CONJUGATION METHODS

This application claims priority of United States provisional application no. 61/183,774, filed June 3, 2009, the entire disclosure of which is expressly incorporated by reference herein.

FIELD OF THE INVENTION

[01] This invention relates to a novel method of conjugating an effector group (e.g., a cytotoxic agent) or a reporter group (e.g., a radiolabel) to a cell binding agent, such as an antibody or a fragment thereof, via a bifunctional linker. More specifically, this invention relates to a novel method of conjugating an effector group (e.g., maytansinoids) or a reporter group (e.g., a radiolabel) to a cell binding agent (e.g., an antibody or a fragment thereof) via a bifunctional linker such that the process eliminates the steps that result in formation of undesired hydrolyzed species or undesired cross-linked species formed due to intra-molecular or inter-molecular reactions.

BACKGROUND OF THE INVENTION

[02] Conjugates of cell binding agents, such as antibodies, with effector groups, such as small cytotoxic agents or cytotoxic proteins, are of immense interest for the development of anti-cancer therapeutics (Richart, A. D., and Tolcher, A. W., 2007, Nature Clinical Practice, 4, 245-25). These conjugates are tumor-specific due to the high specificity of the selected antibodies toward antigens expressed on the cell surface of tumor cells. Upon specific binding to the tumor cell, the antibody-cytotoxic agent conjugate is internalized and degraded inside the target cancer cell thereby releasing the

active cytotoxic agent that inhibits essential cellular functions such as microtubule dynamics or DNA replication resulting in the killing of the cancer cell. Various linkers have been employed to link the antibodies with cytotoxic agents with the goal of enhancing the delivery of the agent inside the cell upon internalization and processing of the conjugate, while maintaining the desired stability of the conjugate in plasma. These linkers include disulfide linkers designed with different degrees of steric hindrance to influence their reduction kinetics with intracellular thiol, cleavable peptide linkers such as valine-citrulline linkage, and non-cleavable linkers such as thioether linkage (Widdison, W., et al., J. Med. Chem., 2006, 49, 4392-4408; Erickson, H., et al., Cancer Res., 2006, 66, 4426-4433).

[03] Conjugates of cell binding agents such as antibodies with labels or reporter groups are useful for tumor-imaging applications in cancer patients, immunoassay applications for diagnosis of various diseases, cancer therapy using radioactive nuclide-ligand conjugates, and affinity chromatography applications for purification of bioactive agents such as proteins, peptides, and oligonucleides. The labels or reporter groups that are conjugated with cell-binding agents include fluorophores, and affinity labels such as biotin.

[04] The conventional method of conjugation of the cell-binding agent such as an antibody (Ab) with an effector group (e.g., a cytotoxic agent) or a reporter group (e.g., a radiolabel) linked via a non-reducible linkage (such as thioether linkage) employs two distinct reaction steps with the antibody and necessitates the use of purification steps. In the first reaction step, the antibody is reacted with a heterobifunctional linker bearing two different reactive groups (e.g., X and Y). For example, in one approach, the reaction of

an antibody's reactive residues (such as lysine amino residues) with the X reactive group (such as *N*-hydroxysuccinimide ester) of the heterobifunctional reagent results in the incorporation of the linker with Y reactive group at one or more reactive residues in the antibody (such as lysine amino residues). The initially modified antibody product must be purified from the excess linker or hydrolyzed linker reagent before the next step can occur. In the second reaction step, the linker-modified antibody containing the Y reactive group (such as maleimide or haloacetamide) is reacted with the effector such as an effector group (C) (e.g., a cytotoxic agent) containing a reactive group such as thiol to generate the antibody-effector conjugate, which is again purified in an additional purification step (see, e.g., U.S. patents 5,208,020, 5,416,064, or 5,024,834). Thus, in the above process, at least two purification steps are needed.

[05] Another approach that involves two reaction and purification steps to conjugate antibody with an effector or reporter group uses the reaction of thiol residues in antibody (generated via modification of antibody with thiol-generating reagents such as 2-iminothiolane, or via mutagenesis to incorporate non-native cysteine residues, or via reduction of native disulfide bonds) with a homobifunctional linker Y-L-Y containing Y reactive groups (such as maleimide or haloacetamide).

[06] Major drawbacks of incorporating a reactive group Y such as maleimide (or haloacetamide) in an antibody or peptide are the propensity of the reactive maleimide (or haloacetamide) groups to undergo intra- or inter-molecular reaction with the native histidine, lysine, tyrosine, or cysteine residues in antibody or peptide (Papini, A. et al., Int. J. Pept. Protein Res., 1992, 39, 348-355; Ueda, T. et al., Biochemistry, 1985, 24, 6316-6322), and aqueous inactivation of the Y maleimide group. The undesired intra-

molecular or inter-molecular reaction of maleimide (or haloacetamide) groups Y incorporated in antibody with the native histidine, lysine, or cysteine residues in antibody, and aqueous inactivation of the Y maleimide group before the second reaction with the effector or reporter group C give rise to cross-linked proteins or heterogeneous conjugates and lower the efficiency of the second reaction with the effector or reporter group C. The heterogeneous conjugate product—cross-linked protein or peptide generated from the undesired reaction of the initially incorporated group Y (such as maleimide group) with native groups in the antibody or peptides (such as histidine, lysine, tyrosine, or cysteine), or with inactive maleimide residues generated by aqueous inactivation—may have inferior activity and stability than the desired homogeneous conjugate product.

[07] Processes for conjugating antibodies to thiol-containing cytotoxic agents via disulfide linkages have been described previously (see, e.g., U.S. patents 5,208,020, 5,416,064, 6,441,163, U.S. Patent Publication No. 2007/0048314 A1). These processes involve the initial reaction of antibody with a heterobifunctional reagent, followed by a second reaction with a thiol-containing cytotoxic agent. An alternative process has been described in U.S. patent 6,441,163 B1 in which the disulfide-linked reactive ester of the cytotoxic agent is first purified and then reacted with the antibody, but which involves an additional reaction and purification step starting from the thiol group-containing cytotoxic agent before the reaction step with the antibody.

[08] A further drawback of the current process to make conjugates of cell binding agents is the need for two purification steps, which lowers the overall yield and also makes the process cumbersome and uneconomical for scale-up.

[09] In view of the foregoing, there is a need in the art to develop improved methods of preparing cell-binding agent-drug conjugate compositions that are of substantially high purity and can be prepared avoiding cumbersome steps and by reducing time and cost to the user. The invention provides such a method. These and other advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

SUMMARY OF THE INVENTION

[10] The present invention describes a conjugation method for preparing non-reducible, thioether-linked conjugates of the formula C-L-CBA, wherein C represents an effector or reporter molecule (e.g., a cytotoxic agent or a radiolabel), L is a linker and CBA is a cell binding agent (e.g., an antibody or a fragment thereof), by utilizing a direct reaction of the thiol-containing cytotoxic agent (e.g., maytansinoids) with a hetero-or a homo-bifunctional reagent, (e.g., cleavable or a non-cleavable linker) followed by mixing of the unpurified reaction mixture with a cell binding agent (e.g., an antibody or a fragment thereof), thereby generating the non-reducible, thioether-linked conjugate by a process that is more efficient, has a high yield, and is amenable for scale up. Another important advantage is that such conjugation method yields thioether-linked non-reducible conjugates with no inter-chain protein cross-linking or inactivated residues (e.g., maleimide or haloacetamide residues). The novel methods disclosed in this application can be applied to the preparation of any conjugate represented by the above formula.

BRIEF DESCRIPTION OF THE DRAWINGS

[11] Figure 1 shows conjugation of antibody with a reaction mixture of the maytansinoid DM1 (or DM4) and Maleimide-PEG_n-NHS linker

[12] Figure 2 shows reducing SDS-PAGE of Ab-(PEG_4-Mal)-DM4 conjugates prepared using the method described in this invention versus conjugates prepared using the traditional 2-step method. Each sample lane contained 10 μg protein; the gel was stained with Coomassie Blue. Lanes 1 and 2 contained molecular weight markers. Lane 3 contained conjugate prepared by the traditional two-step method with 6.1 DM4 per Ab. Lane 4 contained conjugate prepared by the method described in this invention and contained 6.2 DM4 per Ab.

[13] Figure 3 shows Protein LabChip electrophoresis of Ab-(PEG_4-Mal)-DM4 conjugates prepared using the method described in this invention versus conjugates prepared using the traditional 2-step method. A. Protein LabChip electrophoresis under reducing condition (Agilent 2100 Bioanalyzer/Agilent Protein 230 kit) of Ab-(PEG_4-Mal)-DM4 conjugates. Lane 1: molecular weight markers; lane 2: Ab-PEG_4-Mal-DM4, 6.2 D/Ab, synthesized using the method described in this invention; lane 3: Ab-PEG_4-Mal-DM4, 6.1 D/Ab, synthesized using the 2 step conjugation method; lane 4: unconjugated Ab (0.24 microgram total protein in each lane). The upper marker, system peak and lower marker bands represent external markers added from kit. B. Quantitation of protein bands from Protein LabChip electrophoresis.

[14] Figure 4 shows MS of Ab-(PEG_4-Mal)-DM4 conjugates prepared using the method described in this invention versus conjugates prepared using the traditional 2-step method. A. MS of conjugate prepared by the traditional two-step method with 6.1 DM4

per Ab. Due to significant heterogeneity of the conjugate the MS peaks could not be resolved well. B. MS of conjugate prepared by the method described in this invention and contained 6.2 DM4 per Ab. Due to homogeneity of the conjugate, the MS peaks were well resolved.

[15] Figure 5 shows binding of an anti-CanAg antibody-PEG_4-Mal-DM1 conjugate with 6.7 DM1 per antibody (prepared using the method described in this invention) versus binding of unmodified antibody toward CanAg antigen-expressing COLO205 cells. The binding was measured in fluoresence units.

[16] Figure 6 shows *in vitro* cytotoxicity of an anti-CanAg Antibody-PEG_4-Mal-DM1 conjugate with 6.7 DM1 per antibody (prepared using the method described in this invention) toward CanAg antigen-expressing COLO205 cells. The conjugate was added to COLO205 cells, and after 5 days of continuous incubation with the conjugate, the viability of the cells was measured using WST-8 assay. A control experiment to demonstrate the specificity of the conjugate was carried out using an excess of unconjugated anti-CanAg antibody to block the binding and cytotoxicity of the conjugate toward target cancer cells.

[17] Figure 7 shows conjugation of antibody with a reaction mixture of DM1 (or DM4) and Maleimide-Sulfo-NHS linker.

[18] Figure 8 shows reducing SDS-PAGE of Ab-(Sulfo-Mal)-DM1 conjugates prepared using the method described in this invention versus conjugates prepared using the traditional 2-step method. Each sample lane contained 10 μg protein; the gel was stained with Coomassie Blue. Lane 1 contained molecular weight markers. Lanes 3 and 5 contained conjugates prepared by the method described in this invention and contained

3.6 and 5.6 DM1 per Ab, respectively. Lanes 2 and 4 contained conjugates prepared by the traditional two-step method and contained 4.0 and 5.7 DM1 per Ab, respectively.

[19] Figure 9 shows Protein LabChip electrophoresis of Ab-(Sulfo-Mal)-DM1 conjugate prepared using the method described in this invention versus conjugate prepared using the traditional 2-step method. A. Protein LabChip electrophoresis under reducing condition (Agilent 2100 Bioanalyzer/Agilent Protein 230 kit) of Ab-(Sulfo-Mal)-DM1 conjugates. Lane 1: molecular weight markers; lane 2: unconjugated Ab; lane 3: Ab-Sulfo-Mal-DM1, 5.7 D/Ab, synthesized using the 2 step conjugation method; lane 4: Ab-Sulfo-Mal-DM1, 5.6 D/Ab, synthesized using the method described in this invention; 0.22 microgram total protein loaded per well. The upper marker, system peak and lower marker bands represent external markers added from kit (0.24 microgram total protein per well). B. Quantitation of protein bands from Protein LabChip electrophoresis.

