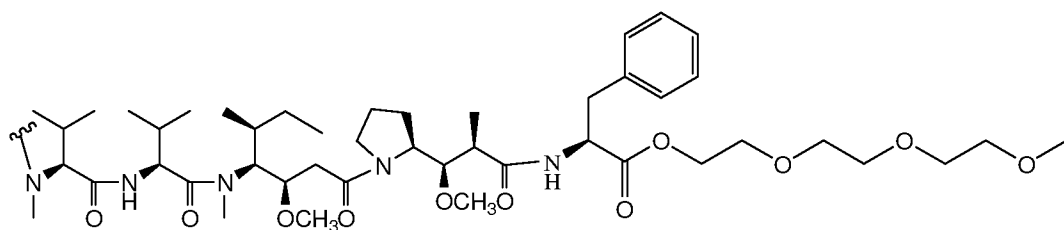
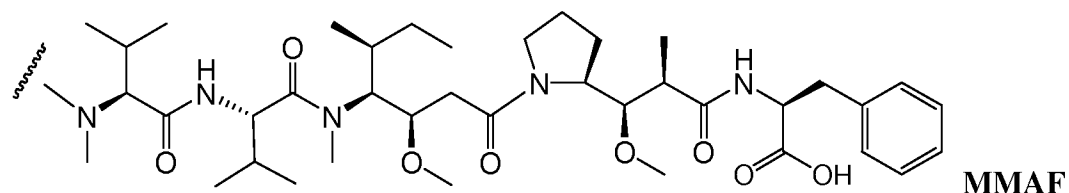
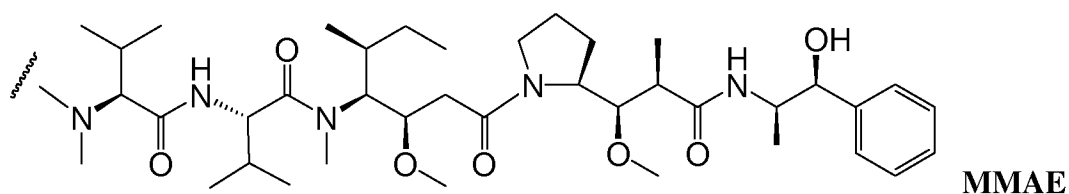
[0128] In an exemplary embodiment, R¹⁰ is -phenyl.

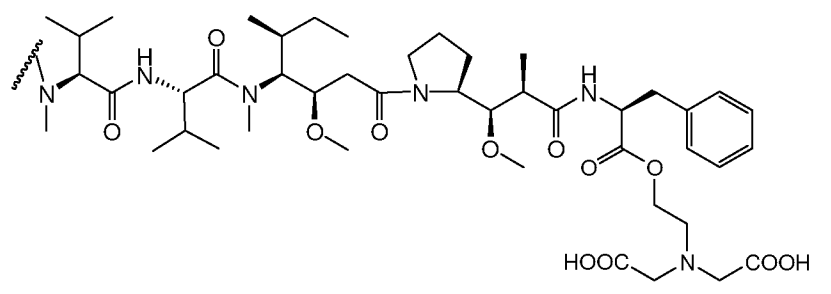
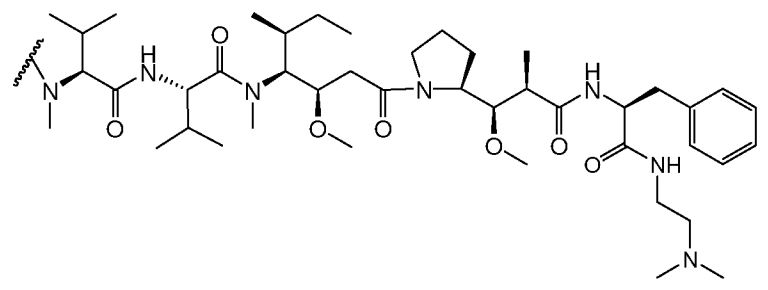
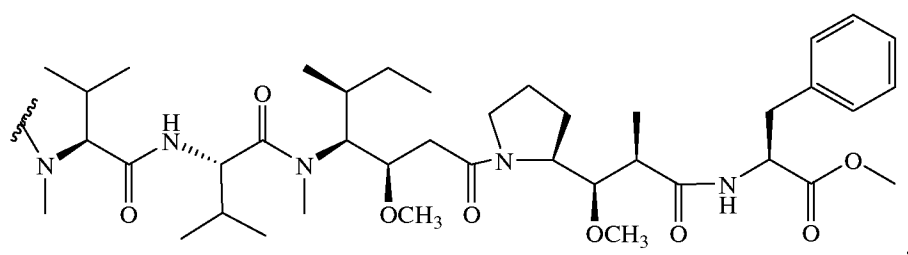
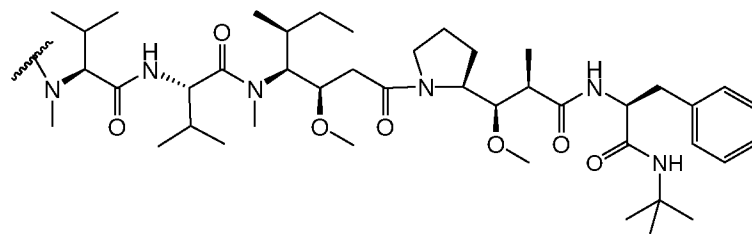
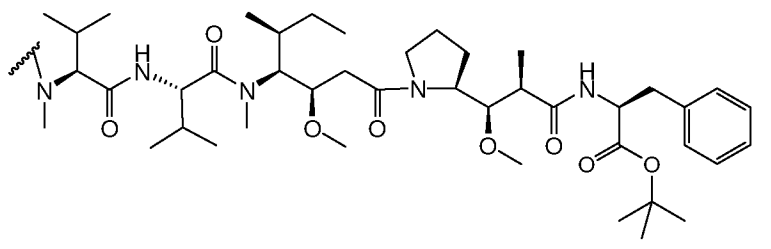
[0129] In an exemplary embodiment, when Z is -O-, R¹¹ is H, methyl or t-butyl.

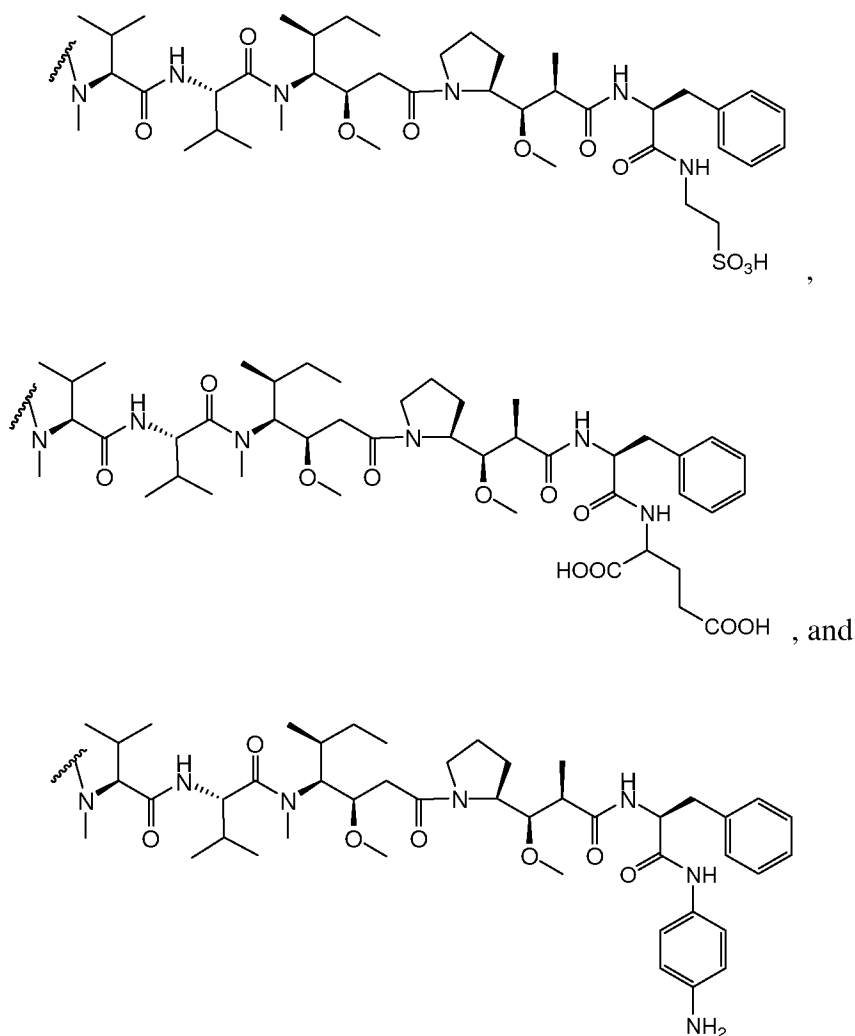
[0130] In one embodiment, when Z is -NH, R¹¹ is -CH(R¹⁵)₂, wherein R¹⁵ is -(CH₂)_n-N(R¹⁶)₂, and R¹⁶ is -C₁-C₈ alkyl or -(CH₂)_n-COOH.

[0131] In another embodiment, when Z is -NH, R¹¹ is -CH(R¹⁵)₂, wherein R¹⁵ is -(CH₂)_n-SO₃H.

[0132] Illustrative Drug units (-D) include the drug units having the following structures:



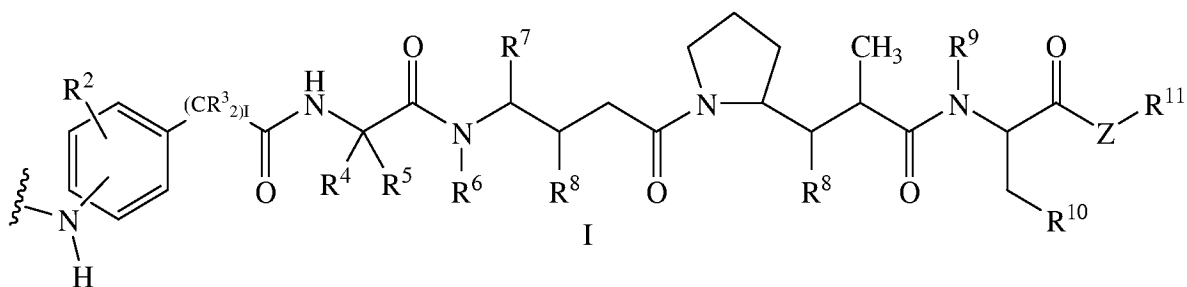




and pharmaceutically acceptable salts or solvates thereof.

[0133] In one aspect, hydrophilic groups, such as but not limited to triethylene glycol esters (TEG) can be attached to the Drug Unit at R¹¹. Without being bound by theory, the hydrophilic groups assist in the internalization and non-agglomeration of the Drug Unit.

[0134] In another aspect, the Drug unit is an amino-benzoic acid derivative of an auristatin of the following formula:



wherein, independently at each location:

R^2 is $-H$, $-C_1-C_8$ alkyl, $-O-(C_1-C_8$ alkyl), $-halogen$, $-NO_2$, $-COOH$, or $-C(O)OR^{11}$;

each R^3 is selected independently from $-H$ or $-C_1-C_8$ alkyl;

I is an integer ranging from 0-10;

R^4 is $-H$, $-C_1-C_8$ alkyl, $-C_3-C_8$ carbocycle, $-aryl$, $-C_1-C_8$ alkyl- $aryl$, $-C_1-C_8$ alkyl- $(C_3-C_8$ carbocycle), $-C_3-C_8$ heterocycle or $-C_1-C_8$ alkyl- $(C_3-C_8$ heterocycle), and R^5 is $-H$ or $-methyl$; or R^4 and R^5 jointly have the formula $-(CR^aR^b)_n$, wherein R^a and R^b are independently selected from $-H$, $-C_1-C_8$ alkyl or $-C_3-C_8$ carbocycle and n is selected from 2, 3, 4, 5 or 6, and form a ring with the carbon atom to which they are attached;

R^6 is $-H$ or $-C_1-C_8$ alkyl;

R^7 is $-H$, $-C_1-C_8$ alkyl, $-C_3-C_8$ carbocycle, $aryl$, $-C_1-C_8$ alkyl- $aryl$, $-C_1-C_8$ alkyl- $(C_3-C_8$ carbocycle), $-C_3-C_8$ heterocycle or $-C_1-C_8$ alkyl- $(C_3-C_8$ heterocycle);

each R^8 is independently selected from $-H$, $-OH$, $-C_1-C_8$ alkyl, $-C_3-C_8$ carbocycle, $-O-alkyl-(C_1-C_8$ carbocycle) or $-O-(C_1-C_8$ alkyl);

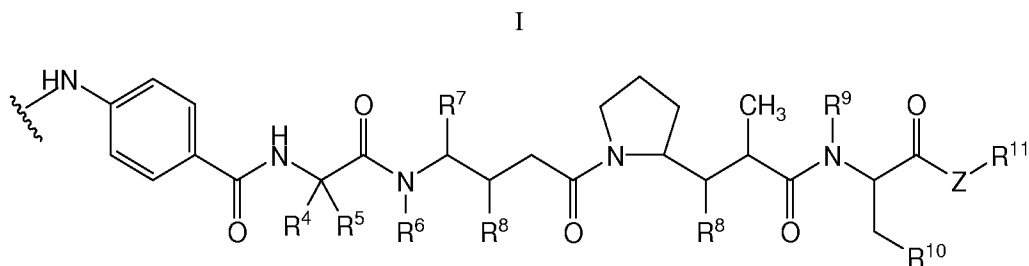
R^9 is $-H$ or $-C_1-C_8$ alkyl;

R^{10} is $aryl$ or $-C_3-C_8$ heterocycle;

Z is $-O-$, $-S-$, $-NH-$, or $-NR^{12}-$ where R^{12} is C_1-C_8 alkyl or $aryl$; and

R^{11} is $-H$, C_1-C_8 alkyl, $aryl$, $-C_3-C_8$ heterocycle, $-(CH_2CH_2O)_r-H$, $-(CH_2CH_2O)_r-CH_3$, or $-(CH_2CH_2O)_r-CH_2CH_2C(O)OH$; wherein r is an integer ranging from 1-10.