[20] Figure 10 shows LC-MS comparison of Antibody-(Sulfo-Mal)-DM1 conjugate prepared by the method described in this invention versus by the traditional two-step conjugation method. A. MS of conjugate with 3.6 DM1/Ab prepared using the method described in this invention shows a homogeneous conjugate with 1-6 DM1-bearing discrete conjugate peaks. B. MS of conjugate with 4.0 DM1/Ab prepared by the traditional two-step conjugation method. The MS for the conjugate prepared by the traditional two-step method shows peaks corresponding to conjugates, and conjugates with hydrolyzed or cross-linked linkers (such as conjugate with 2 DM1, plus one L, 2L, and 3 L), indicating a heterogeneous product.

[21] Figure 11 shows binding of an anti-CanAg antibody-Sulfo-Mal-DM1 conjugate with 5.6 DM4 per antibody (prepared using the method described in this invention) versus binding of unmodified antibody toward CanAg antigen-expressing COLO205 cells. The binding was measured in fluoresence units.

[22] Figure 12 shows *in vitro* cytotoxicity of an anti-CanAg Antibody-Sulfo-Mal-DM1 conjugate with 5.6 DM4 per antibody (prepared using the method described in this invention) toward CanAg antigen-expressing COLO205 cells. The conjugate was added to COLO205 cells and after 5 days of continuous incubation with the conjugate, the viability of the cells was measured using WST-8 assay. A control experiment to demonstrate the specificity of the conjugate was carried out using an excess of unconjugated anti-CanAg antibody to block the binding and cytotoxicity of the conjugate toward target cancer cells.

[23] Figure 13 shows conjugation of antibody with a reaction mixture of DM1 (or DM4) and Sulfo-NHS SMCC linker.

[24] Figure 14 shows reducing SDS-PAGE of Ab-(SMCC)-DM1 conjugate prepared using the method described in this invention versus conjugate prepared using the traditional 2-step method. Each sample lane contained 10 microgram total protein; the gel was stained with Coomassie Blue. Lane 1 contains molecular weight markers, Lane 2 contains unconjugated Ab, Lane 3 contains conjugate prepared by the traditional two-step method with 3.1 DM1 per Ab and Lane 4 contains conjugate prepared by the method described in this invention with 3.1 DM1 per Ab.

[25] Figure 15 shows Protein LabChip electrophoresis of Ab-(SMCC)-DM1 conjugate prepared using the method described in this invention versus conjugate prepared using

the traditional 2-step method. A. Protein LabChip electrophoresis under reducing condition (Agilent 2100 Bioanalyzer/Agilent Protein 230 kit) of Ab-SMCC-DM1 conjugates. Lane 1: molecular weight markers; lane 2: Ab-SMCC-DM1, 3.1 D/Ab, synthesized using the method described in this patent; lane 3: unconjugated Ab; lane 4: Ab-SMCC-DM1, 3.1 D/Ab, synthesized using the 2 step conjugation method; (0.24 microgram total protein in each lane). The upper marker, system peak and lower marker bands represent external markers added from kit. B. Quantitation of protein bands from Protein LabChip electrophoresis.

[26] Figure 16 shows LC-MS comparison of Antibody-(SMCC)-DM1 conjugate prepared by the method described in this invention versus conjugate prepared by the traditional two-step conjugation method. A. MS of conjugate prepared by the sequential two-step method with 3.1 DM1 per Ab. Each major conjugate peak has associated side peaks due to the presence of hydrolyzed and cross-linked linker fragments. B. MS of conjugate prepared by the method described in this invention with 3.1 DM1 per Ab. Due to homogeneity of the conjugate, the MS peaks were well resolved.

[27] Figure 17 shows proposed mechanisms for inter-chain cross-linking and maleimide inactivation during conjugation by the traditional 2-step method.

[28] Figure 18 shows non-reducing SDS PAGE of Ab-(Sulfo-Mal)-DM4 conjugate prepared using the method described in this invention and quenching of free DM4 thiol (after the initial DM4 + NHS-Sulfo-Mal heterobifunctional reagent coupling reaction) using 4-maleimidobuytric acid prior to the antibody conjugation reaction. Each sample contained 10 μg protein; the gel was stained with Coomassie Blue. Lanes 1 and 5 contained molecular weight markers. Lane 2 contained Ab alone. Lane 3 contained

conjugate prepared by the method described in this invention without addition of 4-maleimidobuytric acid. Lane 4 contained conjugate prepared by the method described in this invention with addition of 4-maleimidobutyric acid after the initial DM4 + NHS-Sulfo-Mal heterobifunctional reagent (prior to the antibody conjugation step).

[29] Figure 19 shows preparation of disulfide-linked conjugate of antibody using a reaction mixture of DM1 (or DM4) and SPDB linker.

[30] Figure 20 shows preparation of antibody-maytansinoid conjugate with both disulfide- and non-cleavable PEG_4-Mal linkers via antibody conjugation with an unpurified reaction mixture of DM1 (or DM4) and both SPDB and NHS-PEG_4-Mal linkers.

[31] Figure 21 shows MS of antibody-maytansinoid conjugate with both disulfide- and non-cleavable PEG_4-Mal linkers (prepared by conjugation of antibody with an unpurified reaction mixture of DM1, or DM4, and both SPDB and NHS-PEG_4-Mal linkers).

[32] Figure 22 shows the conjugation of antibody with a reaction mixture of DM1 (or DM4) and SMCC linker.

[33] Figure 23 shows the MS of antibody-SMCC-DM1 conjugate prepared using SMCC by the method described in this invention, containing average 3.1 DM1 per antibody.

[34] Figure 24 shows the preparation of disulfide-linked conjugate of antibody using a reaction mixture of DM1 (or DM4) and SSNPB linker.

[35] Figure 25 shows the conjugation of antibody with a reaction mixture of DM1 (or DM4) and heterobifunctional linker with aliphatic linear carbon chain.

DETAILED DESCRIPTION OF THE INVENTION

[36] Reference will now be made in detail to certain embodiments of the invention, examples of which are illustrated in the accompanying structures and formulas. While the invention will be described in conjunction with the enumerated embodiments, it will be understood that they are not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents which may be included within the scope of the present invention as defined by the claims. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention.

[37] This invention describes a novel method of conjugating a thiol-containing effector (e.g., a cytotoxic agent) or a reporter group (e.g., a radiolabel) with a cell binding agent (e.g., an antibody), in which the thiol-group containing effector or reporter is first reacted with a bifunctional linker reagent in organic, aqueous, or mixed organic/aqueous solvent, followed by reaction of the unpurified reaction mixture with the cell binding agent in organic, aqueous or mixed organic/aqueous solvents.

legend abbreviations

[38] Abbreviations which have been used in the descriptions of the Schemes and the Examples that follow are:

[39] C = Effector or a reporter group (e.g., a cytotoxic agent or a radiolabel)

[40] L = Linker (e.g., cleavable or a non-cleavable linker)

[41] X = amine-reactive group (e.g., *N*-hydroxysuccinimide ester (NHS ester), sulfo-NHS ester, *p*-nitrophenol ester, tetrafluorosulfonate phenyl ester, 1-hydroxy-2-nitro-benzene-4-sulfonic acid ester)

[42] Y = Maleimide, or haloacetamide (iodoacetamide, bromoacetamide)

[43] Y_b is a reactive mixed disulfide group (e.g., 2-pyridyldithio, 4-pyridyldithio, 2-nitro-pyridyldithio, 5-nitro-pyridyldithio, 2-carboxy-5-nitro-pyridyldithio)

[44] X' = amide linkage

[45] Y' = thioether (R-S-R') or selenoether (R-Se-R') linkage

[46] Y_b' = disulfide (R-S-S-R') linkage

[47] In one embodiment of this invention, a process for the preparation of a thioether-linked conjugate of a cell-binding agent with an effector or a reporter molecule is described, the process comprising the following steps: a) contacting a heterobifunctional linker of formula **X-L-Y** with a thiol-containing effector or reporter molecule **C** (e.g., a maytansinoid or a radionuclide) in aqueous solvent, organic solvent, or mixed organic/aqueous reaction mixtures which yields an intermediate product of formula **X-L-Y'-C**; b) mixing of the reaction mixture without purification with a cell-binding agent such as an antibody **(Ab)** to produce a conjugate of formula **Ab-(X'-L-Y'-C)$_m$**, wherein, **L** is a substituted or unsubstituted linear, branched or cyclic alkyl, alkenyl, or alkynyl group bearing from 1-10 carbon atoms, a simple or substituted aryl unit (substituents selected from alkyl, alkoxy, halogen, nitro, fluoro, carboxy, sulfonate, phosphate, amino, carbonyl, piperidino) or a polyethylene glycol containing unit (preferably 1-500 PEG spacer, or more preferably 1-24 PEG spacer, or still more preferably 2-8 PEG spacer); **X**

and Y are amine or thiol-reactive group such as *N*-hydroxysuccinimide ester and maleimide or haloacetamide; **Ab** is an antibody; **m** is an integer from 1-20; **X'** is modified X site (e.g., an amide linkage) upon reaction with antibody; Y' is modified Y site (e.g., thioether linkage) upon reaction with, for example, a cytotoxic agent or a radiolabel of the effector or reporter group; and c) purification of the conjugate by tangential flow filtration, dialysis, or chromatography (e.g., gel filtration, ion-exchange chromatography, hydrophobic interaction chromatography) or a combination thereof. Preferably, Y is a thiol-reactive group selected from maleimide or haloacetamide. Preferably, L is a linear or branched alkyl group with 1-6 carbons or 2-8 PEG spacer. Preferably, C is a cytotoxic agent selected from a maytansinoid, a CC-1065 analog, a taxane, a DNA-binding agent, and more preferably it is a maytansinoid.

[48] This reaction sequence represented in formulae 1 and 2:

X-L-Y + C → X-L-Y'-C (1)

Ab + X-L-Y'-C (unpurified from reaction 1) → Ab-(X'-L-Y'-C)$_m$ (2)

does not involve any purification of the intermediate product **X-L-Y'-C**, and therefore provides the advantage of directly mixing it with the antibody (the unpurified intermediate product is added to the antibody or, the antibody is added to the unpurified intermediate product) thereby making the method advantageous for conjugation because it eliminates the need of a cumbersome purification step. Importantly, this method yields homogeneous conjugate with no inter-chain protein cross-linking or inactivated maleimide residues, in contrast to the inter-chain protein cross-linking and inactivated maleimide residues observed in conjugates prepared by the traditional two step reaction and purification sequence.

[49] The reaction 1 can be carried out at high concentrations of the heterobifunctional linker, X-L-Y, and the effector or reporter group C in aqueous solvent, organic solvent, or organic/aqueous reaction mixtures, resulting in faster reaction rates than at lower concentrations in aqueous solutions for conjugates prepared by the traditional two step reaction and purification sequence.

[50] The intermediate product X-L-Y'-C generated in reaction 1 can be stored unpurified in a frozen state, at low temperatures in aqueous solvent at appropriate low pH (e.g., pH ~4-6), in organic solvents, or in mixed organic/aqueous mixtures, or in lyophilized state, for prolonged periods and can be mixed later with the antibody solution for the final conjugation reaction at a higher pH value of about 4-9, therefore adding to the convenience of this reaction sequence. The intermediate product can be diluted as required with organic solvent or with aqueous buffer, or a mixture of organic solvent and aqueous buffer prior to mixing with the cell binding agent. The term "about" as used herein in connection with a numerical should be understood to refer to all such numbers, including all numbers and small variations therefrom. The reaction of the intermediate product X-L-Y'-C with antibody can be carried out at pH values of about 4 to about pH 9, preferably in the pH range of about 5 to 8.7, more preferably, in the pH range of about 6.5 to about 8.5, such as, pH 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, and 8.5, a pH range therein or small variations therefrom. The buffers used for the reaction of the antibody with the intermediate product X-L-Y'-C in the preferential pH range of about 6.5 to 8.5 are buffers with pK_a values around this pH range, such as phosphate and HEPES buffer. These preferred buffers should not have

primary or secondary amino groups, or other reactive groups, that can react with linker X (such as *N*-hydroxysuccinimide ester).

[51] A stoichiometric or a slight excess of C over the heterobifunctional linker X-L-Y is used in the first reaction to ensure that all Y group (such as maleimide) is reacted before the unpurified mixture is added to the antibody. An optional additional treatment with a quenching reagent (such as 4-maleimidobutyric acid, 3-maleimidopropionic acid, or *N*-ethylmaleimide, or iodoacetamide, or iodoacetamidopropionic acid) can be done to ensure that any unreacted C is quenched before mixing with the antibody to minimize any unwanted thiol-disulfide interchange reaction with the native antibody disulfide groups. Upon quenching with polar, charged thiol-quenching reagents (such as 4-maleimidobutyric acid or 3-maleimidopropionic acid), the excess, unreacted C is converted into a polar, charged adduct that can be easily separated from the covalently-linked conjugate. Optionally, the final reaction mixture 2, before purification, is treated with nucleophiles, such as amino group containing nucleophiles (e.g., lysine, taurine, hydroxylamine) to quench any unreacted linker (X-L-Y'-C).

[52] An alternative method for the reaction of antibody with the unpurified initial reaction mixture of maytansinoids (DMx) and heterobifunctional linker involves mixing the initial reaction mixture of DMx and heterobifunctional linker (upon completion of the DMx-linker reaction) with antibody at low pH (pH ~5) followed by addition of buffer or base to increase the pH to about 6.5-8.5 for the conjugation reaction.

[53] This new method is applied to the preparation of an antibody conjugate with the cytotoxic maytansinoid drug. The antibody-maytansinoid conjugates prepared using this method outlined in the reaction sequence 1-2 unexpectedly were much superior in

homogeneity compared to the conjugates prepared by the traditional two step reaction and purification sequence, based on characterization of the conjugates by reducing SDS-PAGE, protein LabChip electrophoresis, and mass spectrometry. The conjugation method described in this invention that involves the reaction sequence **1-2** also does not require any intermediate purification step and is therefore significantly more convenient than the traditional two-step method.

[54] In a second embodiment of the invention, a process for the preparation of a thioether-linked conjugate of a cell-binding agent with an effector or reporter molecule is described comprising the following steps: a) contacting a homobifunctional linker of formula Y-L-Y with a thiol- or amine-containing effector or reporter group C (such as a cytotoxic agent) in aqueous solvent, organic solvent, or mixed aqueous/organic reaction mixtures to yield Y-L-Y'-C, b) mixing of the reaction mixture without purification with an antibody in aqueous solution or aqueous/organic mixture to produce a conjugate of formula Ab-$(Y'\text{-}L\text{-}Y'\text{-}C)_m$, wherein, L is as defined above; Y is a thiol- or amine-reactive group such as a maleimide or haloacetamide, or *N*-hydroxysuccinimide or sulfo *N*-hydroxysuccinimide; Ab is an antibody; m is an integer from 1 to 20; Y' is the modified Y site (such as a thioether or amide linkage) upon reaction with antibody or a modified Y site (such as a thioether or amide linkage) upon reaction with the cytotoxic agent or effector or reporter group, and c) purification of the conjugate by tangential flow filtration, dialysis, or chromatography (gel filtration, ion-exchange chromatography, hydrophobic interaction chromatography) or a combination thereof. The reaction sequence represented in formulae **3** and **4**:

Y-L-Y + C → Y-L-Y'-C (3)

Ab + Y-L-Y'-C (unpurified from reaction 3) → Ab-(Y'-L-Y'-C)$_m$ (4)

does not involve any purification of the intermediate product Y-L-Y'-C, and therefore is an advantageous method for conjugation.

[55] In a third embodiment, a process for the preparation of a disulfide-linked conjugate of a cell binding agent with an effector or reporter molecule is described that comprises of the following steps: a) contacting a heterobifunctional linker of formula X-L-Y_b with the effector or reporter group C (such as a cytotoxic agent) in aqueous solvent, organic solvent or mixed organic/aqueous reaction mixtures to yield intermediate product X-L-Y_b'-C; b) mixing of the reaction mixture without purification with the antibody in an aqueous solution or aqueous/organic mixture to produce a conjugate of formula Ab-(X'-L-Y_b'-C)$_m$, wherein, L is as described above; Y_b is a reactive disulfide such as a pyridyl disulfide or a nitro-pyridyl disulfide; X is an amine-reactive group such as *N*-hydroxysuccinimide ester or sulfo *N*-hydroxysuccinimide ester; Ab is an antibody; m is an integer from 1 to 20; X' is modified X site (such as amide linkage) upon reaction with antibody; Y_b' is modified Y_b site (disulfide) upon reaction with the cytotoxic agent or effector or reporter group; and c) purification of the conjugate by tangential flow filtration, dialysis, or chromatography (gel filtration, in-exchange chromatography, hydrophobic interaction chromatography) or a combination thereof. The reaction sequence is represented in formulae **5** and **6**:

X-L-Y_b + C → X-L-Y_b'-C (**5**)

Ab + X-L-Y_b'-C (unpurified from reaction **5**) → Ab-(X'-L-Y_b'-C)$_m$ (**6**)

[56] In a fourth embodiment, a process for the preparation of conjugates of antibody with effector or reporter groups with two types of linkers —non-cleavable (thioether

linkage) and cleavable (disulfide linkage)—comprising the following steps is described: a) contacting X-L-Y and X-L-Y_b linkers with the cytotoxic agent C to generate intermediate compounds of formulae X-L-Y'-C and X-L-Y_b'-C, b) mixing of the reaction mixtures without purification with the antibody either in a sequence or simultaneously as indicated in reaction formulae 7-9:

X-L-Y + C → X-L-Y'-C (7)

X-L-Y_b + C → X-L-Y_b'-C (8)

Ab + X-L-Y'-C + X-L-Y_b'-C (unpurified from reactions 7-8)

→ Ab-(X'-L-Y'-C)$_m$(X'-L-Y_b'-C)$_{m'}$ (9)

to provide a conjugate Ab-(X'-L-Y'-C)$_m$(X'-L-Y_b'-C)$_{m'}$, wherein, the definitions of X, L, Y', C, Y_b', and m are as given above, and m' is an integer from 1 to 20; and c) purification of the conjugate by tangential flow filtration, dialysis, or chromatography (gel filtration, ion-exchange chromatography, hydrophobic interaction chromatography) or a combination thereof. These two linker effector intermediates (X-L-Y'-C and X-L-Y_b'-C) are mixed without purification with the antibody in different sequences (first X-L-Y'-C then X-L-Y_b'-C, or first X-L-Y_b'-C then X-L-Y'-C or simultaneously) in various ratio.

[57] The reactions 1, 3, 5, and 7-8, can be carried out at high concentrations of the bifunctional linker (X-L-Y, X-L-Y_b, or Y-L-Y) and the effector or reporter group C in aqueous solvent, organic solvent, or organic/aqueous reaction mixtures, resulting in faster reaction rates than at lower concentrations in aqueous solutions for conjugates prepared by the traditional two step reaction and purification sequence where the solubility of reagents is limiting.

[58] The intermediate products X-L-Y'-C, or Y-L-Y'-C, or X-L-Y_b'-C generated in reactions **1**, **3**, **5**, and **7-8** can be stored unpurified in a frozen state, at low temperatures in aqueous solvent at appropriate pH, in organic solvents, or in mixed organic/aqueous mixtures, or in lyophilized state, for prolonged periods and can be added later to the antibody solution for the final conjugation reaction, therefore adding to the convenience of this reaction sequence.

[59] A stoichiometric or a slight excess of C over the heterobifunctional linker X-L-Y, or Y-L-Y, or X-L-Y_b is used in the first reaction to ensure that all Y group (such as maleimide) is reacted before the unpurified mixture is added to the antibody. An optional additional treatment with a quenching reagent (such as 4-maleimidobutyric acid, or 3-maleimidopropionic acid, or *N*-ethylmaleimide, or iodoacetamide, or iodoacetic acid) can be done to ensure that any unreacted group (such as thiol) in C is quenched before the addition to the antibody to minimize any unwanted thiol-disulfide interchange reaction with the native antibody disulfide groups. The quenching of the excess C using a charged, polar thiol-quenching reagent, after the initial reaction of C with the bifunctional linker, converts excess C into a highly polar, water-soluble adduct that is easily separated from the covalently-linked conjugate by gel filtration, dialysis, or TFF. The final conjugate product does not contain any non-covalently associated C. Optionally, the final reaction mixtures **2**, **4**, **6**, and **9**, before purification, are treated with nucleophiles, such as, amino group containing nucleophiles (e.g., lysine, taurine, hydroxylamine) to quench any unreacted linkers (X-L-Y'-C, Y-L-Y'-C, or X-L-Y_b'-C).

[60] An alternate method of the reaction of antibody with the unpurified initial reaction mixture of DMx and bifunctional linker involves mixing the initial reaction mixture of

DMx and bifunctional linker (upon completion of the DMx-linker reaction) with antibody at low pH (pH ~5) followed by addition of buffer or base to increase the pH to about 6.5-8.5 for the conjugation reaction.

[61] Multiple copies of more than one type of effector can be conjugated to the antibody by adding two or more linker-effector intermediates derived from two or more different effectors, without purification, to the antibody either in a sequence or simultaneously.

EFFECTOR GROUP(S)

[62] The terms Effector group or Effector molecule are used interchangeably and the term **"Effector group(s)"** or "Effector molecule(s)", as used herein, is meant to include cytotoxic agents. In certain respects, it may be desirable that the effector groups or molecules are attached by spacer arms of various lengths to reduce potential steric hindrance. Multiple copies of more than one type of effector can be conjugated to the antibody by adding two or more linker-effector intermediates derived from two or more different effectors, without purification, to the antibody either in a sequence or simultaneously.

[63] Cytotoxic agents that can be used in the present invention include chemotherapeutic agents or structural analogues of chemotherapeutic agents. "Chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents, such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines, such as benzodopa, carboquone, meturedopa, and uredopa;

ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics, such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin .gamma1and calicheamicin theta I, see, e.g., Angew Chem Intl. Ed. Engl. 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromomophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin; chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, nitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid

analogues, such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimnidine analogs such as, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens, such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals, such as aminoglutethimide, mitotane, trilostane; folic acid replenisher, such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfomithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids, such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the

above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors, such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens, such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; siRNA and pharmaceutically acceptable salts, acids or derivatives of any of the above. Other chemotherapeutic agents that can be used with the present invention are disclosed in US Publication No. 20080171040 or US Publication No. 20080305044 and are incorporated in their entirety by reference.

[64] In a preferred embodiment, chemotherapeutic cytotoxic agents are essentially small molecule cytotoxic agents. A "small molecule drug" is broadly used herein to refer to an organic, inorganic, or organometallic compound that may have a molecular weight of for example 100 to 1500, more suitably from 120 to 1200, favorably from 200 to 1000, and typically having a molecular weight of less than about 1000. Small molecule cytotoxic agents of the invention encompass oligopeptides and other biomolecules having a molecular weight of less than about 1000. Small molecule cytotoxic agents are well characterized in the art, such as in WO05058367A2, European Patent Application Nos. 85901495 and 8590319, and in U.S. Patent No. 4,956,303, among others and are incorporated in their entirety by reference.

[65] Preferable small molecule cytotoxic agents are those that allow for linkage to the cell-binding agent. The invention includes known cytotoxic agents as well as those that may become known. Especially preferred small molecule cytotoxic agents include cytotoxic agents.

[66] The cytotoxic agent may be any compound that results in the death of a cell, or induces cell death, or in some manner decreases cell viability, wherein each cytotoxic agent comprises a thiol moiety.

[67] Preferred cytotoxic agents are maytansinoid compounds, taxane compounds, CC-1065 compounds, daunorubicin compounds and doxorubicin compounds, pyrrolobenzodiazepine dimers, calicheamicins, auristatins and analogues and derivatives thereof, some of which are described below.