[0135] In some embodiments, the Drug unit is of the following formula:



wherein, independently at each location:

R^4 is -H, -C₁-C₈ alkyl, -C₃-C₈ carbocycle, -aryl, -C₁-C₈ alkyl-aryl, -C₁-C₈ alkyl-(C₃-C₈ carbocycle), -C₃-C₈ heterocycle or -C₁-C₈ alkyl-(C₃-C₈ heterocycle), and R^5 is -H or -methyl; or R^4 and R^5 jointly have the formula $-(CR^aR^b)_n-$, wherein R^a and R^b are independently selected from -H, -C₁-C₈ alkyl or -C₃-C₈ carbocycle and n is selected from 2, 3, 4, 5 or 6, and form a ring with the carbon atom to which they are attached;

R^6 is -H or -C₁-C₈ alkyl;

R^7 is -H, -C₁-C₈ alkyl, -C₃-C₈ carbocycle, aryl, -C₁-C₈ alkyl-aryl, -C₁-C₈ alkyl-(C₃-C₈ carbocycle), -C₃-C₈ heterocycle or -C₁-C₈ alkyl-(C₃-C₈ heterocycle);

each R^8 is independently selected from -H, -OH, -C₁-C₈ alkyl, -C₃-C₈ carbocycle, -O-alkyl-(C₁-C₈ carbocycle) or -O-(C₁-C₈ alkyl);

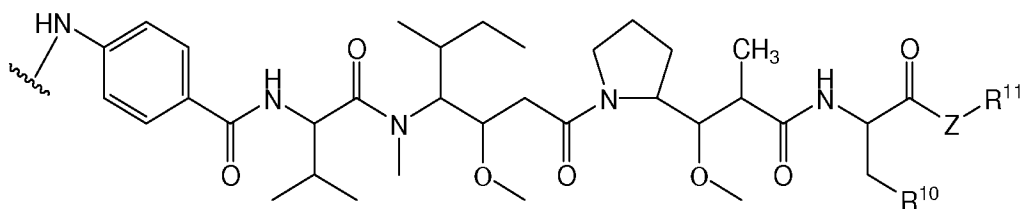
R^9 is -H or -C₁-C₈ alkyl;

R^{10} is aryl or -C₃-C₈ heterocycle;

Z is -O-, -S-, -NH-, or -NR¹²- where R^{12} is C₁-C₈ alkyl or aryl; and

R^{11} is -H, C₁-C₈ alkyl, aryl, -C₃-C₈ heterocycle, $-(CH_2CH_2O)_r-H$, $-(CH_2CH_2O)_r-CH_3$, or $-(CH_2CH_2O)_r-CH_2CH_2C(O)OH$; wherein r is an integer ranging from 1-10.

[0136] In some embodiments, the Drug unit is of the following formula:



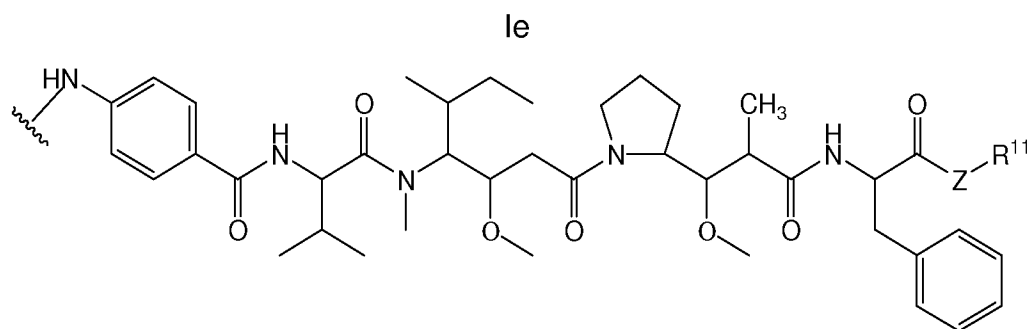
wherein, independently at each location:

R^{10} is selected from aryl group or $-C_3-C_8$ heterocycle;

Z is $-O-$, $-S-$, $-NH-$, or $-NR^{12}-$ where R^{12} is C_1-C_8 alkyl or aryl; and

R^{11} is selected from $-H$, C_1-C_8 alkyl, aryl, $-C_3-C_8$ heterocycle, $-(CH_2CH_2O)_r-H$, $-(CH_2CH_2O)_r-CH_3$, or $-(CH_2CH_2O)_r-CH_2CH_2C(O)OH$; wherein r is an integer ranging from 1-10.

[0137] In some embodiments, the Drug unit is of the following formula:

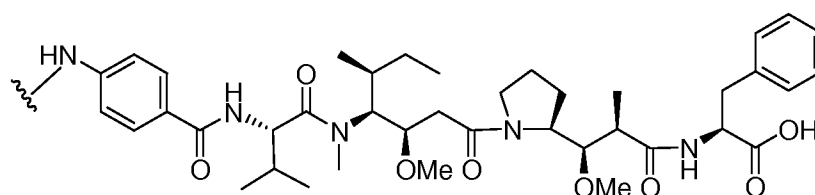


wherein:

Z is $-O-$, $-S-$, $-NH-$, or $-NR^{12}-$ where R^{12} is C_1-C_8 alkyl or aryl; and

R^{11} is $-H$, C_1-C_8 alkyl, aryl, $-C_3-C_8$ heterocycle, $-(CH_2CH_2O)_r-H$, $-(CH_2CH_2O)_r-CH_3$, or $-(CH_2CH_2O)_r-CH_2CH_2C(O)OH$; wherein r is an integer ranging from 1-10.

[0138] In some embodiments, the Drug unit is of the following formula:



[0139] In some embodiments, the Drug is not a radioisotope or is not radiolabeled. In some embodiments, the Drug unit is not TZT-1027.

[0140] In certain embodiments, the Drug is an antimetabolite. The antimetabolite can be, for example, a purine antagonist (*e.g.*, azothioprine or mycophenolate mofetil), a dihydrofolate reductase inhibitor (*e.g.*, methotrexate), acyclovir, gancyclovir, zidovudine, vidarabine, ribavarin, azidothymidine, cytidine arabinoside, amantadine, dideoxyuridine, iododeoxyuridine, poscarnet, or trifluridine.

[0141] In other embodiments, the Drug is tacrolimus, cyclosporine or rapamycin. In further embodiments, the Drug is aldesleukin, alemtuzumab, alitretinoin, allopurinol, altretamine, amifostine, anastrozole, arsenic trioxide, bexarotene, bexarotene, calusterone, capecitabine, celecoxib, cladribine, Darbepoetin alfa, Denileukin diftitox, dexrazoxane, dromostanolone propionate, epirubicin, Epoetin alfa, estramustine, exemestane, Filgrastim, floxuridine, fludarabine, fulvestrant, gemcitabine, gemtuzumab ozogamicin, goserelin, idarubicin, ifosfamide, imatinib mesylate, Interferon alfa-2a, irinotecan, letrozole, leucovorin, levamisole, meclorethamine or nitrogen mustard, megestrol, mesna, methotrexate, methoxsalen, mitomycin C, mitotane, nandrolone phenpropionate, oprelvekin, oxaliplatin, pamidronate, pegademase, pegaspargase, pegfilgrastim, pentostatin, pipobroman, plicamycin, porfimer sodium, procarbazine, quinacrine, rasburicase, Rituximab, Sargramostim, streptozocin, tamoxifen, temozolomide, teniposide, testolactone, thioguanine, toremifene, Tositumomab, Trastuzumab, tretinoin, uracil mustard, valrubicin, vinblastine, vincristine, vinorelbine and zoledronate.

[0142] In some embodiments, the Drug moiety is an immunomodulatory agent. The immunomodulatory agent can be, for example, gancyclovir, etanercept, tacrolimus, cyclosporine, rapamycin, cyclophosphamide, azathioprine, mycophenolate mofetil or methotrexate. Alternatively, the immunomodulatory agent can be, for example, a glucocorticoid (*e.g.*, cortisol or aldosterone) or a glucocorticoid analogue (*e.g.*, prednisone or dexamethasone).

[0143] In some embodiments, the immunomodulatory agent is an anti-inflammatory agent, such as arylcarboxylic derivatives, pyrazole-containing derivatives, oxicam derivatives and nicotinic acid derivatives. Classes of anti-inflammatory agents include, for example, cyclooxygenase inhibitors, 5-lipoxygenase inhibitors, and leukotriene receptor antagonists.

[0144] Suitable cyclooxygenase inhibitors include meclofenamic acid, mefenamic acid, carprofen, diclofenac, diflunisal, fenbufen, fenoprofen, ibuprofen, indomethacin, ketoprofen, nabumetone, naproxen, sulindac, tenoxicam, tolmetin, and acetylsalicylic acid.

[0145] Suitable lipoxygenase inhibitors include redox inhibitors (*e.g.*, catechol butane derivatives, nordihydroguaiaretic acid (NDGA), masoprocol, phenidone, lanopalen, indazolinones, naphazatrom, benzofuranol, alkylhydroxylamine), and non-redox inhibitors (*e.g.*, hydroxythiazoles, methoxyalkylthiazoles, benzopyrans and derivatives thereof, methoxytetrahydropyran, boswellic acids and acetylated derivatives of boswellic acids, and quinolinemethoxyphenylacetic acids substituted with cycloalkyl radicals), and precursors of redox inhibitors.

[0146] Other suitable lipoxygenase inhibitors include antioxidants (*e.g.*, phenols, propyl gallate, flavonoids and/or naturally occurring substrates containing flavonoids, hydroxylated derivatives of the flavones, flavonol, dihydroquercetin, luteolin, galangin, orobol, derivatives of chalcone, 4,2',4'-trihydroxychalcone, ortho-aminophenols, N-hydroxyureas, benzofuranols, ebselen and species that increase the activity of the reducing selenoenzymes), iron chelating agents (*e.g.*, hydroxamic acids and derivatives thereof, N-hydroxyureas, 2-benzyl-1-naphthol, catechols, hydroxylamines, carnosol trolox C, catechol, naphthol, sulfasalazine, zyleuton, 5-hydroxyanthranilic acid and 4-(omega-arylalkyl)phenylalkanoic acids), imidazole-containing compounds (*e.g.*, ketoconazole and itraconazole), phenothiazines, and benzopyran derivatives.

[0147] Yet other suitable lipoxygenase inhibitors include inhibitors of eicosanoids (*e.g.*, octadecatetraenoic, eicosatetraenoic, docosapentaenoic, eicosahexaenoic and docosahexaenoic acids and esters thereof, PGE1 (prostaglandin E1), PGA2 (prostaglandin A2), viprostol, 15-monohydroxyeicosatetraenoic, 15-monohydroxy-eicosatrienoic and 15-monohydroxyeicosapentaenoic acids, and leukotrienes B5, C5 and D5), compounds interfering with calcium flows, phenothiazines, diphenylbutylamines, verapamil, fuscoidin, curcumin, chlorogenic acid, caffeic acid, 5,8,11,14-eicosatetrayenoic acid (ETYA), hydroxyphenylretinamide, lonapalen, esculin, diethylcarbamazine, phenantrolin, baicalein, proxidromil, thioethers, diallyl sulfide and di-(1-propenyl) sulfide.

[0148] Leukotriene receptor antagonists include calcitriol, ontazolast, Bayer Bay-x-1005, Ciba-Geigy CGS-25019C, ebselen, Leo Denmark ETH-615, Lilly LY-293111, Ono ONO-4057, Terumo TMK-688, Boehringer Ingelheim BI-RM-270, Lilly LY 213024, Lilly LY 264086, Lilly LY 292728, Ono ONO LB457, Pfizer 105696, Purdue Frederick PF 10042, Rhone-Poulenc Rorer RP 66153, SmithKline Beecham SB-201146, SmithKline Beecham SB-201993, SmithKline Beecham SB-209247, Searle SC-53228, Sumitomo SM 15178, American Home Products WAY 121006, Bayer Bay-o-8276, Warner-Lambert CI-987, Warner-Lambert CI-987BPC-15LY 223982, Lilly LY 233569, Lilly LY-255283, MacroNex MNX-160, Merck and Co. MK-591, Merck and Co. MK-886, Ono ONO-LB-448, Purdue Frederick PF-5901, Rhone-Poulenc Rorer RG 14893, Rhone-Poulenc Rorer RP 66364, Rhone-Poulenc Rorer RP 69698, Shionogi S-2474, Searle SC-41930, Searle SC-50505, Searle SC-51146, Searle SC-52798, SmithKline Beecham SK&F-104493, Leo Denmark SR-2566, Tanabe T-757 and Teijin TEI-1338.

[0149] Methods of determining whether a Drug or Ligand-Drug conjugate compound exerts a cytostatic or cytotoxic effect on a cell are known. Illustrative examples of such methods for Ligand Drug conjugate compound are described *infra*. Generally, the cytotoxic or cytostatic activity of a Ligand Drug conjugate compound can be measured by: exposing mammalian cells expressing a target protein of the

Ligand Drug conjugate compound in a cell culture medium; culturing the cells for a period from about 6 hours to about 5 days; and measuring cell viability. Cell-based *in vitro* assays can be used to measure viability (proliferation), cytotoxicity, and induction of apoptosis (caspase activation) of the Ligand Drug conjugate compound.

[0150] For determining whether a Ligand Drug conjugate compound exerts a cytostatic effect, a thymidine incorporation assay may be used. For example, cancer cells expressing a target antigen at a density of 5,000 cells/well of a 96-well plated can be cultured for a 72-hour period and exposed to 0.5 μ Ci of ^{3} H-thymidine during the final 8 hours of the 72-hour period. The incorporation of ^{3} H-thymidine into cells of the culture is measured in the presence and absence of the Ligand Drug conjugate compound.