[68] Other cytotoxic agents, which are not necessarily small molecules, such as siRNA, are also encompassed within the scope of the instant invention. For example, siRNAs can be linked to the crosslinkers of the present invention by methods commonly used for the modification of oligonucleotides (see, for example, US Patent Publications 20050107325 and 20070213292). Thus the siRNA in its 3' or 5'-phosphoromidite form is reacted with one end of the crosslinker bearing a hydroxyl functionality to give an ester bond between the siRNA and the crosslinker. Similarly reaction of the siRNA phosphoramidite with a crosslinker bearing a terminal amino group results in linkage of the crosslinker to the siRNA through an amine. siRNA are described in detail in U.S. Patent Publication Numbers: 20070275465, 20070213292, 20070185050, 20070161595, 20070054279, 20060287260, 20060035254, 20060008822, 20050288244, 20050176667, which are incorporated herein in their entirety by reference.

Maytansinoids

[69] Maytansinoids that can be used in the present invention are well known in the art and can be isolated from natural sources according to known methods or prepared synthetically according to known methods.

[70] Examples of suitable maytansinoids include maytansinol and maytansinol analogues. Examples of suitable maytansinol analogues include those having a modified aromatic ring and those having modifications at other positions.

[71] Specific examples of suitable analogues of maytansinol having a modified aromatic ring include:

[72] (1) C-19-dechloro (U.S. Patent No. 4,256,746) (prepared by LAH reduction of ansamitocin P2);

[73] (2) C-20-hydroxy (or C-20-demethyl) +/-C-19-dechloro (U.S. Patent Nos. 4,361,650 and 4,307,016) (prepared by demethylation using *Streptomyces* or *Actinomyces* or dechlorination using LAH); and

[74] (3) C-20-demethoxy, C-20-acyloxy (-OCOR), +/-dechloro (U.S. Patent No. 4,294,757) (prepared by acylation using acyl chlorides).

[75] Specific examples of suitable analogues of maytansinol having modifications of other positions include:

[76] (1) C-9-SH (U.S. Patent No. 4,424,219) (prepared by the reaction of maytansinol with H2S or P2S5);

[77] (2) C-14-alkoxymethyl (demethoxy/CH2OR) (U.S. Patent No. 4,331,598);

[78] (3) C-14-hydroxymethyl or acyloxymethyl (CH2OH or CH2OAc) (U.S. Patent No. 4,450,254) (prepared from Nocardia);

[79] (4) C-15-hydroxy/acyloxy (U.S. Patent No. 4,364,866) (prepared by the conversion of maytansinol by Streptomyces);

[80] (5) C-15-methoxy (U.S. Patent Nos. 4,313,946 and 4,315,929) (isolated from Trewia nudiflora);

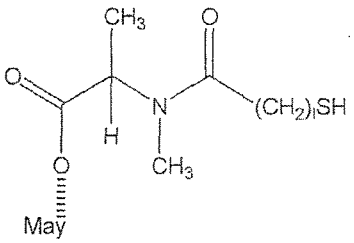
[81] (6) C-18-N-demethyl (U.S. Patent Nos. 4,362,663 and 4,322,348) (prepared by the demethylation of maytansinol by *Streptomyces*); and

[82] (7) 4,5-deoxy (U.S. Patent No. 4,371,533) (prepared by the titanium trichloride/LAH reduction of maytansinol).

[83] The synthesis of thiol-containing maytansinoids useful in the present invention is fully disclosed in U.S. Patent Nos. 5,208,020, 5,416,064, and U. S. Patent Application No. 20040235840.

[84] Maytansinoids with a thiol moiety at the C-3 position, the C-14 position, the C-15 position or the C-20 position are all expected to be useful. The C-3 position is preferred and the C-3 position of maytansinol is especially preferred. Also preferred are an *N*-methyl-alanine-containing C-3 thiol moiety maytansinoid, and an *N*-methyl-cysteine-containing C-3 thiol moiety maytansinoid, and analogues of each.

[85] Specific examples of *N*-methyl-alanine-containing C-3 thiol moiety maytansinoid derivatives useful in the present invention are represented by the formulae **M1**, **M2**, **M3**, **M6** and **M7**.

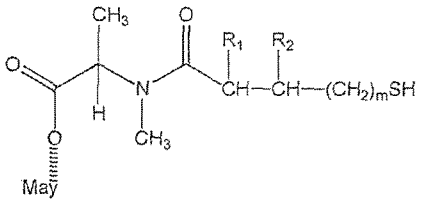


M1

wherein:

l is an integer of from 1 to 10; and

May is a maytansinoid.



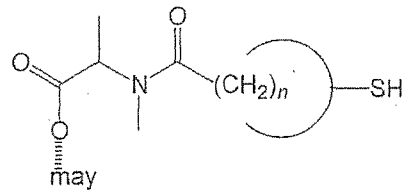
M2

wherein:

R_1 and R_2 are H, CH_3 or CH_2CH_3, and may be the same or different;

m is 0, 1, 2 or 3; and

May is a maytansinoid.

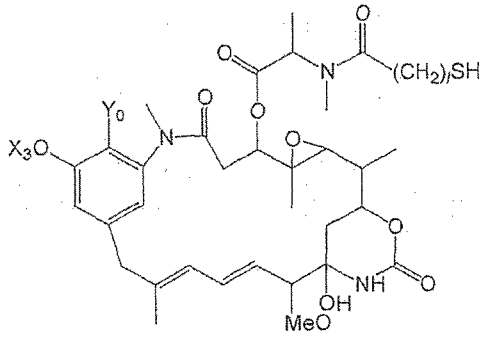


M3

wherein:

n is an integer of from 3 to 8; and

May is a maytansinoid.



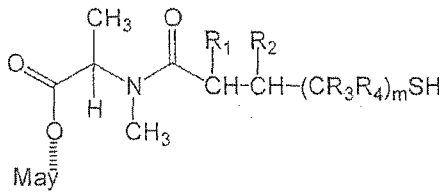
M6

wherein:

l is 1, 2 or 3;

Y_0 is Cl or H; and

X_3 is H or CH_3.



M7

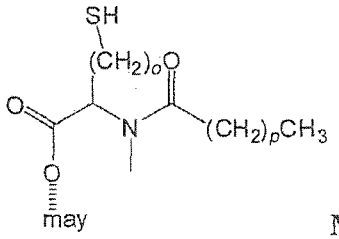
wherein:

R_1, R_2, R_3, R_4 are H, CH_3 or CH_2CH_3, and may be the same or different;

m is 0, 1, 2 or 3; and

May is a maytansinoid.

[86] Specific examples of N-methyl-cysteine-containing C-3 thiol moiety maytansinoid derivatives useful in the present invention are represented by the formulae **M4** and **M5**.



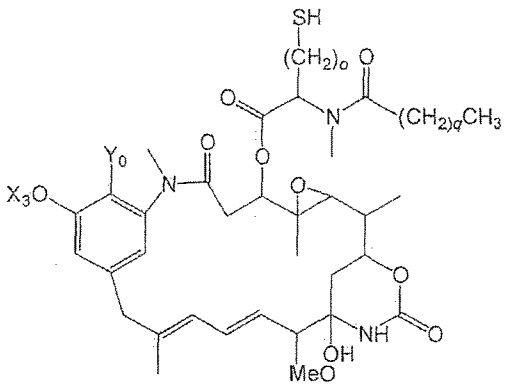
M4

wherein:

o is 1, 2 or 3;

p is an integer of 0 to 10; and

May is a maytansinoid.



M5

wherein:

o is 1, 2 or 3;

q is an integer of from 0 to 10;

Y_0 is Cl or H; and

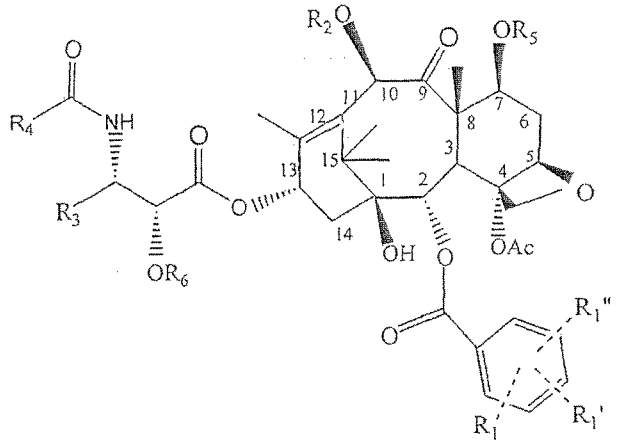
X_3 is H or CH_3.

[87] Preferred maytansinoids are those described in U.S. Patent Nos. 5,208,020; 5,416,064; 6,333.410; 6,441,163; 6,716,821; RE39,151 and 7,276,497.

Taxanes

[88] The cytotoxic agent according to the present invention may also be a taxane.

[89] Taxanes that can be used in the present invention have been modified to contain a thiol moiety. Some taxanes useful in the present invention have the formula T1 shown below:



T1

[90] Preferred taxoids are those described in U.S. Patent Nos. 6,340,701; 6,372,738; 6.436,931; 6,596,757; 6,706,708; 7,008,942; 7,217,819 and 7,276,499.

[91] *CC-1065 analogues*

[92] The cytotoxic agent according to the present invention may also be a CC-1065 analogue.

[93] According to the present invention, the CC-1065 analogues contain an A subunit and a B or a B-C subunit. Preferred CC-1065 analogs are those described in U.S. Patent Nos. 5,475,092; 5,595,499; 5,846,545; 6,534,660; 6,586,618; 6,756,397 and 7,049,316.

Daunorubicin/Doxorubicin Analogues

[94] The cytotoxic agent according to the present invention may also be a daunorubicin analogue or a doxorubicin analogue.

[95] The daunorubicin and doxorubicin analogues of the present invention can be modified to comprise a thiol moiety. The modified doxorubicin/daunorubicin analogues of the present invention, which have a thiol moiety, are described in WO 01/38318. The modified doxorubicin/daunorubicin analogues can be synthesized according to known methods (*see, e.g.*, U.S. Patent No. 5,146,064).

[96] Auristatin include auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE) and are described in U.S. Patent No. 5,635,483, *Int. J. Oncol.* 15:367-72 (1999); Molecular Cancer Therapeutics, vol. 3, No. 8, pp. 921-932 (2004); U.S. Application Number 11/134826. U.S. Patent Publication Nos. 20060074008, 2006022925.

[97] The cytotoxic agents according to the present invention include pyrrolobenzodiazepine dimers that are known in the art (US Patent Nos 7,049,311; 7,067,511; 6,951,853; 7,189,710; 6,884,799; 6,660,856).

Analogues and derivatives

[98] One skilled in the art of cytotoxic agents will readily understand that each of the cytotoxic agents described herein can be modified in such a manner that the resulting compound still retains the specificity and/or activity of the starting compound. The skilled artisan will also understand that many of these compounds can be used in place of the cytotoxic agents described herein. Thus, the cytotoxic agents of the present invention include analogues and derivatives of the compounds described herein.

REPORTER GROUP(S)

[99] The terms Reporter group or Reporter molecule are used interchangeably and the term "Reporter group(s)" or "Reporter molecule(s)", as used herein, refers to a substance which is delivered to the specific substance or cells by the specific affinity portion of the reagent, for a diagnostic or therapeutic purpose; examples are radioisotopes, paramagnetic contrast agents, and anti-cancer agents. Various labels or reporter groups are useful for tumor-imaging applications in cancer patients, immunoassay applications

for diagnosis of various diseases, cancer therapy using radioactive nuclide-ligand conjugates, and affinity chromatography applications for purification of bioactive agents such as proteins, peptides, and oligonucleides. The labels or reporter groups that are conjugated with cell-binding agents include fluorophores, and affinity labels such as biotin. Such reporter group references can be found in US publication number 2007/0092940. Reporter groups including, for example, biotin or fluorescein can also be attached to a PEG conjugate moiety. A number of suitable reporter groups are known in the art, e.g., U.S. Pat. No. 4,152,411 and Hirschfeld U.S. Pat. No. 4,166,105, U.S. Pat. No. 5,223,242, U.S. Pat. No. 5,501,952, US publication 20090136940 and are incorporated in their entirety by reference.

LINKERS

[100] The conjugates may be prepared by *in vitro* methods. In order to link a drug to the cell-binding agent, a linking group is used. Suitable linking groups are well known in the art and include non-cleavable or cleavable linkers. A non-cleavable linker is any chemical moiety that is capable of linking a cytotoxic agent to a cell-binding agent in a stable, covalent manner. Non-cleavable linkers are substantially resistant to acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage. Examples of non-cleavable linkers include linkers having an N-succinimidyl ester, N-sulfosuccinimidyl ester moiety, maleimido- or haloacetyl-based moiety for reaction with the drug, the reporter group or the cell binding agent. Crosslinking reagents comprising a maleimido-based moiety include N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), N-succinimidyl-4-(N-

maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate), which is a "long chain" analog of SMCC (LC-SMCC), κ-maleimidoundecanoic acid N-succinimidyl ester (KMUA), γ-maleimidobutyric acid N-succinimidyl ester (GMBS), ε-maleimidocaproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-(α-maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-(β-maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), and N-(p-maleimidophenyl)isocyanate (PMPI). Cross-linking reagents comprising a haloacetyl-based moiety include N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl iodoacetate (SIA), N-succinimidyl bromoacetate (SBA), and N-succinimidyl 3-(bromoacetamido)propionate (SBAP).

[101] Other crosslinking reagents lacking a sulfur atom that form non-cleavable linkers can also be used in the inventive method. Such linkers can be derived from dicarboxylic acid based moieties. Suitable dicarboxylic acid based moieties include, but are not limited to, α,ω-dicarboxylic acids of the general formula shown below:

$HOOC\text{-}X_l\text{-}Y_n\text{-}Z_m\text{-}COOH$

[102] wherein X is a linear or branched alkyl, alkenyl, or alkynyl group having 2 to 20 carbon atoms, Y is a cycloalkyl or cycloalkenyl group bearing 3 to 10 carbon atoms, Z is a substituted or unsubstituted aromatic group bearing 6 to 10 carbon atoms, or a substituted or unsubstituted heterocyclic group wherein the hetero atom is selected from N, O or S, and wherein l, m, and n are each 0 or 1, provided that l, m, and n are all not zero at the same time.

[103] Many of the non-cleavable linkers disclosed herein are described in detail in U.S. Patent publication number 20050169933.

[104] Cleavable linkers are linkers that can be cleaved under mild conditions, i.e. conditions under which the activity of the cytotoxic agent is not affected. Many known linkers fall in this category and are described below.

[105] Acid-labile linkers are linkers cleavable at acid pH. For example, certain intracellular compartments, such as endosomes and lysosomes, have an acidic pH (pH 4-5), and provide conditions suitable to cleave acid-labile linkers.

[106] Linkers that are photo-labile are useful at the body surface and in many body cavities that are accessible to light. Furthermore, infrared light can penetrate tissue.

[107] Some linkers can be cleaved by peptidases. Only certain peptides are readily cleaved inside or outside cells, see e.g. Trouet et al., 79 Proc. Natl. Acad. Sci. USA, 626-629 (1982), Umemoto et al. 43 Int. J. Cancer, 677-684 (1989), and lysosomal-hydrolase cleavable valine-citrulline linkage (US Patent 6,214,345 B1). Furthermore, peptides are composed of .alpha.-amino acids and peptidic bonds, which chemically are amide bonds between the carboxylate of one amino acid and the .alpha.-amino group of a second amino acid. Other amide bonds, such as the bond between a carboxylate and the .epsilon.-amino group of lysine, are understood not to be peptidic bonds and are considered non-cleavable.

[108] Some linkers can be cleaved by esterases. Again only certain esters can be cleaved by esterases present inside or outside cells. Esters are formed by the condensation of a carboxylic acid and an alcohol. Simple esters are esters produced with simple alcohols, such as aliphatic alcohols, and small cyclic and small aromatic alcohols. For example, the present inventors found no esterase that cleaved the ester at C-3 of

maytansine, since the alcohol component of the ester, maytansinol, is very large and complex.

[109] Preferred cleavable linker molecules include, for example, N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (see, e.g., Carlsson et al., *Biochem. J., 173:* 723-737 (1978)), N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB) (see, e.g., U.S. Patent 4,563,304), N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP) (see, e.g., CAS Registry number 341498-08-6), and other reactive cross-linkers, such as those described in U.S. Patent 6,913,748, which is incorporated herein in its entirety by reference.

[110] Other linkers which can be used in the present invention include charged linkers or hydrophilic linkers and are described in U.S. Patent Application Nos., 12/433,604 and 12/433,668, respectively, which are incorporated herein in its entirety by reference.

CELL BINDING AGENTS

[111] The cell-binding agents used in this invention are proteins (e.g., immunoglobulin and non-immunoglobulin proteins) which bind specifically to target antigens on cancer cells. These cell-binding agents include:

-antibodies including:

-resurfaced antibodies (U.S. patent no. 5,639,641);

-humanized or fully human antibodies (Humanized or fully human antibodies are selected from, but not limited to, huMy9-6, huB4, huC242, huN901, DS6, CD38, IGF-IR, CNTO 95, B-B4, trastuzumab, bivatuzumab, sibrotuzumab, and rituximab (see, e.g., U.S. Patent Nos. 5,639,641, 5,665,357, and 7,342,110, International Patent Application WO 02/16,401, U.S. publication number 20060045877, U.S. publication number

20060127407, U.S. publication number 20050118183, Pedersen et al., (1994) *J. Mol. Biol.* 235, 959-973, Roguska et al., (1994) *Proceedings of the National Academy of Sciences*, Vol 91, 969-973, Colomer et al., *Cancer Invest., 19:* 49-56 (2001), Heider et al., *Eur. J. Cancer, 31A*: 2385-2391 (1995), Welt et al., *J. Clin. Oncol., 12:* 1193-1203 (1994), and Maloney et al., *Blood, 90:* 2188-2195 (1997)); and

-fragments of antibodies such as sFv, Fab, Fab', and $F(ab')_2$ that preferentially bind to a target cell (Parham, *J. Immunol.* 131:2895-2902 (1983); Spring et al, *J. Immunol.* 113:470-478 (1974); Nisonoff et al, *Arch. Biochem. Biophys.* 89:230-244 (1960));

[112] Additional cell-binding agents include other cell binding proteins and polypeptides exemplified by, but not limited to:

-Ankyrin repeat proteins (DARPins; Zahnd et al., J. Biol. Chem., 281, 46, 35167-35175, (2006); Binz, H.K., Amstutz, P. & Pluckthun, A. (2005) Nature Biotechnology, 23, 1257-1268) or ankyrin-like repeats proteins or synthetic peptides described, for example, in U.S. publication number 20070238667; U.S. Patent No. 7,101,675; WO/2007/147213; WO/2007/062466);

-interferons (e.g. α, β, γ);

-lymphokines such as IL-2, IL-3, IL-4, IL-6;

-hormones such as insulin, TRH (thyrotropin releasing hormones), MSH (melanocyte-stimulating hormone), steroid hormones, such as androgens and estrogens; and

-growth factors and colony-stimulating factors such as EGF, TGF-α, IGF-1, G-CSF, M-CSF and GM-CSF (Burgess, *Immunology Today* 5:155-158 (1984)).

[113] Where the cell binding agent is an antibody it binds to an antigen that is a polypeptide and may be a transmembrane molecule (e.g. receptor) or a ligand such as a growth factor. Exemplary antigens include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor vmc, factor IX, tissue factor (TF), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β.; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF);

transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-beta1, TGF-β2, TGF- β3, TGF-β4, or TGF- β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the HIV envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM, alpha-V subunit of a heterodimeric human integrin receptor; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

[114] Preferred antigens for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD46; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150.95, VLA-4, ICAM-1, VCAM, alpha4/beta7 integrin, and alpha v/beta3 integrin including either alpha or beta subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; tissue factor (TF); TGF-β.; alpha interferon (alpha-IFN); an interleukin, such as IL-8; IgE; blood group antigens Apo2, death receptor; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C etc. The most preferred targets herein are IGF-IR, CanAg, EGF-R, EphA2, MUC1, MUC16, VEGF, TF, CD19, CD20, CD22,

CD33, CD37, CD38, CD40, CD44, CD56, CD138, CA6, Her2/neu, CRIPTO (a protein produced at elevated levels in a majority of human breast cancer cells), alpha v/beta3 integrin, alpha v/beta5 integrin, TGF-β, CD11a, CD18, Apo2 and C24.

[115] Monoclonal antibody techniques allow for the production of specific cell-binding agents in the form of monoclonal antibodies. Particularly well known in the art are techniques for creating monoclonal antibodies produced by immunizing mice, rats, hamsters or any other mammal with the antigen of interest such as the intact target cell, antigens isolated from the target cell, whole virus, attenuated whole virus, and viral proteins such as viral coat proteins. Sensitized human cells can also be used. Another method of creating monoclonal antibodies is the use of phage libraries of sFv (single chain variable region), specifically human sFv (*see, e.g.*, Griffiths et al, U.S. patent no. 5,885,793; McCafferty et al, WO 92/01047; Liming et al, WO 99/06587.)

[116] Selection of the appropriate cell-binding agent is a matter of choice that depends upon the particular cell population that is to be targeted, but in general monoclonal antibodies and fragments thereof that preferentially bind to a target cell are preferred, if an appropriate one is available.

[117] For example, the monoclonal antibody My9 is a murine IgG_{2a} antibody that is specific for the CD33 antigen found on Acute Myeloid Leukemia (AML) cells (Roy et al. *Blood* 77:2404-2412 (1991)) and can be used to treat AML patients. Similarly, the monoclonal antibody anti-B4 is a murine IgG_1, that binds to the CD19 antigen on B cells (Nadler et al, *J. Immunol.* 131:244-250 (1983)) and can be used if the target cells are B cells or diseased cells that express this antigen such as in non-Hodgkin's lymphoma or chronic lymphoblastic leukemia. Similarly, the antibody N901 is a murine monoclonal

IgG_1 antibody that binds to CD56 found on small cell lung carcinoma cells and on cells of other tumors of neuroendocrine origin (Roy et al. *J. Nat. Cancer Inst.* 88:1136-1145 (1996)), huC242 antibody that binds to the CanAg antigen, Trastuzumab that binds to HER2/neu, and anti-EGF receptor antibody that binds to EGF receptor.

PURIFICATION METHODS

[118] The conjugate, i.e., the finalized product, of the present invention is purified to remove any unreacted or unconjugated effector or reporter molecule, or unreacted linker or unconjugated, hydrolyzed linker. The purification method can be a tangential flow filtration (TFF, also known as cross flow filtration, ultrafiltration, or diafiltration), gel filtration, adsorptive chromatography, selective precipitation, or combinations thereof. The adsorptive chromatography methods include ion-exchange chromatography, hydroxyapatite chromatography, hydrophobic interaction chromatography (HIC), hydrophobic charge induction chromatography (HCIC), mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, and reversed phase chromatography. For example, the conjugate Ab-$(X'\text{-}L\text{-}Y'\text{-}C)_m$ described in formula **2** is purified from unreacted C or unreacted/hydrolyzed linker X-L-Y or X-L-Y'-C. Similarly, the conjugates described in formulae **4**, **6**, and **9** are purified. Such methods of purification are known to one of skill in the art and can be found, for example, in US Publication No. 2007/0048314.