[0151] For determining cytotoxicity, necrosis or apoptosis (programmed cell death) can be measured. Necrosis is typically accompanied by increased permeability of the plasma membrane; swelling of the cell, and rupture of the plasma membrane. Apoptosis is typically characterized by membrane blebbing, condensation of cytoplasm, and the activation of endogenous endonucleases. Determination of any of these effects on cancer cells indicates that a Ligand Drug conjugate compound is useful in the treatment of cancers.

[0152] Cell viability can be measured by determining in a cell the uptake of a dye such as neutral red, trypan blue, or ALAMARTM blue (see, e.g., Page *et al.*, 1993, *Intl. J. Oncology* 3:473-476). In such an assay, the cells are incubated in media containing the dye, the cells are washed, and the remaining dye, reflecting cellular uptake of the dye, is measured spectrophotometrically. The protein-binding dye sulforhodamine B (SRB) can also be used to measure cytotoxicity (Skehan *et al.*, 1990, *J. Natl. Cancer Inst.* 82:1107-12).

[0153] Alternatively, a tetrazolium salt, such as MTT, is used in a quantitative colorimetric assay for mammalian cell survival and proliferation by detecting living, but not dead, cells (see, e.g., Mosmann, 1983, *J. Immunol. Methods* 65:55-63).

[0154] Apoptosis can be quantitated by measuring, for example, DNA fragmentation. Commercial photometric methods for the quantitative *in vitro* determination of DNA fragmentation are available. Examples of such assays, including TUNEL (which detects incorporation of labeled nucleotides in fragmented DNA) and ELISA-based assays, are described in *Biochemica*, 1999, no. 2, pp. 34-37 (Roche Molecular Biochemicals).

[0155] Apoptosis can also be determined by measuring morphological changes in a cell. For example, as with necrosis, loss of plasma membrane integrity can be determined by measuring uptake of certain dyes (*e.g.*, a fluorescent dye such as, for example, acridine orange or ethidium bromide). A method for measuring apoptotic cell number has been described by Duke and Cohen, *Current Protocols in Immunology* (Coligan *et al.* eds., 1992, pp. 3.17.1-3.17.16). Cells also can be labeled with a DNA dye (*e.g.*, acridine orange, ethidium bromide, or propidium iodide) and the cells observed for chromatin condensation and margination along the inner nuclear membrane. Other morphological changes that can be measured to determine apoptosis include, *e.g.*, cytoplasmic condensation, increased membrane blebbing, and cellular shrinkage.

[0156] The presence of apoptotic cells can be measured in both the attached and “floating” compartments of the cultures. For example, both compartments can be collected by removing the supernatant, trypsinizing the attached cells, combining the preparations following a centrifugation wash step (*e.g.*, 10 minutes at 2000 rpm), and detecting apoptosis (*e.g.*, by measuring DNA fragmentation). (See, *e.g.*, Piazza *et al.*, 1995, *Cancer Research* 55:3110-16).

[0157] The effects of Ligand Drug conjugate compounds can be tested or validated in animal models. A number of established animal models of cancers are known to the skilled artisan, any of which can be used to assay the efficacy of a Ligand Drug conjugate compound. Non-limiting examples of such models are described *infra*. Moreover, small animal models to examine the *in vivo* efficacies of Ligand Drug conjugate compounds can be created by implanting human tumor cell

lines into appropriate immunodeficient rodent strains, *e.g.*, athymic nude mice or SCID mice.

LIGAND DRUG CONJUGATE COMPOUNDS

[0158] The Ligand unit (L) has at least one functional group that can form a bond with a functional group of a Linker unit. Useful functional groups that can be present on a Ligand unit, either naturally, via chemical manipulation or via engineering, include, but are not limited to, sulfhydryl (-SH), amino, hydroxyl, carboxy, the anomeric hydroxyl group of a carbohydrate, and carboxyl. In some embodiments, a Ligand unit functional group is a sulfhydryl group. The sulfhydryl group is typically a solvent accessible sulfhydryl group, such as a solvent accessible sulfhydryl group on a cysteine residue. Sulfhydryl groups can be generated by reduction of an intramolecular or intermolecular disulfide bond of a Ligand. Sulfhydryl groups also can be generated by reaction of an amino group of a lysine moiety of a Ligand using 2-iminothiolane (Traut's reagent) or another sulfhydryl generating reagent.

[0159] In some embodiments, one or more sulfhydryl groups are engineered into a Ligand unit, such as by amino acid substitution. For example, a sulfhydryl group can be introduced into a Ligand unit. In some embodiments, a sulfhydryl group is introduced by an amino acid substitution of serine or threonine to a cysteine residue, and/or by addition of a cysteine residue into a Ligand unit (an engineered cysteine residue). In some embodiments, the cysteine residue is an internal cysteine residue, *i.e.*, not located at the N-terminus or C-terminus of the Ligand moiety.

[0160] In an exemplary embodiment, a cysteine residue can be engineered into an antibody heavy or light variable region (*e.g.*, of an antibody fragment, such as a diabody) by amino acid substitution. The amino acid substitution is typically introduced into the framework region and is located distal to the epitope-binding face of the variable region. For example, the amino acid substitution can be at least 10 angstroms, at least 20 angstroms or at least 25 angstroms from the epitope-binding face or the CDRs. Suitable positions for substitution of a cysteine residue can be

determined based on the known or predicted three dimensional structures of antibody variable regions. (See generally Holliger and Hudson, 2005, *Nature BioTechnology* 23(9):1126-1136.) In exemplary embodiments, a serine to cysteine amino acid substitution is introduced at amino acid position 84 of the V_H region and/or position 14 of the V_L region (according to the numbering system of Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th edition, (Bethesda, MD, NIH) 1991).

[0161] To control the number of Drug or Linker unit-Drug units attached to a Ligand unit, one or more cysteine residues can be eliminated by amino acid substitution. For example, the number of solvent accessible cysteine residues in an immunoglobulin hinge region can be reduced by amino acid substitution of cysteine to serine residues.

[0162] In some embodiments, a Ligand unit contains 1, 2, 3, 4, 5, 6 7 or 8 solvent-accessible cysteine residues. In some embodiments, a Ligand unit contains 2 or 4 solvent-accessible cysteine residues.

COMPOSITIONS AND METHODS OF ADMINISTRATION

[0163] The Ligand Drug conjugate compounds can be in any form that allows for the compound to be administered to a patient. For example, the compound can be in the form of a liquid or solid. Typical routes of administration include, without limitation, parenteral, topical, oral, sublingual, rectal, vaginal, ocular, intra-tumor, and intranasal. Parenteral administration includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. In one aspect, the compositions are administered parenterally. In yet another aspect, the compounds are administered intravenously.

[0164] Pharmaceutical compositions can be formulated so as to allow a compound to be bioavailable upon administration of the composition to a patient. Compositions can take the form of one or more dosage units, where for example, a tablet can be a single dosage unit.

[0165] Materials used in preparing the pharmaceutical compositions can be non-toxic in the amounts used. It will be evident to those of ordinary skill in the art that the optimal dosage of the active ingredient(s) in the pharmaceutical composition will depend on a variety of factors. Relevant factors include, without limitation, the type of animal (*e.g.*, human), the particular form of the compound, the manner of administration, and the composition employed.

[0166] The pharmaceutically acceptable carrier or vehicle can be particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) can be liquid, with the compositions being, for example, an oral syrup or injectable liquid.

[0167] When intended for oral administration, the composition is preferably in solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

[0168] As a solid composition for oral administration, the composition can be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition typically contains one or more inert diluents. In addition, one or more of the following can be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrans, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin, a flavoring agent such as peppermint, methyl salicylate or orange flavoring, and a coloring agent.

[0169] When the composition is in the form of a capsule, *e.g.*, a gelatin capsule, it can contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol, cyclodextrin or a fatty oil.

[0170] The composition can be in the form of a liquid, *e.g.*, an elixir, syrup, solution, emulsion or suspension. The liquid can be useful for oral administration or for delivery by injection. When intended for oral administration, a composition can

comprise one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition for administration by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent can also be included.

[0171] The liquid compositions, whether they are solutions, suspensions or other like form, can also include one or more of the following: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which can serve as the solvent or suspending medium, polyethylene glycols, glycerin, cyclodextrin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as amino acids, acetates, citrates or phosphates; detergents, such as nonionic surfactants, polyols; and agents for the adjustment of tonicity such as sodium chloride or dextrose. A parenteral composition can be enclosed in ampoule, a disposable syringe or a multiple-dose vial made of glass, plastic or other material. Physiological saline is an exemplary adjuvant. An injectable composition is preferably sterile.

[0172] The amount of the compound that is effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the compositions will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

[0173] The compositions comprise an effective amount of a compound such that a suitable dosage will be obtained. Typically, this amount is at least about 0.01% of a compound by weight of the composition. When intended for oral administration, this amount can be varied to range from about 0.1% to about 80% by

weight of the composition. In one aspect, oral compositions can comprise from about 4% to about 50% of the compound by weight of the composition. In yet another aspect, present compositions are prepared so that a parenteral dosage unit contains from about 0.01% to about 2% by weight of the compound.

[0174] For intravenous administration, the composition can comprise from about 0.01 to about 100 mg of a compound per kg of the animal's body weight. In one aspect, the composition can include from about 1 to about 100 mg of a compound per kg of the animal's body weight. In another aspect, the amount administered will be in the range from about 0.1 to about 25 mg/kg of body weight of a compound.

[0175] Generally, the dosage of a compound administered to a patient is typically about 0.01 mg/kg to about 100 mg/kg of the subject's body weight. In some embodiments, the dosage administered to a patient is between about 0.01 mg/kg to about 15 mg/kg of the subject's body weight. In some embodiments, the dosage administered to a patient is between about 0.1 mg/kg and about 15 mg/kg of the subject's body weight. In some embodiments, the dosage administered to a patient is between about 0.1 mg/kg and about 20 mg/kg of the subject's body weight. In some embodiments, the dosage administered is between about 0.1 mg/kg to about 5 mg/kg or about 0.1 mg/kg to about 10 mg/kg of the subject's body weight. In some embodiments, the dosage administered is between about 1 mg/kg to about 15 mg/kg of the subject's body weight. In some embodiments, the dosage administered is between about 1 mg/kg to about 10 mg/kg of the subject's body weight.

[0176] The compound or compositions can be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.). Administration can be systemic or local. Various delivery systems are known, e.g., encapsulation in liposomes, microparticles, microcapsules, capsules, etc., and can be used to administer a compound. In certain embodiments, more than one compounds or composition is administered to a patient.

[0177] In specific embodiments, it can be desirable to administer one or more compounds or compositions locally to the area in need of treatment. This can be achieved, for example, and not by way of limitation, by local infusion during surgery; topical application, *e.g.*, in conjunction with a wound dressing after surgery; by injection; by means of a catheter; by means of a suppository; or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a cancer, tumor or neoplastic or pre-neoplastic tissue. In another embodiment, administration can be by direct injection at the site (or former site) of a manifestation of an autoimmune disease.

[0178] In certain embodiments, it can be desirable to introduce one or more compounds or compositions into the central nervous system by any suitable route, including intraventricular and intrathecal injection. Intraventricular injection can be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

[0179] In yet another embodiment, the compound or compositions can be delivered in a controlled release system, such as but not limited to, a pump or various polymeric materials can be used. In yet another embodiment, a controlled-release system can be placed in proximity of the target of the compound or compositions, *e.g.*, the brain, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer (1990, *Science* 249:1527-1533) can be used.

[0180] The term “carrier” refers to a diluent, adjuvant or excipient, with which a compound is administered. Such pharmaceutical carriers can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The carriers can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica,

urea, and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents can be used. In one embodiment, when administered to a patient, the compound or compositions and pharmaceutically acceptable carriers are sterile. Water is an exemplary carrier when the compounds are administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

[0181] The present compositions can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, suspensions, or any other form suitable for use. Other examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[0182] In an embodiment, the compounds are formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to animals, particularly human beings. Typically, the carriers or vehicles for intravenous administration are sterile isotonic aqueous buffer solutions. Where necessary, the compositions can also include a solubilizing agent. Compositions for intravenous administration can optionally comprise a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where compound is to be administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the compound is administered by injection, an ampoule of sterile

water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

[0183] Compositions for oral delivery can be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions can contain one or more optionally agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions can be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for orally administered compounds. In these later platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time-delay material such as glycerol monostearate or glycerol stearate can also be used.

[0184] The compositions can be intended for topical administration, in which case the carrier may be in the form of a solution, emulsion, ointment or gel base. If intended for transdermal administration, the composition can be in the form of a transdermal patch or an iontophoresis device. Topical formulations can comprise a concentration of a compound of from about 0.05% to about 50% w/v (weight per unit volume of composition), in another aspect, from 0.1% to 10% w/v.

[0185] The composition can include various materials that modify the physical form of a solid or liquid dosage unit. For example, the composition can include materials that form a coating shell around the active ingredients. The materials that form the coating shell are typically inert, and can be selected from, for

example, sugar, shellac, and other enteric coating agents. Alternatively, the active ingredients can be encased in a gelatin capsule.

[0186] Whether in solid or liquid form, the present compositions can include a pharmacological agent used in the treatment of cancer, an autoimmune disease or an infectious disease.

THERAPEUTIC USES OF THE CONJUGATE COMPOUNDS

[0187] The Ligand Drug conjugate compounds are useful for treating cancer and other diseases in which Glypican-3 is expressed or overexpressed, relative to normal (*i.e.*, non-cancerous tissue). In some embodiments, the conjugate compounds are administered alone. In other embodiments, the conjugate compounds are co-administered with another therapeutic agent. In some embodiments, the conjugate compounds coadministered with standard of care chemotherapeutics.