UNDESIRED HYDROLYZED LINKER OR PROTEIN CROSS-LINKING IN CONJUGATES

[119] Traditional conjugation methods employing the initial reaction of a protein with a heterobifunctional linker with reactive maleimide or haloacetamide residue suffer from two major drawbacks: (i) the conjugate product may consist of hydrolyzed linker, due to aqueous inactivation of the incorporated linker in the antibody before reaction with the effector or reporter molecule; and (ii) inter-or intrachain cross-linking of conjugate, due to reaction of maleimide (or haloacetamide) group with the native histidine, lysine, tyrosine, or cysteine residues in protein or peptide (A. Papini et al., Int. J. Pept. Protein Res., 1992, 39, 348-355; T. Ueda et al., Biochemistry, 1985, 24, 6316-6322). Such interchain cross-linking in antibody would result in various non-reducible covalent linkages between the heavy and light chains, or between two heavy chains, which would be apparent in reducing SDS-PAGE analysis as bands of higher molecular weights than the expected heavy and light chain bands. Such interchain or intrachain cross-linking in antibody would also be apparent by MS as peaks of aberrant masses different than the expected masses of antibody plus linked reporter or effector groups. Unlike traditional conjugation methods, the method described in this application results in conjugates with high homogeneity with no substantial interchain cross-linking or hydrolyzed linker.

[120] All references cited herein and in the examples that follow are expressly incorporated by reference in their entireties.

EXAMPLES

[121] The following examples, which are illustrative only, are not intended to limit the present invention.

Example 1. Conjugation of antibody with cytotoxic agent DM1/DM4 using heterobifunctional linker Maleimide-PEG$_n$-NHS by this method (Figure 1) versus traditional two-step method.

[122] Stock solutions of DM1 [$N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine], or DM4 [$N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl)maytansine] (DMx) thiol and the Maleimide-PEG$_n$-NHS bifunctional linker were made up in *N,N*-dimethylacetamide (DMA) at concentrations of 30-60 mM. The linker and DMx thiol were mixed together in DMA containing up to 50% v/v of 200 mM succinate buffer, 2 mM EDTA, pH 5.0 to give a molar ratio of DMx to linker of 1.6:1 and a final concentration of DMx equal to 15 mM. After mixing, the reaction mixture was left for 1-4 h and then an aliquot of the reaction mixture was diluted 10 fold and its absorbance measured at 302-320 nm to determine the presence of any remaining unreacted maleimide using the extinction coefficient (ε) of maleimide at 302 nm = 620 M^{-1} cm^{-1}, and ε320 nm ~ 450 M^{-1} cm^{-1}. (Additional reverse phase HPLC analysis of a frozen aliquot of the reaction mixture was carried out later with absorbance monitoring at 302 nm and 252 nm to verify complete disappearance of linker maleimide and formation of the desired linker-DMx reagent at the time of addition of the reaction mixture to antibody). When no further maleimide was present by UV, an aliquot of the reaction mixture was added without purification to a solution of antibody in phosphate buffer (pH

7.5) under final conjugation conditions of 4 mg/ml Ab, 90% phosphate buffer/10% DMA, pH 7.5. The conjugation reaction was allowed to proceed at ambient temperature for 2 h. Ab-DMx conjugate was purified from the excess small-molecule DMx and linker reactants using a G25 gel filtration column equilibrated in pH 7.5 phosphate buffer, or using tangential flow filtration (TFF). The conjugation mixture was further kept at 4 °C for 2 days in pH 7.5 buffer to allow the dissociation of any DMx species attached to antibody non-covalently or via labile linkage. The conjugate was then dialyzed overnight into pH 5.5 histidine/glycine buffer and then filtered through a 0.22 μm filter for final storage. The number of DMx molecule per Ab molecule (average) in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DMx and antibody at these two wavelengths.

[123] Several different reaction conditions were used for the initial reaction of DMx thiol with the heterobifunctional maleimide-PEG_4-NHS reagent: 50% DMA/50% aqueous 200 mM succinate buffer pH 5.0, 2 mM EDTA (v/v); or 60% DMA/40% 200 mM succinate buffer pH 5.0, 2 mM EDTA (v/v); or 100% DMA with 1.5 molar equivalents of an organic base (for example N,N'-diisopropyl ethylamine, DIPEA, or 4-methylmorpholine) per mol DM4 thiol.

[124] In one series of experiments, the molar equivalent of DMx to maleimide-PEG_4-NHS linker (purchased from Pierce Endogen) was varied from 1.2 - 2.4, and the reaction time was 30 min. The number of DMx/Ab measured on purified conjugates were measured as a function of added equivalents of DMx per linker. Conditions of 1.2 - 2.0 equivalents of DM1/Linker gave conjugates with similar DMx/Ab loads, indicating that the undesired reaction of the DMx thiol at the NHS ester side of the linker is not a

significant problem. The amount of cross-linking present in the final conjugates was also analyzed by reducing SDS PAGE showing that the presence of cross-linked contaminants decreases significantly with increasing DM1/linker ratio.

[125] One optional quenching step using maleimide or haloacetamide reagents (such as maleimidobutyric acid, or maleimidopropionic acid, or *N*-ethylmaleimide, or iodoacetamide, or iodoacetic acid) was introduced after the completion of the initial DMx and heterobifunctional linker reaction (before the addition of the reaction mixture to the antibody) to quench the excess DMx thiol group in order to prevent any unwanted reaction of DMx thiol with the antibody.

[126] An alternate method of the reaction of antibody with the unpurified initial reaction mixture of DMx and heterobifunctional linker involved mixing the initial reaction mixture of DMx and heterobifunctional linker (upon completion of the DMx-linker reaction) with antibody at low pH (pH ~5) followed by the addition of buffer or base to increase the pH to 6.5-8.5 for the conjugation reaction.

[127] An antibody-PEG_4-Mal-DM1 or DM4 conjugate was made by the traditional two step conjugation method for comparison with the conjugation method described in this invention. The humanized antibody at a concentration of 8 mg/ml in pH 7.5 phosphate buffer (50 mM potassium phosphate, 50 mM sodium chloride, 2 mM EDTA, pH 7.5) and 5% DMA was modified with excess heterobifunctional maleimide-PEG_4-NHS linker reagent (purchased from Pierce Endogen). After 2 h at 25 °C, the modified antibody was gel purified by G25 chromatography to remove excess unreacted, unincorporated linker. The recovery of purified Ab was determined by UV absorbance at 280 nm. The number of linked maleimide groups in the modified Ab was determined using a small aliquot of

modified antibody by addition of a known amount of thiol (such as 2-mercaptoethanol), added in excess over the maleimide, to react with the maleimide residues in the modified antibody and then assaying the remaining thiol by Ellman's assay using DTNB reagent (extinction coefficient of TNB thiolate at 412 nm = 14150 M^{-1} cm^{-1}; Riddles, P. W. et al., Methods Enzymol., 1983, 91, 49-60; Singh, R., Bioconjugate Chem., 1994, 5, 348-351). The conjugation of modified Ab with DM1 or DM4 thiol was carried out at an Ab concentration of 2.5 mg/ml in a reaction mixture consisting of 95% phosphate buffer pH 7.5 (50 mM potassium phosphate, 50 mM sodium chloride, 2 mM EDTA, pH 7.5) and 5% DMA. An excess of 1.7 molar equivalents of DM1 or DM4 thiol was added per mol of linked maleimide on the Ab. After reacting overnight at 25 °C, the conjugate was sterile filtered using a 0.22 μm filter and gel purified from excess unreacted DM1 or DM4 by a G25 column equilibrated in phosphate buffer pH 7.5 (50 mM potassium phosphate, 50 mM sodium chloride, 2 mM EDTA, pH 7.5). The purified conjugate was held at 4 °C for 2 days in phosphate buffer pH 7.5 (50 mM potassium phosphate, 50 mM sodium chloride, 2 mM EDTA, pH 7.5) to allow for the dissociation of any DM1 or DM4 species attached to antibody non-covalently or via a labile linkage. The conjugate was subsequently dialyzed for 2 days in histidine/glycine buffer pH 5.5 (130 mM glycine/10 mM histidine, pH 5.5) and sterile filtered using a 0.22 μm filter. The number of DM1 or DM4 molecules per Ab molecule in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm using known extinction coefficients for DM1/DM4 and Ab at these two wavelengths.

[128] Reducing SDS PAGE was carried out on conjugate and antibody samples using the NuPage electrophoresis system with a 4 -12% Bis Tris Gel (Invitrogen). Heat

denatured and reduced samples were loaded at 10 μg/lane. The reducing SDS-PAGE of the conjugates prepared using the method described in this invention showed only the expected heavy and light chain bands (50 kDa and 25 kDa respectively) as the major bands (Figure 2). In contrast, the conjugates prepared by the traditional two-step conjugation method showed undesired cross-linked bands with molecular weights of 75, 100, 125, and 150 kDa, presumably corresponding to inter-chain cross-linked species HL, H_2, H_2L, and H_2L_2 respectively (Figure 2).

[129] Protein LabChip electrophoresis analysis (under reducing condition) of the antibody-PEG_4-Mal-DM4 conjugate prepared by the method described in this invention showed the expected heavy and light chain bands with percentages of 58 and 30% (of total protein), which are similar to those for unconjugated antibody of 65 and 30% respectively (Figure 3). In contrast, the conjugate prepared using the traditional two-step conjugation method showed heavy and light bands of only 16 and 8% respectively, and major bands of higher molecular weights ranging from 94-169 kDa presumably due to inter-chain cross-linking. Based on the quantitative Protein LabChip analysis, the conjugate prepared by the method described in this application is highly superior to that prepared using the conventional two-step process (Figure 3).

[130] The MS analysis of the conjugates prepared by the method described in this invention showed discrete DMx-antibody conjugate peaks for antibody bearing increasing numbers of maytansinoid molecules per antibody molecule (Figure 4). In contrast, the MS of the conjugate obtained using the traditional 2-step method was nearly unresolved suggesting inhomogeneity of the conjugate preparation presumably due to cross-linking or inactivated maleimide linker. Based on MS, therefore, the conjugate

prepared using the method described in this invention is superior to that synthesized by the traditional two-step method.

[131] The binding of an anti-CanAg Ab-PEG_4-Mal-DM1 conjugate prepared by the method described in this invention was measured by flow cytometry using the antigen-expressing COLO205 cells, and was found to be similar to that of unconjugated antibody suggesting that the conjugation had no detrimental effect on the binding of the antibody (Figure 5). The cytotoxic activity of the anti-CanAg Ab-PEG_4-Mal-DM1 conjugate prepared by the method described in this invention was measured in vitro using COLO205 colon cancer cells expressing the CanAg antigen (Figure 6). The antigen-expressing cancer cells were plated at around 1000 cells/well in a 96 well plate in cell culture media containing fetal bovine serum and exposed to varying concentrations of Ab-DMx conjugate. After a 5 day exposure to the conjugate, the viable cells remaining in each well were measured using the WST-8 assay (Dojindo Molecular Technologies). As shown in Figure 6, the anti-CanAg Ab-PEG_4-Mal-DM1 conjugate prepared using this method was highly potent at low concentrations toward CanAg antigen-expressing COLO205 colon cancer cells. The cytotoxicity of the anti-CanAg Ab-PEG_4-Mal-DM1 conjugate prepared by the method described in this invention was specific to COLO205 cells as it could be blocked by the addition of excess, unconjugated antibody.

Example 2. Conjugation of antibody with DM1/DM4 using maleimide-Sulfo-NHS linker by this method (Figure 7) versus traditional sequential two-step method.

[132] Stock solutions of DMx thiol and the maleimide-Sulfo-NHS heterobifunctional linker were made up in *N,N*-dimethylacetamide (DMA) at concentrations of 30-60 mM.

The linker and DMx thiol were mixed together in DMA containing up to 40 % v/v of 200 mM succinate buffer, 2 mM EDTA, pH 5.0 to give a ratio of DMx to linker of 1.6 and a final concentration of DMx equal to 15 mM. After mixing, the reaction was left for 1-4 h and then an aliquot of the reaction mixture was diluted 10 fold to measure the absorbance at 302-320 nm for assessing the completion of reaction and the absence of maleimide. (Additional reverse phase HPLC analysis of a frozen aliquot of the reaction mixture was carried out later with absorbance monitoring at 302 nm and 252 nm to verify complete disappearance of linker maleimide and formation of the desired linker-DMx reagent at the time of addition of the reaction mixture to antibody). When no further maleimide was present by UV, an aliquot of the reaction mixture was added to a mixture of antibody in phosphate buffer (pH 7.5) under final conjugation conditions of 4 mg/ml Ab, 90% phosphate buffer/10% DMA, pH 7.5. The conjugation reaction was allowed to proceed at ambient temperature for 2 h. The Ab-DMx conjugate was purified from excess unreacted DMx and unconjugated linker products using a G25 gel filtration column equilibrated in pH 7.5 phosphate buffer or by tangential flow filtration. The conjugate was kept at 4 °C for 2 days in pH 7.5 buffer to allow the dissociation of any DMx species attached to antibody non-covalently or via labile linkage. The conjugate was then dialyzed overnight into pH 5.5 histidine/glycine buffer and then filtered through a 0.22 μm filter for final storage. The number of DMx molecules per Ab antibody molecule (average) in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DMx and antibody at these two wavelengths.

[133] For comparison, the Ab-Sulfo-Mal-DMx conjugates were prepared using the traditional 2-step conjugation method. The antibody (Ab) at a concentration of 8 mg/ml in pH 7.5 phosphate buffer/5% DMA buffer was modified with excess bifunctional maleimide-Sulfo-NHS linker. The reaction was allowed to proceed at 20 °C for 2 h and then the modified Ab was purified away from excess unreacted linker using G25 chromatography. The recovery of purified Ab was determined by UV absorbance at 280 nm. The number of linked maleimide groups in the modified Ab was determined using a small aliquot of modified antibody by addition of a known amount of thiol (such as 2-mercaptoethanol), added in excess over the maleimide, to react with the maleimide residues in the modified antibody and then assaying the remaining thiol by Ellman's assay using DTNB reagent (extinction coefficient of TNB thiolate at 412 nm = 14150 M^{-1} cm^{-1}; Riddles, P. W. et al., Methods Enzymol., 1983, 91, 49-60; Singh, R., Bioconjugate Chem., 1994, 5, 348-351) . The conjugation of modified Ab with DMx was carried out at an antibody concentration of 2.5 mg/ml in 95% pH 7.5 phosphate buffer/5% DMA (v/v), with 1.7 molar equivalents of DMx thiol added per mol of linked maleimide in the Ab. The reaction was left for 8-24 h at 18 °C and the conjugate was separated from excess, unreacted DMx via G25 size-exclusion chromatography. After purification the conjugate was kept at 4 °C for 2 days in pH 7.5 buffer to allow the dissociation of any DMx species attached to antibody non-covalently or via labile linkage. The conjugate was then dialyzed overnight into pH 5.5 histidine/glycine buffer and then filtered through a 0.22 μm filter for final storage. The number of DMx molecule per Ab molecule in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DMx and antibody at these two wavelengths.

[134] Reducing SDS PAGE was carried out using conjugate and antibody samples (10 μg/lane) using the NuPage electrophoresis system (Invitrogen) with a NuPage 4 -12% Bis Tris Mini Gel and NuPAGE MOPS SDS running buffer (Figure 8). Bands on the gel with molecular weights of 75, 125, and 150 kDa were indicative of inter-chain cross-linked species (HL, H_2L and H_2L_2 respectively). A comparison of Ab-Sulfo-Mal-DM1 conjugates with ~4 DM1/Ab (lane 3, by this method, and lane 2, by traditional 2-step conjugation method, respectively) and ~6 DM1/Ab (lane 5, by this method, and lane 4, by traditional 2-step conjugation method, respectively) clearly shows that conjugates made via the method described in this invention (lanes 3 and 5) have much smaller proportion of high molecular weight cross-linked species than conjugates made by the traditional 2-step method (lanes 2 and 4).

[135] Protein LabChip electrophoresis analysis (under reducing condition) of the antibody-Sulfo-Mal-DM1 conjugate prepared by the method described in this invention showed the heavy and light chain major bands with percentages of 70 and 28% (of total protein), which are similar to those for unconjugated antibody of 70 and 30% respectively (Figure 9). In contrast, the conjugate prepared using the traditional two-step method showed heavy and light bands of only 53 and 23% respectively, and major bands of higher molecular weights ranging from 99-152 kDa presumably due to inter-chain cross-linking. Based on the quantitative Protein LabChip analysis, the conjugate prepared by the method described in this application is much superior in terms of lack of inter-chain cross-linking compared to that prepared using the conventional two-step process (Figure 9).

[136] The Ab-Sulfo-mal-DM1 conjugates with similar drug loads made via the method described in this invention and by the traditional two step method were compared by size exclusion LC/MS analysis (Figure 10). The conjugates made via the method described in this invention show the desired MS spectrum containing only the expected distribution of peaks with mass equal to Ab-(linker-DMx)$_n$. In the case of conjugates made using the traditional two-step method, the major peaks in the spectra all contain one or more hydrolyzed or cross-linked linker fragments in addition to the desired Ab-(linker-DMx)$_n$ moieties. The putative mechanism of the inter-chain cross-linking or aqueous inactivation of maleimide in the traditional 2-step reaction sequence is shown in Figure 17, whereby the incorporated maleimide (or haloacetamide) residue from the initial reaction of antibody with the heterobifunctional linker can react with intramolecular (or intermolecular) histidine, lysine, tyrosine, or cysteine residues resulting in inter-chain cross-linking, or the initially incorporated maleimide (or haloacetamide) residue can get inactivated (such as by hydrolytic maleimide ring cleavage or by aqueous addition to maleimide) and therefore become unavailable for the rapid reaction with thiol-bearing effector or reporter group. Thus the LC-MS analysis clearly shows that the method described in this invention has the advantage of producing homogeneous conjugate with little or no hydrolyzed or cross-linked linker fragments attached to antibody.

[137] The binding of an anti-CanAg Ab-Sulfo-Mal-DM1 conjugate with 5.6 maytansinoid load per antibody molecule (average) prepared by the method described in this invention was measured by flow cytometry using the antigen-expressing COLO205 cells, and was found to be similar to that of unconjugated antibody suggesting that the conjugation did not affect the binding of the antibody to target antigen (Figure 11). The

cytotoxic activity of the anti-CanAg Ab-Sulfo-Mal-DM1 conjugate prepared by the method described in this invention was measured *in vitro* using COLO205 colon cancer cells expressing the CanAg antigen (Figure 12). The antigen-expressing cancer cells were plated at around 1000 cells/well in a 96 well plate in cell culture media containing fetal bovine serum and exposed to varying concentrations of Ab-DMx conjugate. After a 5 day exposure to the conjugate, the viable cells remaining were measured using the WST-8 assay (Dojindo Molecular Technologies). As shown in Figure 12, the anti-CanAg Ab-Sulfo-Mal-DM1 conjugate prepared using this method was highly potent at low concentrations toward CanAg antigen-expressing COLO205 colon cancer cells. The cytotoxicity of this conjugate was specific as it could be blocked by competition with excess, unconjugated antibody.

[138] An alternative method of conjugation using the method described in this invention involved a quenching step using maleimide or haloacetamide reagents (such as 4-maleimidobutyric acid, or 3-maleimidopropionic acid, or *N*-ethylmaleimide, or iodoacetamide, or iodoacetic acid) after the completion of the initial DMx and heterobifunctional linker reaction (before the addition of the reaction mixture to the antibody) to quench the excess DMx thiol group in order to prevent any unwanted reaction of DMx thiol with the antibody. In a specific example, following completion of the initial DMx and heterobifunctional linker reaction (before the addition of the reaction mixture to the antibody), 4-maleimidobutyric acid was added to quench the excess DMx thiol group in order to prevent any unwanted reaction of DMx thiol with the antibody during the conjugation reaction. To a reaction mixture of DM4 and Sulfo-Mal-NHS heterobifunctional reagent that contained an excess of DM4 (3 mM), upon completion of

the desired DM4 thiol coupling to the maleimide group of the heterobifunctional reagent, a two-fold molar excess of 4-maleimidobutyric acid (6 mM) was added to the reaction mixture at ambient temperature for 20 minutes to quench the remaining DM4 from the initial coupling reaction. Without purification of the reaction mixture, an aliquot was mixed with a solution of antibody in phosphate buffer (pH 7.5) under final conjugation conditions of 4 mg/ml Ab, 90% aqueous phosphate buffer/10%DMA, pH 7.5. The conjugation reaction was allowed to proceed at ambient temperature for 2 h. The antibody-DM4 conjugate was purified from the excess small-molecule DM4 and linker reactants using a G25 gel filtration column equilibrated in pH 7.5 phosphate buffer. The conjugation mixture was further kept at 4 °C for 2 days in pH 7.5 buffer to allow the dissociation of any DMx species attached to antibody non-covalently or via labile linkage. The conjugate was then dialyzed overnight into pH 5.5 histidine/glycine buffer and filtered through a 0.22 μm filter for final storage. The average number of DM4 molecules per Ab molecule in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DM4 and antibody at these two wavelengths. The conjugate samples were analyzed by non-reducing SDS PAGE using the NuPage electrophoresis system with a 4–12% Bis Tris Gel (Invitrogen). The heat-denatured samples were loaded at 10 μg/lane. The non-reducing SDS-PAGE of the conjugate prepared using the method described in this invention (without quenching) showed evidence of a light chain band (~25 kDa) and half-antibody band (heavy-light chain; ~75 kDa) (Figure 18). On the other hand, the conjugate prepared using the method described in this invention which was treated with 4-maleimidobutyric acid (to cap excess DMx thiol) had significantly lower amounts of

these undesirable bands (at levels comaparable to unmodifed antibody sample). Another advantage of the quenching of the initial DMx and heterobifunctional reaction mixture (before conjugation with the antibody) by thiol-quenching reagents such as 4-maleimidobutyric acid is that during the antibody conjugation reaction there is no "free" DMx (DM1 or DM4) species and therefore the final conjugate after purification does not contain "free" or unconjugated DMx species. The DMx-adduct with 4-maleimidobutyric acid (or other polar thiol-quencing reagents) is more water soluble than DMx and therefore can be more easily separated from the covalently linked antibody-DMx conjugate.

Example 3. Conjugation of antibody with maytansinoid (DM1/DM4) using sulfo-NHS-SMCC linker (Figure 13).

[139] Stock solutions of DM1 or DM4 thiol (DMx) and the sulfo-SMCC heterobifunctional linker with sulfo-NHS group (purchased from Pierce Endogen; Figure 13) were prepared in DMA at concentrations of 30-60 mM. Linker and DM1 or DM4 thiol were mixed together in DMA containing up to 40% v/v of aqueous 200 mM succinate buffer, 2 mM EDTA, pH 5.0 to give a ratio of DM1 or DM4 (DMx) to linker of 1.6:1 and a final concentration of DMx of 6 mM. After mixing, the reaction was left for 1-4 h at ambient temperature and then an aliquot of the reaction mixture was diluted 10-fold to measure absorbance at 302-320 nm to assess whether all of the maleimide had reacted. (Additional reverse phase HPLC analysis of a frozen aliquot of the reaction mixture was carried out later with monitoring at 302 nm and 252 nm to verify complete disappearance of linker maleimide and formation of the desired sulfo-NHS-linker-Mal-

DMx reagent at the time of addition of the reaction mixture to antibody). When no further maleimide was present by UV an aliquot of the reaction was added to an aqueous solution of an antibody in phosphate buffer (pH 7.5) under final conjugation conditions of 4 mg/ml Ab, 90% phosphate buffer (aqueous)/10% DMA (v/v), pH 7.5. The conjugation reaction was allowed to proceed at ambient temperature for 2 h. Ab-DMx conjugate was purified from excess unreacted reagent and excess DMx using a G25 gel filtration column equilibrated in pH 7.5 phosphate buffer (aqueous). Conjugate was kept at 4 °C for 2 days in pH 7.5 buffer to allow the dissociation of any DMx species attached to Ab non-covalently or via labile linkage. The conjugate was then dialyzed overnight into pH 5.5 histidine/glycine buffer and then filtered through a 0.22 μm filter for final storage. The number of DMx molecule per Ab molecule in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DMx and antibody at these two wavelengths.