TREATMENT OF CANCER

[0188] The conjugate compounds are useful for inhibiting the multiplication of a tumor cell or cancer cell, causing apoptosis in a tumor or cancer cell, or for treating cancer in a patient. The compounds can be used accordingly in a variety of settings for the treatment of cancers. The conjugate compounds provide conjugation-specific tumor or cancer targeting, thus reducing potential toxicity of the Drug. The linker stabilizes the conjugate compounds in blood, yet is cleavable by proteases within the cell, liberating the Drug(s).

[0189] In some embodiments, the cancer (or carcinoma) is a cancer expressing or over-expressing GPC-3 (*i.e.*, relative to normal tissue), such as liver (hepatocellular), breast, lung (*e.g.*, squamous cell carcinoma or adenocarcinoma), uterine (*e.g.*, endometrial adenocarcinoma), ovarian (*e.g.*, ovarian adenocarcinoma), skin (*e.g.*, melanoma), colorectal, gastric, neuroblastoma, Wilm's tumor, teratocarcinoma or muscle (rhabdomyosarcoma).

MULTI-MODALITY THERAPY FOR CANCER

[0190] Cancers, including, but not limited to, a tumor, metastasis, or other disease or disorder characterized by uncontrolled cell growth, can be treated or prevented by administration of a conjugate compound according to the present invention.

[0191] In some embodiments, methods for treating or preventing cancer are provided, including administering to a patient in need thereof an effective amount of a conjugate compound and a chemotherapeutic agent. In one embodiment the chemotherapeutic agent is that with which treatment of the cancer has not been found to be refractory. In another embodiment, the chemotherapeutic agent is that with which the treatment of cancer has been found to be refractory. The conjugate compounds can be administered to a patient that has also undergone surgery as treatment for the cancer.

[0192] In one embodiment, the additional treatment is radiation therapy.

[0193] In a specific embodiment, the conjugate compound is administered concurrently with the chemotherapeutic agent or with radiation therapy. In another specific embodiment, the chemotherapeutic agent or radiation therapy is administered prior or subsequent to administration of a conjugate compound. In some embodiments, the chemotherapeutic agent or radiation therapy is administered at least an hour, five hours, 12 hours, a day, a week, a month, several months (*e.g.*, up to three months), prior or subsequent to administration of a conjugate compound.

[0194] A chemotherapeutic agent can be administered over a series of sessions. Any one or a combination of the following chemotherapeutic agents can be administered (*see infra*). With respect to radiation, any radiation therapy protocol can be used depending upon the type of cancer to be treated. For example, but not by way of limitation, x-ray radiation can be administered; in particular, high-energy megavoltage (radiation of greater than 1 MeV energy) can be used for deep tumors, and electron beam and orthovoltage x-ray radiation can be used. Gamma-ray

emitting radioisotopes, such as radioactive isotopes of radium, cobalt and other elements, can also be administered.

[0195] Additionally, methods of treatment of cancer with a conjugate compound are provided as an alternative to chemotherapy or radiation therapy where the chemotherapy or the radiation therapy has proven or can prove too toxic, *e.g.*, results in unacceptable or unbearable side effects, for the subject being treated. The subject being treated can, optionally, be treated with another cancer treatment such as surgery, radiation therapy or chemotherapy, depending on which treatment is found to be acceptable or bearable.

[0196] The conjugate compounds can also be used in an *in vitro* or *ex vivo* fashion, such as for the treatment of certain cancers.

MULTI-DRUG THERAPY FOR CANCER

[0197] Methods for treating cancer including administering to a patient in need thereof an effective amount of a conjugate compound and another therapeutic agent that is an anti-cancer agent are disclosed.

[0198] Suitable anticancer agents include, but are not limited to, methotrexate, taxol, L-asparaginase, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, cisplatin, carboplatin, mitomycin, dacarbazine, procarbazine, topotecan, nitrogen mustards, cytoxan, etoposide, 5-fluorouracil, BCNU, irinotecan, camptothecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, asparaginase, vinblastine, vincristine, vinorelbine, paclitaxel, and docetaxel.

[0199] In some embodiments, the anti-cancer agent includes, but is not limited to, a drug listed in Table 1.

Table 1

<u>Generic Name</u>	<u>Tradename</u>
Aldesleukin	Proleukin
Alemtuzumab	Campath
alitretinoin	Panretin

allopurinol	Zyloprim
altretamine	Hexalen
amifostine	Ethyol
anastrozole	Arimidex
arsenic trioxide	Trisenox
Asparaginase	Elspar
BCG Live	TICE BCG
bexarotene capsules	Targretin
bexarotene gel	Targretin
bleomycin	Blenoxane
busulfan intravenous	Busulfex
busulfan oral	Myleran
calusterone	Methosarb
capecitabine	Xeloda
carboplatin	Paraplatin
carmustine	BCNU, BiCNU
carmustine with	Gliadel Wafer
Polifeprosan 20 Implant	
Celecoxib	Celebrex
chlorambucil	Leukeran
cisplatin	Platinol
cladribine	Leustatin, 2-CdA
cyclophosphamide	Cytoxan
Cytarabine	Cytosar-U
cytarabine liposomal	DepoCyt
dacarbazine	DTIC-Dome
dactinomycin, actinomycin D	Cosmegen
Darbepoetin alfa	Aranesp
daunorubicin liposomal	DanuoXome
daunorubicin, daunomycin	Daunorubicin
daunorubicin, daunomycin	Cerubidine
Denileukin diftitox	Ontak
dexrazoxane	Zinecard
docetaxel	Taxotere
doxorubicin	Adriamycin, Rubex
doxorubicin	Adriamycin PFS Injection
	intravenous injection
doxorubicin liposomal	Doxil
Dromostanolone Propionate	Dromostanolone
Dromostanolone Propionate	Masterone Injection
Elliott's B Solution	Elliott's B Solution
epirubicin	Ellence
Epoetin alfa	epogen
estramustine	Emcyt
etoposide phosphate	Etopophos
etoposide, VP-16	Vepesid
exemestane	Aromasin
Filgrastim	Neupogen
floxuridine (intraarterial)	FUDR
fludarabine	Fludara
fluorouracil, 5-FU	Adrucil
fulvestrant	Faslodex
gemcitabine	Gemzar
gemtuzumab ozogamicin	Mylotarg
goserelin acetate	Zoladex Implant

goserelin acetate	Zoladex
hydroxyurea	Hydrea
Ibritumomab Tiuxetan	Zevalin
idarubicin	Idamycin
ifosfamide	IFEX
imatinib mesylate	Gleevec
Interferon alfa-2a	Roferon-A
Interferon alfa-2b	Intron A
irinotecan	Camptosar
lenalidomide	Revlimid
letrozole	Femara
leucovorin	Wellcovorin, Leucovorin
levamisole	Ergamisol
lomustine, CCNU	CeeBU
meclorethamine, nitrogen	Mustargen
mustard	
megestrol acetate	Megace
melphalan, L-PAM	Alkeran
melphalan, L-PAM	Alkeran
mercaptopurine, 6-MP	Purinethol
mesna	Mesnex
methotrexate	Methotrexate
methoxsalen	Uvadex
mitomycin C	Mutamycin
mitomycin C	Mitozytrex
mitotane	Lysodren
mitoxantrone	Novantrone
nandrolone phenpropionate	Durabolin-50
Nofetumomab	Verluma
Oprelvekin	Neumega
oxaliplatin	Eloxatin
paclitaxel	Paxene
paclitaxel	Taxol
pamidronate	Aredia
pegademase	Adagen (Pegademase Bovine)
Pegaspargase	Oncaspar
Pegfilgrastim	Neulasta
pentostatin	Nipent
pipobroman	Vercyte
plicamycin, mithramycin	Mithracin
porfimer sodium	Photofrin
procarbazine	Matulane
quinacrine	Atabrine
Rasburicase	Elitek
Rituximab	Rituxan
Sargramostim	Prokine
streptozocin	Zanosar
talc	Sclerosol
tamoxifen	Nolvadex
tamoxifen	Nolvadex
temozolomide	Temodar
teniposide, VM-26	Vumon
testolactone	Teslac
testolactone	Teslac
thalidomide	Thalidomid

thioguanine, 6-TG
 thiotepa
 topotecan
 toremifene
 Tositumomab
 Trastuzumab
 tretinoin, ATRA
 Uracil Mustard
 valrubicin
 vinblastine
 vincristine
 vinorelbine
 vinorelbine
 zoledronate

Thioguanine
 Thioplex
 Hycamtin
 Fareston
 Bexxar
 Herceptin
 Vesanoid
 Uracil Mustard Capsules
 Valstar
 Velban
 Oncovin
 Navelbine
 Navelbine
 Zometa

[0200] In some embodiments, the anti-cancer agent includes, but is not limited to, a drug listed in Table 2.

TABLE 2

Alkylating agents	
Nitrogen mustards:	Cyclophosphamide ifosfamide trofosfamide chlorambucil melphalan
Nitrosoureas:	carmustine (BCNU) lomustine (CCNU)
Alkylsulphonates	busulfan treosulfan
Triazenes:	decarbazine
Platinum containing compounds:	cisplatin carboplatin
Plant Alkaloids	
Vinca alkaloids:	vincristine vinblastine vindesine vinorelbine
Taxanes:	paclitaxel docetaxol
DNA Topoisomerase Inhibitors	
Epipodophyllins:	etoposide teniposide topotecan 9-aminocamptothecin camptothecin

Alkylating agents	
	crisnatol
mitomycins:	mitomycin C
Anti-metabolites	
Anti-folates:	
DHFR inhibitors:	methotrexate trimetrexate
IMP dehydrogenase Inhibitors:	mycophenolic acid tiazofurin ribavirin EICAR
Ribonucleotide reductase Inhibitors:	hydroxyurea deferroxamine
Pyrimidine analogs:	
Uracil analogs	5-Fluorouracil
	floxuridine doxifluridine ratitrexed
Cytosine analogs	cytarabine (ara C) cytosine arabinoside fludarabine
Purine analogs:	mercaptopurine thioguanine
Hormonal therapies:	
Receptor antagonists:	
Anti-estrogen	tamoxifen raloxifene megestrol
LHRH agonists:	goserelin leuprolide acetate
Anti-androgens:	flutamide bicalutamide
Retinoids/Deltoids	
Vitamin D3 analogs:	EB 1089 CB 1093 KH 1060
Photodynamic therapies:	vertoporphin (BPD-MA) phthalocyanine photosensitizer Pc4 demethoxy-hypocrellin A (2BA-2-DMHA)
Cytokines:	Interferon- α Interferon- γ tumor necrosis factor
Others:	Gemcitabine

Alkylating agents	
	Velcade Revamid Thalomid
Isoprenylation inhibitors:	Lovastatin
Dopaminergic neurotoxins:	1-methyl-4-phenylpyridinium ion
Cell cycle inhibitors:	staurosporine
Actinomycins:	Actinomycin D
	dactinomycin
Bleomycins:	bleomycin A2 bleomycin B2 peplomycin
Anthracyclines:	daunorubicin Doxorubicin (adriamycin) idarubicin epirubicin pirarubicin zorubicin mtoxantrone
MDR inhibitors:	verapamil
Ca ²⁺ ATPase inhibitors:	thapsigargin

Examples

[0201] The invention is further described in the following examples, which are not intended to limit the scope of the invention.

Example 1

In Vitro Characterization of anti-Glypican-3 ADC's.

[0202] The cytotoxic activities of the anti-GPC-3 antibodies (1G12, 8H5) with a secondary antibody conjugate were evaluated on the Glypican-3 positive HepG2 and Hep3B cell lines. (The unconjugated antibodies were not observed to have cytotoxic activity on these cell lines.) The secondary antibody, reactive with mouse antibody, was conjugated to eight molecules of drug. (See Klussman *et al.*, 2004, *Bioconjugate Chemistry* 15:765-773, for details.) The ratio of primary to secondary antibody was 1:2.

[0203] Approximately 3,000 - 7,000 cells per well in 150 μ l of culture medium were treated with a single dose of each antibody and ADC. Cytotoxicity assays were carried out for 96 hours after addition of test compounds. Fifty μ l of resazurin dye were added to each well during the last 4 to 8 hours of the incubation to assess viable cells at the end of culture. Dye reduction was determined by fluorescence spectrometry using the excitation and emission wavelengths of 535 nm and 590 nm, respectively. For analysis, the extent of resazurin reduction by the treated cells was compared to that of the untreated control cells.

[0204] Referring to Figure 1, both primary-secondary mAb combinations exhibited a cytotoxic effect on HepG2 (panel A) and Hep3B (panel B) cells. mAb 1G12 in combination with the secondary antibody exhibited a greater cytotoxic effect than mAb 8H5. Referring to Table 3, the IC₅₀'s for the conjugates are shown.

Table 3

In vitro cytotoxicity

(ng/ml)

Cell line	1G12 + 2° ADC	8H5 + 2° ADC	IgG + 2° ADC
HepG2	141	1412	>10,000
Hep3B	170	933	4266

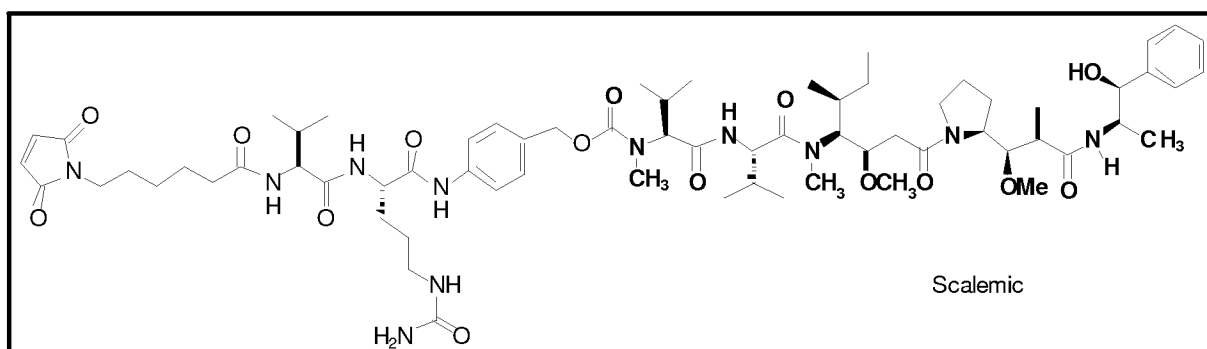
[0205] In contrast, the IgG control had no effect on Hep3B or HepG2 cells over a range of 0.1 to 100,000 ng/ml.

Example 2

Preparation of a Glypican-3 Antibody Drug Conjugate

[0206] One milligram of the 1G12 mAb (BioMosaics catalog # 037, lot # 2575) was provided in a formulation of 50% glycerol. Four cycles of concentration and resuspension in PBS were performed with a MicroCon centrifugal concentration device, with each cycle representing an approximate 50x diafiltration. Assuming a molecular weight of 146 kD and an extinction coefficient of $1.45 \text{ AU} \cdot \text{mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$, the recovery from this procedure was 0.9 mg, or 6.2 nmol of 1G12 at a final concentration of 3.0 mg/mL.

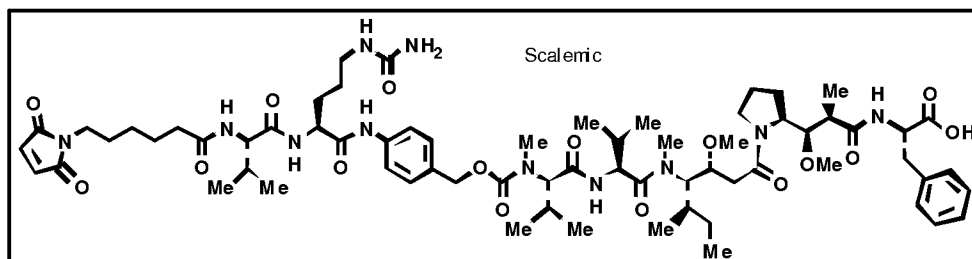
[0207] The auristatins MMAE and MMAF were conjugated to the purified antibody as follows. The concentrated and purified antibody was incubated at 37 °C for 90 minutes with 15 nmol of TCEP (representing a 25% excess of reductant for the desired reduction level of 4 free thiols per antibody) with 1 mM DTPA as a divalent cation scavenger. The reduction level was determined by performing a microscale test conjugation with the following test compound:



The drug loading distribution was characterized by HIC chromatography. This mAb exhibited a reduction pattern occasionally seen with murine antibodies, wherein the distribution is weighted at 0 and 10 drugs per antibody, with 4- and 6-loaded antibody being represented at lower levels. However, the mean drug loading was approximately 4, as desired.

[0208] The partially reduced mAb was conjugated with MMAF by addition of 51 μL of DMSO to the approximately 300 μL of mAb solution, followed by 2.1 μL of

a 17.4 mM DMSO solution of the following compound, maleimidocaproyl-Val-Cit-MMAF (37 nmol).



[0209] The conjugation reaction was allowed to proceed for 45 minutes at 0 °C, at which time an aliquot was analyzed by HIC to confirm complete conjugation. Residual maleimidocaproyl-Val-Cit-MMAF was quenched by addition of 1.8 µL of 100 mM N-acetyl cysteine. The reaction mixture was then diafiltered against PBS five times with a centrifugal concentration device to remove DMSO, unreacted drug, and other small-molecule contaminants resulting from the conjugation process.

Example 3

In Vitro Characterization of anti-Glypican-3 ADC's.

[0210] The cytotoxic activity of the antibody drug conjugate 1G12-mc-vc-MMAF was evaluated on the Glypican-3 positive cell HepG2 and Hep3B cell lines and control cell lines HCT116 and HREC. To evaluate the cytotoxicity, approximately 3,000-7,000 cells per well in 150 μ l of culture medium then treated with a single dose of ADC. Cytotoxicity assays were carried out for 96 hours after addition of test compounds. Fifty μ l of resazurin dye was added to each well during the last 4 to 8 hours of the incubation to assess viable cells at the end of culture. Dye reduction was determined by fluorescence spectrometry using the excitation and emission wavelengths of 535nm and 590nm, respectively. For analysis, the extent of resazurin reduction by the treated cells was compared to that of the untreated control cells.

[0211] Cell surface Glypican-3 expression levels were quantified with a DAKO QIFIKIT flow cytometric indirect immunofluorescence assay (DAKO A/S, Glostrup, Denmark) using 1G12 or 8H5 as the primary antibody. Briefly, cells were detached with versene and washed once with complete media then PBS. 5×10^5 cells/sample were incubated with a saturating concentration (10 $\mu\text{g/ml}$) of primary antibody for 60 minutes at 4°C. After washes, FITC-conjugated secondary antibody (1:50 dilution) was added for 45 minutes at 4°C. QIFIKIT standard beads were simultaneously labeled with the secondary antibody. Binding of antibodies was analyzed by flow cytometry and specific antigen density was calculated by subtracting background antibody equivalent from antibody-binding capacity based on a standard curve of log mean fluorescence intensity versus log antigen binding capacity.

[0212] Referring to Figure 2 and Table 4, conjugate 1G12-mc-vc-MMAF has a lower IC_{50} for Hep3B cells than for HepG2 cells. The IC_{50} of the conjugate was several magnitudes of order lower, than for control cells (HCT116 and HREC cells).

Table 4

In vitro cytotoxicity

Cell line	IC_{50} (ng/ml)	Antibody Binding Capacity (sites/cell)
HepG2	32	150,000
Hep3B	7.5	140,000
HCT116	>10,000	700
HREC	>10,000	0

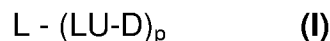
[0213] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

[0214] No license is expressly or implicitly granted to any patent or patent applications referred to or incorporated herein. The discussion above is descriptive, illustrative and exemplary and is not to be taken as limiting the scope defined by any appended claims.

[0215] Various references, including patent applications, patents, and scientific publications, are cited herein, the disclosures of each of which is incorporated herein by reference in its entirety.

Claims:

1. A method for treating target cancer cells expressing human GPC-3 comprising administering to a patient in need thereof an effective amount of a ligand-drug conjugate compound of the following formula:



or a pharmaceutically acceptable salt or solvate thereof;

wherein:

L is a Ligand unit that specifically binds to GPC-3; and

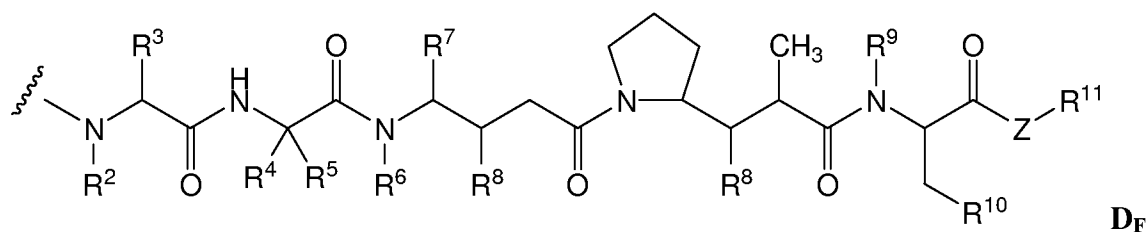
(LU-D) is a Linker unit-Drug unit moiety, wherein:

LU- is a Linker unit, and

-D is a Drug unit having cytostatic or cytotoxic activity against the target cells; and

p is an integer from 1 to about 20.

2. The method of claim 1, wherein the Drug unit (-D) has the following Formula D_F:



wherein, independently at each location:

R² is H or C₁-C₈ alkyl;

R³ is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

R⁴ is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

R⁵ is H or methyl;

or R^4 and R^5 jointly form a carbocyclic ring and have the formula $-(CR^aR^b)_n-$ wherein R^a and R^b are independently selected from H, C_1 - C_8 alkyl or C_3 - C_8 carbocycle and n is selected from 2, 3, 4, 5 or 6;

R^6 is H or C_1 - C_8 alkyl;

R^7 is H, C_1 - C_8 alkyl, C_3 - C_8 carbocycle, aryl, C_1 - C_8 alkyl-aryl, C_1 - C_8 alkyl- $(C_3$ - C_8 carbocycle), C_3 - C_8 heterocycle or C_1 - C_8 alkyl- $(C_3$ - C_8 heterocycle);

each R^8 is independently selected from H, OH, C_1 - C_8 alkyl, C_3 - C_8 carbocycle or O- $(C_1$ - C_8 alkyl);

R^9 is H or C_1 - C_8 alkyl;

R^{10} is aryl or C_3 - C_8 heterocycle;

Z is O, S, NH, or NR^{12} , wherein R^{12} is C_1 - C_8 alkyl;

R^{11} is H, C_1 - C_{20} alkyl, aryl, C_3 - C_8 heterocycle, $-(R^{13}O)_m-R^{14}$, or $-(R^{13}O)_m-CH(R^{15})_2$;

m is an integer ranging from 1-1000;

R^{13} is C_2 - C_8 alkyl;

R^{14} is H or C_1 - C_8 alkyl;

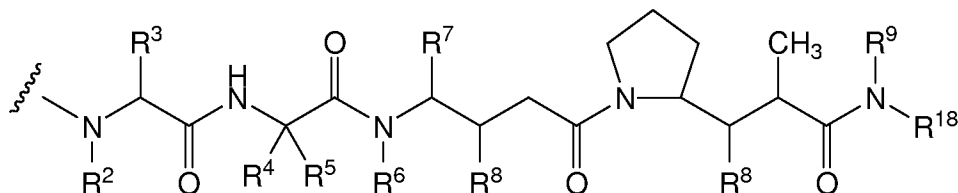
each occurrence of R^{15} is independently H, COOH, $-(CH_2)_n-N(R^{16})_2$, $-(CH_2)_n-SO_3H$, or $-(CH_2)_n-SO_3-C_1$ - C_8 alkyl;

each occurrence of R^{16} is independently H, C_1 - C_8 alkyl, or $-(CH_2)_n$ -COOH;

R^{18} is selected from $-C(R^8)_2-C(R^8)_2$ -aryl, $-C(R^8)_2-C(R^8)_2$ - $(C_3$ - C_8 heterocycle), or $-C(R^8)_2-C(R^8)_2$ - $(C_3$ - C_8 carbocycle); and

p is an integer ranging from 0 to 6.

3. The method of claim 1, wherein the Drug unit (-D) has the following Formula D_E:



wherein, independently at each location:

R^2 is H or C_1-C_8 alkyl;

R^3 is H, C_1-C_8 alkyl, C_3-C_8 carbocycle, aryl, C_1-C_8 alkyl-aryl, C_1-C_8 alkyl- $(C_3-C_8$ carbocycle), C_3-C_8 heterocycle or C_1-C_8 alkyl- $(C_3-C_8$ heterocycle);

R^4 is H, C_1-C_8 alkyl, C_3-C_8 carbocycle, aryl, C_1-C_8 alkyl-aryl, C_1-C_8 alkyl- $(C_3-C_8$ carbocycle), C_3-C_8 heterocycle or C_1-C_8 alkyl- $(C_3-C_8$ heterocycle);

R^5 is H or methyl;

or R^4 and R^5 jointly form a carbocyclic ring and have the formula $-(CR^aR^b)_n-$ wherein R^a and R^b are independently selected from H, C_1-C_8 alkyl or C_3-C_8 carbocycle and n is selected from 2, 3, 4, 5 or 6;

R^6 is H or C_1-C_8 alkyl;

R^7 is H, C_1-C_8 alkyl, C_3-C_8 carbocycle, aryl, C_1-C_8 alkyl-aryl, C_1-C_8 alkyl- $(C_3-C_8$ carbocycle), C_3-C_8 heterocycle or C_1-C_8 alkyl- $(C_3-C_8$ heterocycle);

each R^8 is independently selected from H, OH, C_1-C_8 alkyl, C_3-C_8 carbocycle or $O-(C_1-C_8$ alkyl);

R^9 is H or C_1-C_8 alkyl;

R^{10} is aryl or C_3-C_8 heterocycle;

Z is O, S, NH, or NR^{12} , wherein R^{12} is C_1-C_8 alkyl;

R^{11} is selected from H, C_1-C_{20} alkyl, aryl, C_3-C_8 heterocycle, $-(R^{13}O)_m-$ R^{14} , or $-(R^{13}O)_m-CH(R^{15})_2$;

m is an integer ranging from 1-1000;

R^{13} is C_2-C_8 alkyl;

R^{14} is H or C_1-C_8 alkyl;

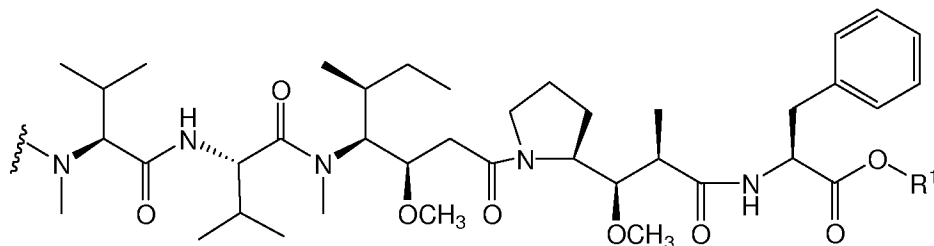
each occurrence of R^{15} is independently H, COOH, $-(CH_2)_n-N(R^{16})_2$, $-(CH_2)_n-SO_3H$, or $-(CH_2)_n-SO_3-C_1-C_8$ alkyl;

each occurrence of R^{16} is independently H, C_1-C_8 alkyl, or $-(CH_2)_n-COOH$;

R^{18} is selected from $-C(R^8)_2-C(R^8)_2$ -aryl, $-C(R^8)_2-C(R^8)_2-(C_3-C_8$ heterocycle), or $-C(R^8)_2-C(R^8)_2-(C_3-C_8$ carbocycle); and

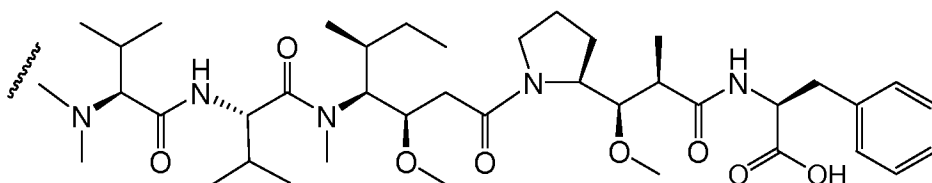
p is an integer ranging from 0 to 6.

4. The method of claim 2, wherein the Drug unit comprises the following formula:

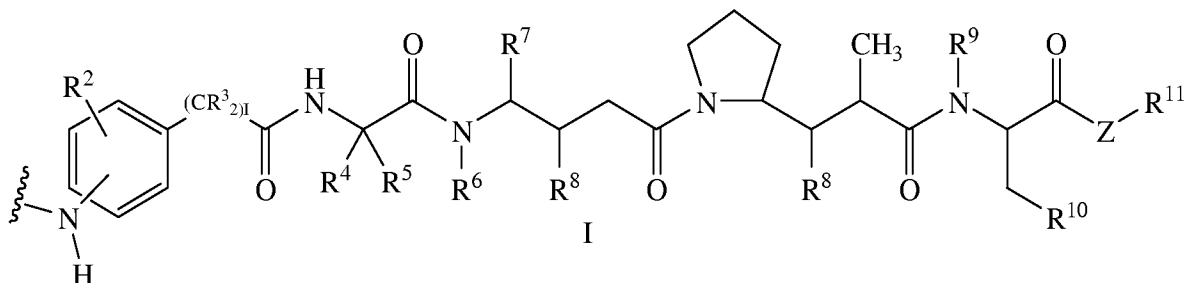


wherein $R^1 = H$ or Me.

5. The method of claim 2, wherein the Drug unit comprises the following formula:



6. The method of claim 1, wherein the Drug unit comprises the following formula:



wherein, independently at each location:

R^2 is -hydrogen - C_1 - C_8 alkyl, -O-(C_1 - C_8 alkyl), -halogen, - NO_2 , -COOH, or -C(O)OR¹¹;

each R^3 is selected independently from -hydrogen or - C_1 - C_8 alkyl;

I is an integer ranging from 0-10;

R^4 is -hydrogen, - C_1 - C_8 alkyl, - C_3 - C_8 carbocycle, -aryl, - C_1 - C_8 alkyl-aryl, - C_1 - C_8 alkyl-(C_3 - C_8 carbocycle), - C_3 - C_8 heterocycle or - C_1 - C_8 alkyl-(C_3 - C_8 heterocycle), and R^5 is -H or -methyl; or R^4 and R^5 jointly have the formula

$-(CR^aR^b)_n-$, wherein R^a and R^b are independently selected from -H, -C₁-C₈ alkyl or -C₃-C₈ carbocycle and n is selected from 2, 3, 4, 5 or 6, and form a ring with the carbon atom to which they are attached;

R^6 is -H or -C₁-C₈ alkyl;

R^7 is -H, -C₁-C₈ alkyl, -C₃-C₈ carbocycle, aryl, -C₁-C₈ alkyl-aryl, -C₁-C₈ alkyl-(C₃-C₈ carbocycle), -C₃-C₈ heterocycle or -C₁-C₈ alkyl-(C₃-C₈ heterocycle);

each R^8 is independently selected from -H, -OH, -C₁-C₈ alkyl, -C₃-C₈ carbocycle, -O-alkyl-(C₁-C₈ carbocycle) or -O-(C₁-C₈ alkyl);

R^9 is -H or -C₁-C₈ alkyl;

R^{10} is aryl or -C₃-C₈ heterocycle;

Z is -O-, -S-, -NH-, or -NR¹²- where R^{12} is C₁-C₈ alkyl or aryl; and

R^{11} is -H, C₁-C₈ alkyl, aryl, -C₃-C₈ heterocycle, $-(CH_2CH_2O)_r-H$, $-(CH_2CH_2O)_r-CH_3$, or $-(CH_2CH_2O)_r-CH_2CH_2C(O)OH$; wherein r is an integer ranging from 1-10.

7. The method of claim 1, further comprising administering a chemotherapeutic agent to the patient.

8. The method of claim 1, wherein the Ligand unit comprises an antibody or antigen-binding fragment.

9. The method of claim 1, wherein the Ligand unit specifically binds to the C-terminal portion of the Glypican-3.

10. The method of claim 1, wherein the Linker unit has the formula:



wherein:

-A- is a Stretcher unit;

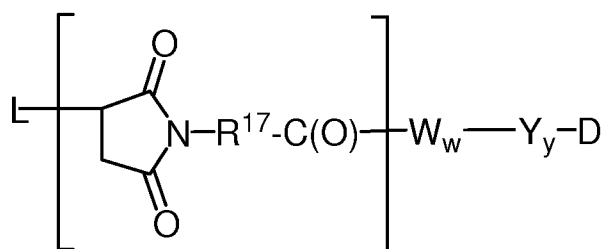
a is 0 or 1;

each -W- is independently an Amino Acid unit;

w is independently an integer ranging from 0 to 12;

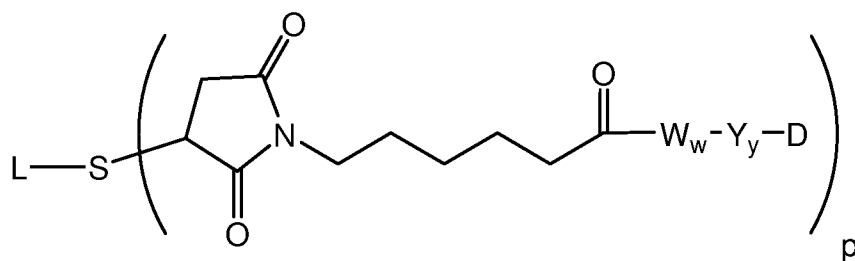
-Y- is a Spacer unit; and
y is 0, 1 or 2.

11. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:

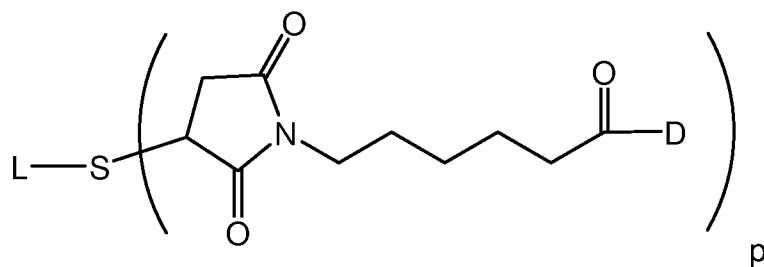


wherein R¹⁷ is C₁-C₁₀ alkylene-, -C₃-C₈ carbocyclo-, -O-(C₁-C₈ alkyl)-, arylene-, -C₁-C₁₀ alkylene-arylene-, -arylene-C₁-C₁₀ alkylene-, -C₁-C₁₀ alkylene-(C₃-C₈ carbocyclo)-, -(C₃-C₈ carbocyclo)-C₁-C₁₀ alkylene-, -C₃-C₈ heterocyclo-, -C₁-C₁₀ alkylene-(C₃-C₈ heterocyclo)-, -(C₃-C₈ heterocyclo)-C₁-C₁₀ alkylene-, -(CH₂CH₂O)_r-, or -(CH₂CH₂O)_r-CH₂-; and r is an integer ranging from 1-10.

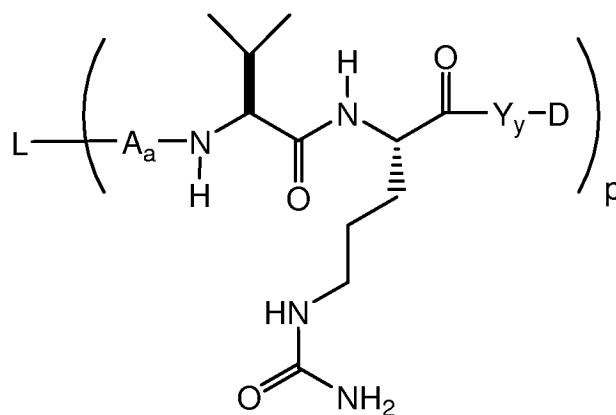
12. The method of claim 10, wherein the ligand-drug- conjugate compound has the formula:



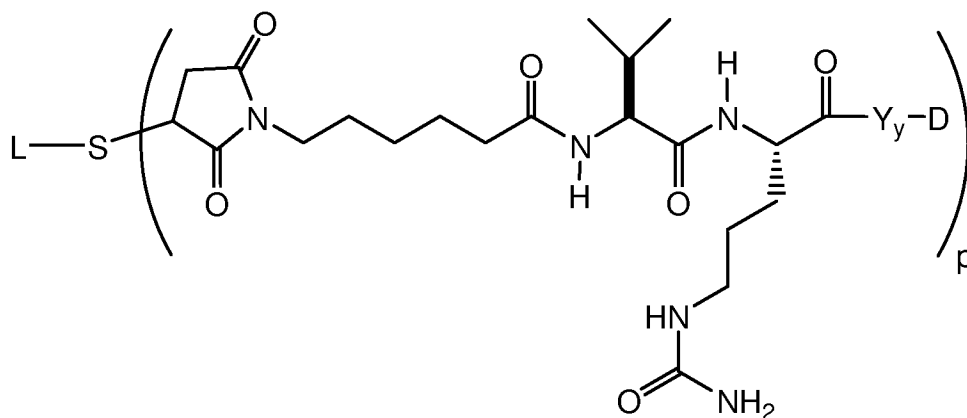
13. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:



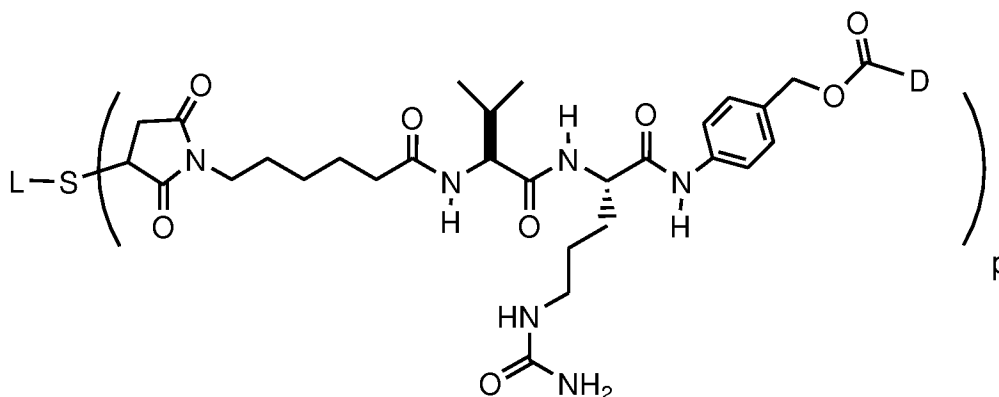
14. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:



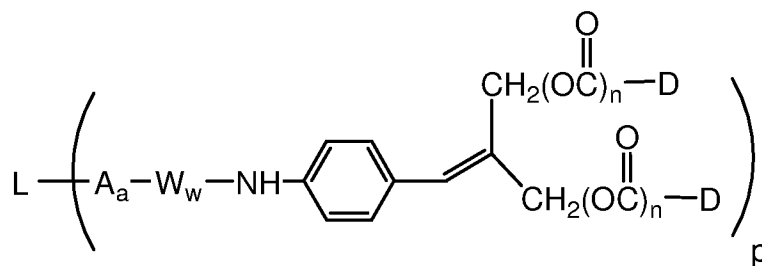
15. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:



16. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:



17. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:



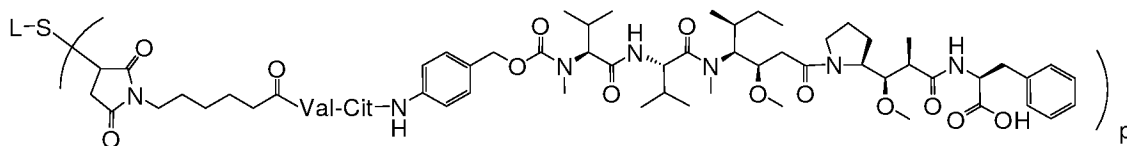
18. The method of claim 10, wherein w is an integer ranging from 2 to 12.

19. The method of claim 10, wherein w is 2.

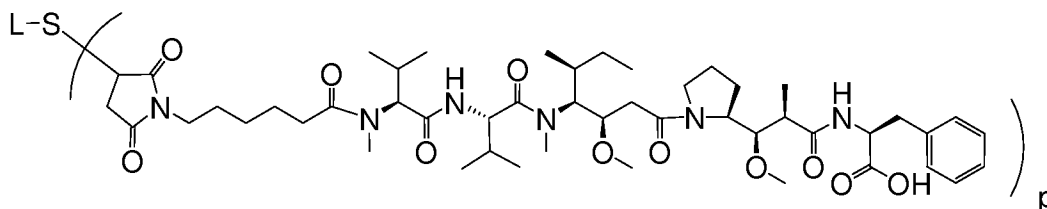
20. The method of claim 10, wherein W_w is -valine-citrulline-.

21. The method of claim 10, wherein W_w is 5-aminovaleric acid, homo-phenylalanine lysine, tetraisoquinolinecarboxylate lysine, cyclohexylalanine lysine, isonepepotic acid lysine, beta-alanine lysine, glycine serine valine glutamine and isonepepotic acid.

22. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:



23. The method of claim 10, wherein the ligand-drug conjugate compound has the formula:



24. The method of claim 1, comprising administering the ligand-drug conjugate compound as a pharmaceutical composition comprising an effective amount of the ligand-drug conjugate compound, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable diluent, carrier or excipient.

25. The method of claim 1, wherein the target cells are a cancer, a tumor or a cell proliferative disorder.

26. The method of claim 1, wherein the patient is a human.

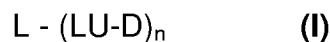
27. The method of claim 16 or 17, wherein the antibody is a growth inhibitory antibody.

28. The method of claim 1, wherein the ligand-drug conjugate compound induces cell death.

29. The method of claim 1, wherein the ligand-drug conjugate compound induces apoptosis.

30. The method of claim 25, wherein the cancer is liver, breast, ovarian, uterine, skin, or colorectal cancer.

31. A medicament for the treatment of a cancer in a patient in need of such treatment, comprising a ligand-drug conjugate compound of the following formula:



or a pharmaceutically acceptable salt or solvate thereof;

wherein:

L is a Ligand unit that specifically binds to an epitope of GPC-3; and

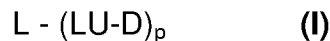
(LU-D) is a Linker unit-Drug unit moiety, wherein:

LU- is a Linker unit, and

-D is a Drug unit having cytostatic or cytotoxic activity against the target cells; and

n is an integer from 1 to about 20.

32. A ligand-drug conjugate compound of the following formula:



or a pharmaceutically acceptable salt or solvate thereof;

wherein:

L is a Ligand unit that specifically binds to GPC-3; and

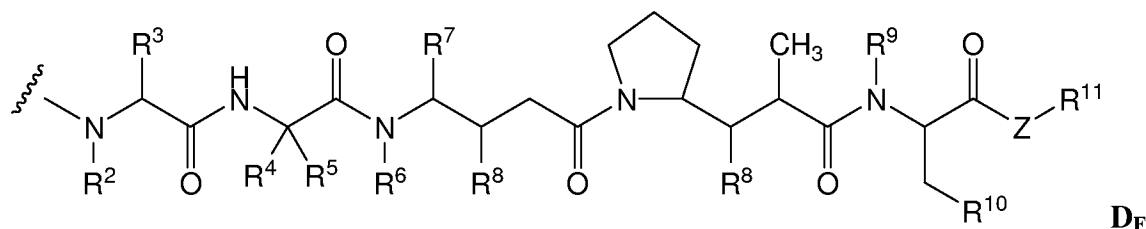
(LU-D) is a Linker unit-Drug unit moiety, wherein:

LU- is a Linker unit, and

-D is a Drug unit; and

n is an integer from 1 to about 20.

33. The ligand-drug conjugate compound of claim 32, wherein the Drug unit (-D) has the following Formula D_F:



wherein, independently at each location:

R^2 is H or C₁-C₈ alkyl;

R^3 is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

R^4 is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

R^5 is H or methyl;

or R^4 and R^5 jointly form a carbocyclic ring and have the formula $-(CR^aR^b)_n-$ wherein R^a and R^b are independently selected from H, C₁-C₈ alkyl or C₃-C₈ carbocycle and n is selected from 2, 3, 4, 5 or 6;

R^6 is H or C₁-C₈ alkyl;

R^7 is H, C₁-C₈ alkyl, C₃-C₈ carbocycle, aryl, C₁-C₈ alkyl-aryl, C₁-C₈ alkyl-(C₃-C₈ carbocycle), C₃-C₈ heterocycle or C₁-C₈ alkyl-(C₃-C₈ heterocycle);

each R^8 is independently selected from H, OH, C₁-C₈ alkyl, C₃-C₈ carbocycle or O-(C₁-C₈ alkyl);

R^9 is H or C₁-C₈ alkyl;

R^{10} is aryl or C₃-C₈ heterocycle;

Z is O, S, NH, or NR¹², wherein R^{12} is C₁-C₈ alkyl;

R^{11} is H, C₁-C₂₀ alkyl, aryl, C₃-C₈ heterocycle, $-(R^{13}O)_m-R^{14}$, or $-(R^{13}O)_m-CH(R^{15})_2$;

m is an integer ranging from 1-1000;

R^{13} is C₂-C₈ alkyl;

R^{14} is H or C₁-C₈ alkyl;

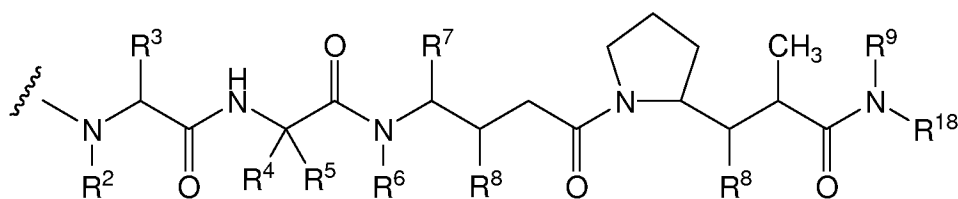
each occurrence of R^{15} is independently H, COOH, $-(CH_2)_n-N(R^{16})_2$, $-(CH_2)_n-SO_3H$, or $-(CH_2)_n-SO_3-C_1-C_8$ alkyl;

each occurrence of R^{16} is independently H, C_1 - C_8 alkyl, or $-(CH_2)_n$ -COOH;

R^{18} is $-C(R^8)_2-C(R^8)_2$ -aryl, $-C(R^8)_2-C(R^8)_2-(C_3$ - C_8 heterocycle), or $-C(R^8)_2-C(R^8)_2-(C_3$ - C_8 carbocycle); and

p is an integer ranging from 0 to 6.

34. The ligand-drug conjugate compound of claim 32, wherein the Drug unit (-D) has the following Formula D_E:



wherein, independently at each location:

R^2 is H or C_1 - C_8 alkyl;

R^3 is H, C_1 - C_8 alkyl, C_3 - C_8 carbocycle, aryl, C_1 - C_8 alkyl-aryl, C_1 - C_8 alkyl- $(C_3$ - C_8 carbocycle), C_3 - C_8 heterocycle or C_1 - C_8 alkyl- $(C_3$ - C_8 heterocycle);

R^4 is H, C_1 - C_8 alkyl, C_3 - C_8 carbocycle, aryl, C_1 - C_8 alkyl-aryl, C_1 - C_8 alkyl- $(C_3$ - C_8 carbocycle), C_3 - C_8 heterocycle or C_1 - C_8 alkyl- $(C_3$ - C_8 heterocycle);

R^5 is H or methyl;

or R^4 and R^5 jointly form a carbocyclic ring and have the formula $-(CR^aR^b)_n$ - wherein R^a and R^b are independently selected from H, C_1 - C_8 alkyl or C_3 - C_8 carbocycle and n is selected from 2, 3, 4, 5 or 6;

R^6 is H or C_1 - C_8 alkyl;

R^7 is H, C_1 - C_8 alkyl, C_3 - C_8 carbocycle, aryl, C_1 - C_8 alkyl-aryl, C_1 - C_8 alkyl- $(C_3$ - C_8 carbocycle), C_3 - C_8 heterocycle or C_1 - C_8 alkyl- $(C_3$ - C_8 heterocycle);

each R^8 is independently selected from H, OH, C_1 - C_8 alkyl, C_3 - C_8 carbocycle or O- $(C_1$ - C_8 alkyl);

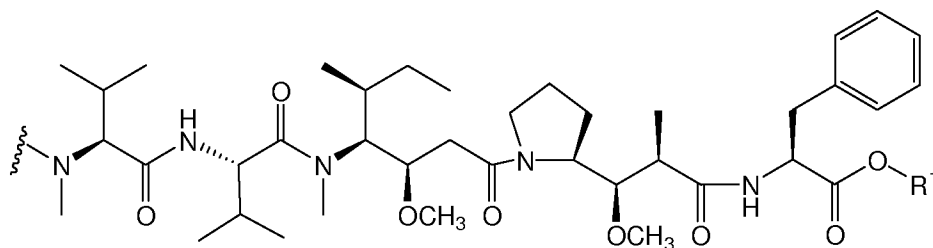
R^9 is H or C_1 - C_8 alkyl;

R^{10} is aryl or C_3 - C_8 heterocycle;

Z is O, S, NH, or NR^{12} , wherein R^{12} is C_1 - C_8 alkyl;

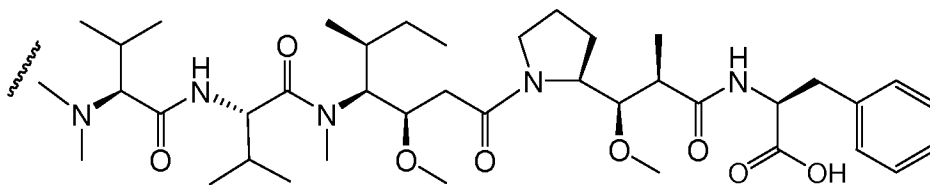
R^{11} is H, C_1 - C_{20} alkyl, aryl, C_3 - C_8 heterocycle, $-(R^{13}O)_m-R^{14}$, or $-(R^{13}O)_m-CH(R^{15})_2$;
 m is an integer ranging from 1-1000;
 R^{13} is C_2 - C_8 alkyl;
 R^{14} is H or C_1 - C_8 alkyl;
each occurrence of R^{15} is independently H, COOH, $-(CH_2)_n-N(R^{16})_2$, $-(CH_2)_n-SO_3H$, or $-(CH_2)_n-SO_3-C_1-C_8$ alkyl;
each occurrence of R^{16} is independently H, C_1 - C_8 alkyl, or $-(CH_2)_n-COOH$;
 R^{18} is $-C(R^8)_2-C(R^8)_2$ -aryl, $-C(R^8)_2-C(R^8)_2$ -(C_3 - C_8 heterocycle), or $-C(R^8)_2-C(R^8)_2$ -(C_3 - C_8 carbocycle); and
 p is an integer ranging from 0 to 6.

35. The ligand-drug conjugate compound of claim 33, wherein the Drug unit comprises the following formula:

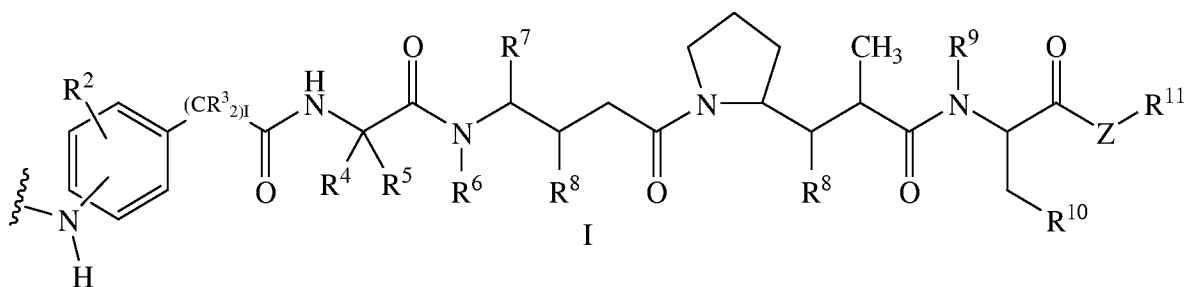


wherein $R^1 = H$ or Me.

36. The ligand-drug conjugate compound of claim 32, wherein the Drug unit comprises the following formula:



37. The ligand-drug conjugate compound of claim 32, wherein the Drug unit comprises the following formula:



wherein, independently at each location:

R^2 is -hydrogen - C_1 - C_8 alkyl, -O-(C_1 - C_8 alkyl), -halogen, - NO_2 , -COOH, or -C(O)OR¹¹;

each R^3 is selected independently from -hydrogen or - C_1 - C_8 alkyl;

I is an integer ranging from 0-10;

R^4 is -hydrogen, - C_1 - C_8 alkyl, - C_3 - C_8 carbocycle, -aryl, - C_1 - C_8 alkyl-aryl, - C_1 - C_8 alkyl-(C_3 - C_8 carbocycle), - C_3 - C_8 heterocycle or - C_1 - C_8 alkyl-(C_3 - C_8 heterocycle), and R^5 is -H or -methyl; or R^4 and R^5 jointly have the formula $-(CR^aR^b)_n$, wherein R^a and R^b are independently selected from -H, - C_1 - C_8 alkyl or - C_3 - C_8 carbocycle and n is selected from 2, 3, 4, 5 and 6, and form a ring with the carbon atom to which they are attached;

R^6 is -H or - C_1 - C_8 alkyl;

R^7 is -H, - C_1 - C_8 alkyl, - C_3 - C_8 carbocycle, aryl, - C_1 - C_8 alkyl-aryl, - C_1 - C_8 alkyl-(C_3 - C_8 carbocycle), - C_3 - C_8 heterocycle or - C_1 - C_8 alkyl-(C_3 - C_8 heterocycle);

each R^8 is independently selected from -H, -OH, - C_1 - C_8 alkyl, - C_3 - C_8 carbocycle, -O-alkyl-(C_1 - C_8 carbocycle) or -O-(C_1 - C_8 alkyl);

R^9 is -H or - C_1 - C_8 alkyl;

R^{10} is aryl or - C_3 - C_8 heterocycle;

Z is -O-, -S-, -NH-, or -NR¹²- where R^{12} is C_1 - C_8 alkyl or aryl; and

R^{11} is selected from -H, C_1 - C_8 alkyl, aryl, - C_3 - C_8 heterocycle, - $(CH_2CH_2O)_r$ -H, - $(CH_2CH_2O)_r$ -CH₃, or - $(CH_2CH_2O)_r$ -CH₂CH₂C(O)OH; wherein r is an integer ranging from 1-10.

38. The ligand-drug conjugate compound of claim 47, wherein the Ligand unit comprises an antibody or an antigen binding fragment of an antibody.

39. The ligand-drug conjugate compound of claim 32, wherein the Linker unit has the formula:

$$\text{---}A_a\text{---}W_w\text{---}Y_y\text{---}$$

wherein:

-A- is a Stretcher unit;

a is 0 or 1;

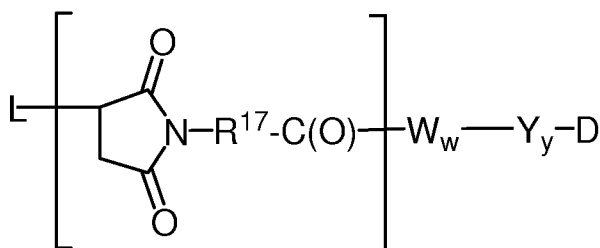
each -W- is independently an Amino Acid unit;

w is independently an integer ranging from 0 to 12;

-Y- is a Spacer unit; and

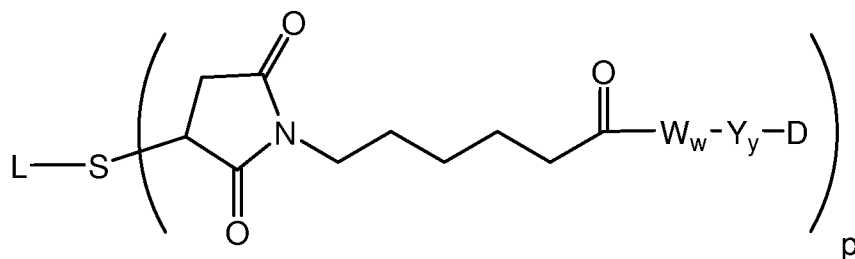
y is 0, 1 or 2.

40. The ligand-drug conjugate compound of claim 32, wherein the ligand-drug conjugate compound has the formula:

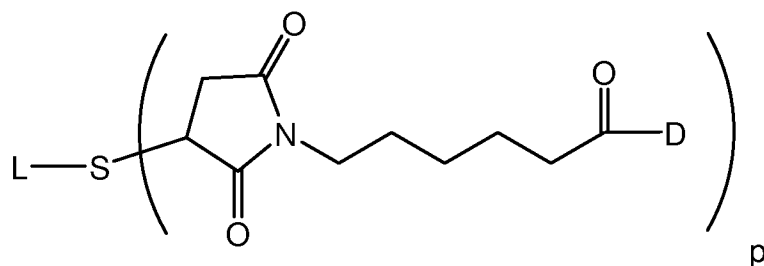


wherein R^{17} is $\text{C}_1\text{-C}_{10}$ alkylene-, $\text{-C}_3\text{-C}_8$ carbocyclo-, $\text{-O-(C}_1\text{-C}_8\text{ alkyl)-}$, arylene-, $\text{-C}_1\text{-C}_{10}$ alkylene-arylene-, arylene- $\text{-C}_1\text{-C}_{10}$ alkylene-, $\text{-C}_1\text{-C}_{10}$ alkylene-($\text{C}_3\text{-C}_8$ carbocyclo)-, $\text{-(C}_3\text{-C}_8\text{ carbocyclo)-C}_1\text{-C}_{10}$ alkylene-, $\text{-C}_3\text{-C}_8$ heterocyclo-, $\text{-C}_1\text{-C}_{10}$ alkylene-($\text{C}_3\text{-C}_8$ heterocyclo)-, $\text{-(C}_3\text{-C}_8\text{ heterocyclo)-C}_1\text{-C}_{10}$ alkylene-, $\text{-(CH}_2\text{CH}_2\text{O)}_r\text{-}$, or $\text{-(CH}_2\text{CH}_2\text{O)}_r\text{-CH}_2\text{-}$; and r is an integer ranging from 1-10.

41. The ligand-drug conjugate compound of claim 32, having the formula:

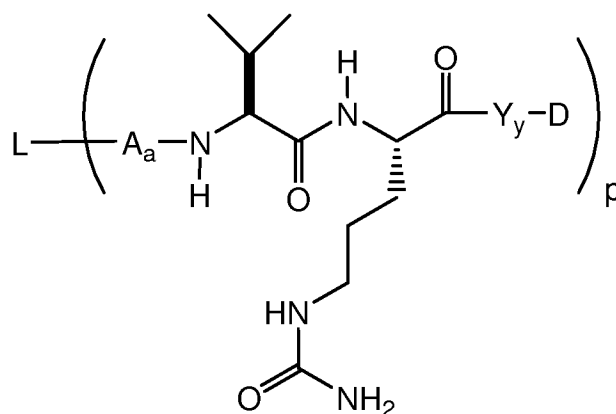


42. The ligand-drug conjugate compound of claim 32, having the formula:

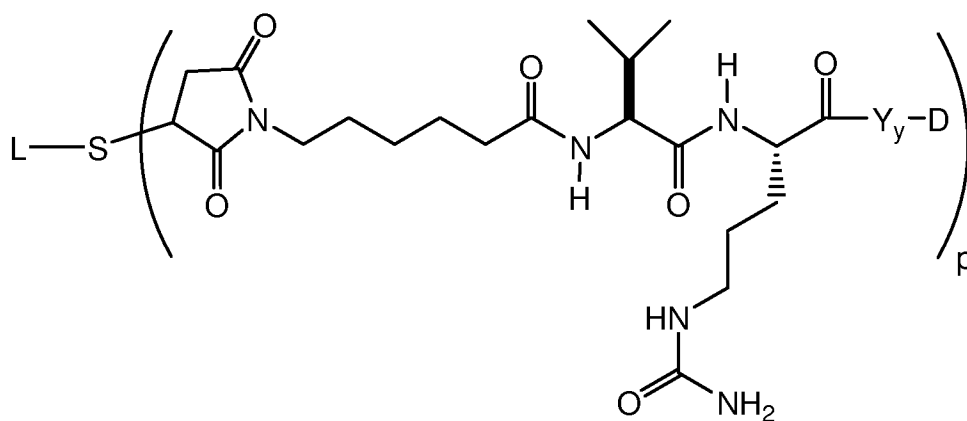


wherein w and y are each 0.

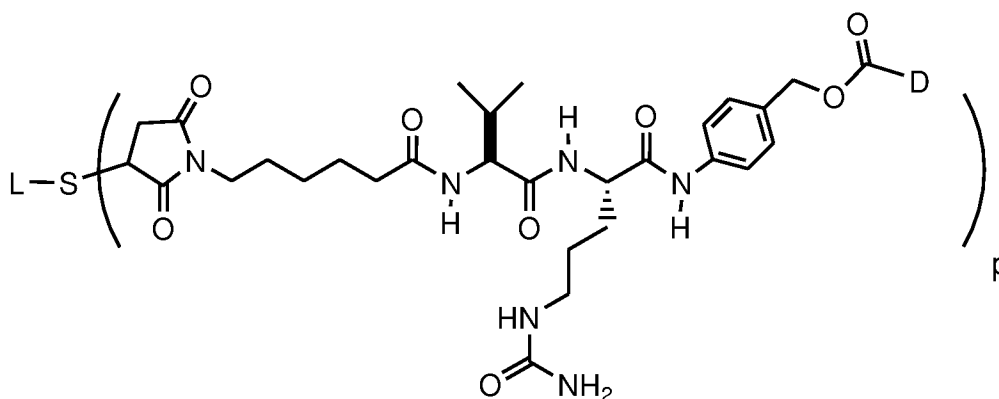
43. The ligand-drug conjugate compound of claim 32, having the formula:



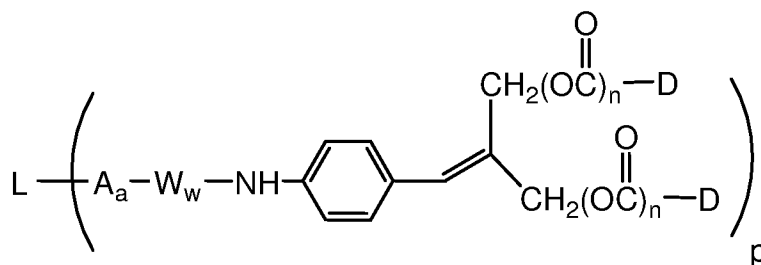
44. The ligand-drug conjugate compound of claim 32, having the formula:



45. The ligand-drug conjugate compound of claim 32, having the formula:

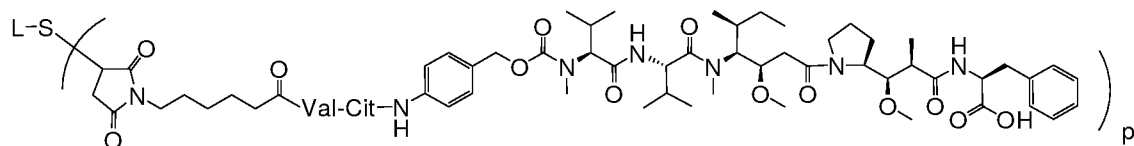


46. The ligand-drug conjugate compound of claim 32, having the formula:



47. The ligand-drug conjugate compound of claim 32, wherein w is an integer ranging from 2 to 12.
48. The ligand-drug conjugate compound of claim 32, wherein w is 2.
49. The ligand-drug conjugate compound of claim 32, wherein W_w is -valine-citrulline-.
50. The ligand-drug conjugate compound of claim 32, wherein W_w is 5-aminovaleric acid, homo-phenylalanine lysine, tetraisoquinolinecarboxylate lysine, cyclohexylalanine lysine, isonepecotic acid lysine, beta-alanine lysine, glycine serine valine glutamine and isonepecotic acid.

51. The ligand-drug conjugate compound of claim 32, wherein the ligand-drug conjugate compound has the formula:



52. The ligand-drug conjugate compound of claim 32, wherein the ligand-drug conjugate compound has the formula:

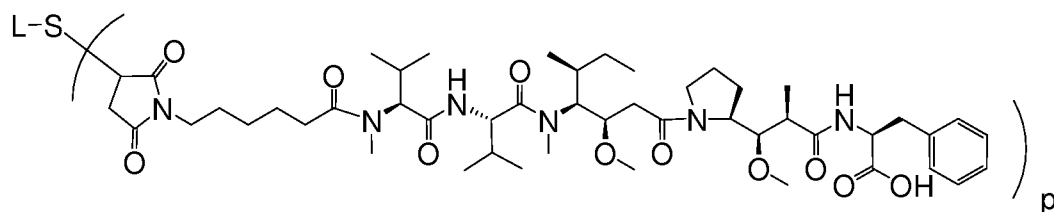
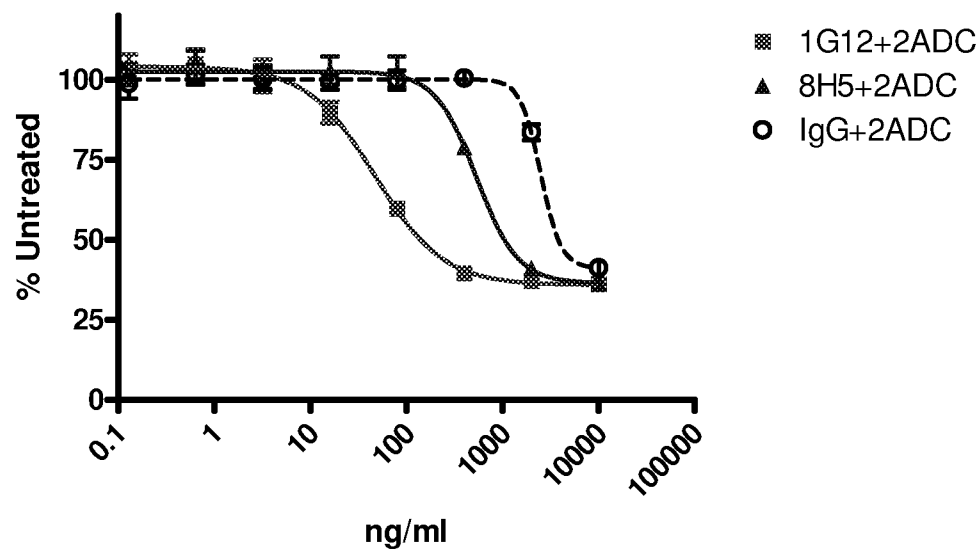
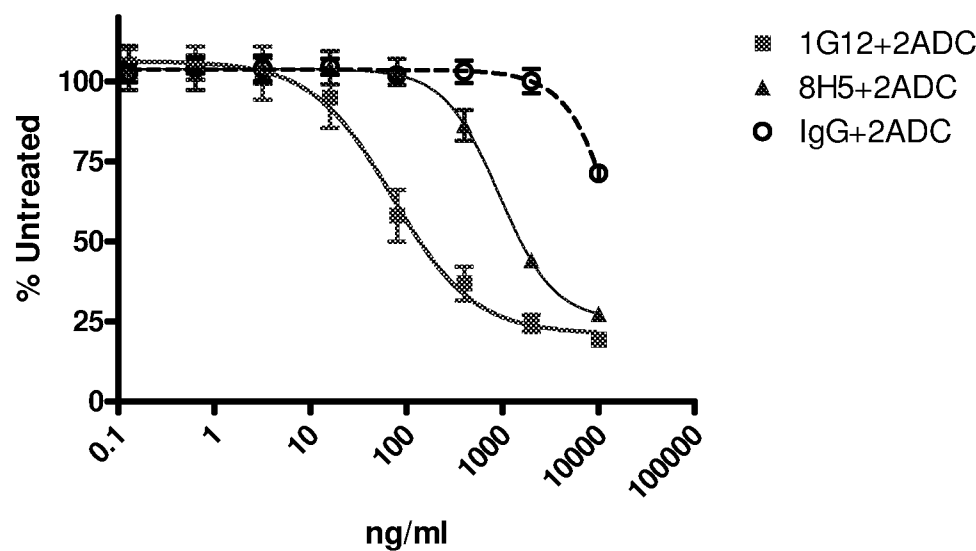


Figure 1.

A. Hep3B



B. HepG2



2/2

Figure 2