[140] For comparison, the Ab-SMCC-DMx conjugates were prepared using the traditional 2-step conjugation method. The antibody (Ab) at a concentration of 8 mg/ml in 95% pH 6.5 phosphate buffer/5% DMA buffer was modified with excess bifunctional sulfo-SMCC linker with sulfo-NHS group (purchased from Pierce Endogen). The reaction was allowed to proceed at 25 °C for 2 h and then the modified Ab was purified away from excess unreacted linker using G25 chromatography. The recovery of purified Ab was determined by UV absorbance at 280 nm. The number of linked maleimide groups in the modified Ab was determined using a small aliquot of modified antibody by addition of a known amount of thiol (such as 2-mercaptoethanol), added in excess over the maleimide, to react with the maleimide residues in the modified antibody and then

assaying the remaining thiol by Ellman's assay using DTNB reagent (extinction coefficient of TNB thiolate at 412 nm = 14150 M^{-1} cm^{-1}; Riddles, P. W. et al., Methods Enzymol., 1983, 91, 49-60; Singh, R., Bioconjugate Chem., 1994, 5, 348-351). The conjugation of modified Ab with DM1 or DM4 was carried out at an antibody concentration of 2.5 mg/ml in 95% pH 6.5 phosphate buffer/5% DMA (v/v), with 1.7 molar equivalents of DM1 or DM4 thiol added per mol of linked maleimide in the Ab. The reaction was left for 8-24 h at 18 °C and the conjugate was separated from excess, unreacted DM1 (or DM4) via G25 chromatography. After purification the conjugate was kept at 4 °C for 2 days in pH 6.5 buffer to allow the hydrolysis of any weakly linked DM1/DM4 species. The conjugate was then dialyzed overnight into pH 5.5 histidine/glycine buffer and then filtered through a 0.22 μm filter for final storage. The number of DM1/DM4 molecules per Ab molecule in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DM1/DM4 and antibody at these two wavelengths.

[141] Reducing SDS PAGE was carried out on conjugate and antibody samples (10 μg/lane) using the NuPage electrophoresis system (Invitrogen) with a NuPage 4 -12% Bis Tris Mini Gel and NuPAGE MOPS SDS running buffer (Figure 14). Bands on the gel with molecular weights of 75, 125, and 150 kDa were indicative of inter-chain cross-linked species (HL, H_2L and H_2L_2 respectively). A comparison of Ab-SMCC-DM1 conjugates with 3.1 D/Ab (lane 4, by this method, and lane 3, by the traditional 2-step method, respectively) clearly shows that conjugate made via the method described in this invention (lane 4) has much fewer high molecular weight cross-linked species than conjugates made by the traditional 2 step method (lane 3).

[142] Protein LabChip electrophoresis analysis (under reducing condition) of the antibody-SMCC-DM1 conjugate prepared by the method described in this invention showed the heavy and light chain major bands with percentages of 67 and 30% (of total protein), which are similar to those for unconjugated antibody of 68 and 30% respectively (Figure 15). In contrast, the conjugate prepared using the traditional two-step method showed heavy and light bands of only 54 and 24% respectively, and major bands of higher molecular weights ranging from 96-148 kDa presumably due to inter-chain cross-linking. Based on the quantitative Protein LabChip analysis, the conjugate prepared by the method described in this application is much superior in terms of lack of inter-chain cross-linking compared to that prepared using the conventional two-step process (Figure 15).

[143] The Ab-SMCC-DM1 conjugates with similar drug loads made via the method described in this invention and by the traditional two step method were compared by size exclusion LC/MS analysis (Figure 16). The conjugate made via the method described in this invention shows the desired MS spectrum containing only the expected distribution of peaks with mass equal to Ab-(linker-DMx)$_n$. In the case of conjugate made using the traditional two-step method the spectrum shows a heterogeneous mixture of species which includes the desired Ab-(linker-DMx)$_n$ species plus additional species containing inactivated maleimide and cross-linked linker fragments. The putative mechanisms of the inter-chain cross-linking and maleimide inactivation in the traditional 2-step reaction sequence are shown in Figure 17 whereby the incorporated maleimide (or haloacetamide residue) from the initial reaction of antibody with the heterobifunctional linker can react with intramolecular (or intermolecular) histidine, lysine, tyrosine, or cysteine residues

resulting in inter-chain cross-linking, or the initially incorporated maleimide (or haloacetamide) residue can get inactivated by hydrolysis or hydration of the maleimide residue before the reaction step with the thiol-bearing DM1 or DM4 (DMx) agent. Thus the LC-MS analysis clearly shows that the method described in this invention has the advantage of producing homogeneous conjugate with little or no inactivated maleimide or cross-linked linker fragments attached to antibody.

Example 4. Conjugation of antibody with DM1/DM4 (DMx) with cleavable, disulfide linkers by this method (Figure 19).

[144] Stock solutions containing DM1 or DM4 thiol (DMx) and heterobifunctional linker 4-(2-pyridyldithio)butanoic acid-*N*-hydroxysuccinimide ester (SPDB) were prepared in DMA at concentrations of 30-60 mM. Linker and DMx thiol were mixed together in DMA containing up to 40% v/v of aqueous 200 mM succinate buffer, 2 mM EDTA, pH 5.0 to give a ratio of DM1 or DM4 (DMx) to linker of 1.6:1 and a final concentration of DMx of 8 mM. After mixing, the reaction was left for 1 h at ambient temperature and then an aliquot of the reaction was added to an aqueous solution of antibody in phosphate buffer (pH 7.5) under final conjugation conditions of 4 mg/ml Ab, 90% phosphate buffer (aqueous)/10% DMA (v/v), pH 7.5. The conjugation reaction was allowed to proceed at ambient temperature for 2 h. The Ab-DMx conjugate was purified from excess unreacted reagent and excess DMx using a G25 gel filtration column equilibrated in pH 7.5 phosphate buffer (aqueous). Conjugate was kept at 4°C for 2 days in pH 7.5 buffer to allow for the dissociation of any DMx species attached to Ab non-covalently or via labile linkage. The conjugate was then dialyzed overnight into pH 5.5

histidine/glycine buffer and then filtered through a 0.22 μm filter for final storage. The number of DMx molecules per Ab molecule on the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm using known extinction coefficients for DMx and antibody at these two wavelengths.

Example 5. Preparation of antibody-DM1/DM4 (Ab-DMx) conjugate with both disulfide- and non-cleavable linkers using this method (Figure 20).

[145] Stock solutions of DM1 or DM4 thiol (DMx) and the NHS-PEG_n-Maleimide heterobifunctional linker were prepared in N,N-dimethylacetamide (DMA) at concentrations of 30-80 mM. The NHS-PEG_4-Maleimide linker and DMx thiol were mixed together in DMA containing up to 40% v/v of 200 mM succinate buffer, 2 mM EDTA, pH 5.0 to give a molar ratio of DMx to linker of 1.6:1 and a final concentration of DMx equal to 8.0 mM. The reaction mixture was left to react for 2 h at ambient temperature. In a separate parallel reaction, SPDB linker and DMx thiol were mixed together and reacted in a similar fashion to the conditions used for NHS-PEG_4-maleimide reaction except for a reaction time of 1 h. After the completion of both reactions and without purification, equal volumes of PEG_4-Mal-DM4 mixture and SPDB-DM4 mixture were combined. An aliquot of the combined reaction mixtures was added without purification to a solution of antibody in phosphate buffer (pH 7.5) under final conjugation conditions of 4 mg/ml Ab, 90% phosphate buffer (aqueous)/10% DMA (v/v), pH 7.5. The conjugation reaction was allowed to proceed at ambient temperature for 2 h. Ab-DMx conjugate was purified from excess unreacted reagents and excess DMx using a G25 gel filtration column equilibrated in pH 7.5 phosphate buffer (aqueous). The

conjugate was kept at 4°C for 2 days in pH 7.5 buffer to allow for the dissociation of DMx species attached to Ab non-covalently or via labile linkage. The conjugate was then dialyzed overnight into pH 5.5 histidine/glycine buffer and then filtered through a 0.22 μm filter for final storage. The number of DMx molecules per Ab molecule on the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm using known extinction coefficients for DMx and antibody at these two wavelengths.

[146] The Ab-(mixed SPDB and PEG_4-Mal linker)-DMx conjugate made via the method described in this invention was tested to determine the percent of incorporation of cleavable versus non-cleavable linker on the Ab by comparing DMx per antibody (D/A) ratio before and after DTT (dithiothreitol) treatment of the conjugate to reduce the disulfide linkage. In order to maintain reaction pH at 7.5 during DTT reduction, the conjugate was first dialyzed into 250 mM HEPES buffer pH 7.5. The conjugate was then reduced by reacting with 25 mM DTT for 20 min at 37°C. After the DTT reaction, the released DMx and DTT were separated from the reaction mixture using a G25 gel filtration column equilibrated in 250 mM HEPES buffer pH 7.5. The average number of DMx molecules per Ab molecule in the purified product was measured by determining the absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DMx and antibody at these two wavelengths. The ratio between D/A of DTT-treated conjugate and D/A of non-DTT treated conjugate was used to calculate the percent of DMx attached to Ab via non-cleavable linkage. Two additional samples, Ab-SPDB-DM4 and Ab-PEG_4-Mal-DM4 conjugates, were treated with DTT as positive and negative controls, respectively. By comparing D/A ratio before and after DTT treatment, the control non-cleavable Ab-PEG_4-Mal-DM4 conjugate showed that approximately all

linkers bound were found to be non-cleavable (93%) as expected. The Ab-(mixed SPDB and PEG_4Mal linker)-DMx conjugate containing both non-cleavable and disulfide linkers made via the method described in this invention had 41% less DMx cleaved by DTT treatment relative to the amount of DMx loss from the Ab-SPDB-DMx conjugate that consists entirely of cleavable linker. This demonstrated that the Ab-(mixed SPDB and PEG_4-Mal)-DMx conjugate made via the method described in this invention was composed of approximately 40% non-cleavable and 60% cleavable linkers. By changing the initial ratio of the non-cleavable and cleavable linker reagents, conjugates of antibody with maytansinoid or other effector can be prepared with different ratio of non-cleavable and cleavable linkers. Figure 21 shows the mass spectrum of deglycosylated conjugate described above, which comprises of antibody with an average of 3.5 maytansinoid molecules per antibody molecule linked via both disulfide linkers (SPDB) and non-cleavable linkers (PEG). The MS shows discrete conjugate species bearing both cleavable and non-cleavable linkers (Figure 21). For example, the conjugate peak designated D2-PEG-SPDB bears one disulfide-linked and one non-cleavable thioether-linked maytansinoid molecule; the conjugate peak designated D3-PEG-2SPDB bears two disulfide-linked and one non-cleavable thioether-linked maytansinoid molecules; and the conjugate peak designated D3-2PEG-SPDB bears one disulfide-linked and two non-cleavable thioether-linked maytansinoid molecules.

Example 6. Conjugation of antibody with maytansinoid using SMCC linker (Figure 22).

[147] Stock solutions of DM1 thiol and SMCC heterobifunctional linker (Pierce) were prepared in DMA at concentrations of 30-60 mM. Linker and DM1 thiol were mixed together in DMA containing up to 50% v/v of aqueous 200 mM succinate buffer, 2 mM EDTA, pH 5.0 to give a ratio of DM1 to linker of 1.4:1 mole equivalent and a final concentration of DM1 of 1 to 6 mM. After mixing, the reaction was left for up to 4 h at ambient temperature and then an aliquot of the reaction mixture was diluted 10-fold to measure absorbance at 302-320 nm to assess whether all of the maleimide had reacted with thiol. When no further maleimide was present by UV, an aliquot of the reaction was added to an aqueous solution of an antibody in phosphate buffer (pH 7.5-8.5) under final conjugation conditions of 2.5 mg/ml Ab, 70-80% phosphate buffer (aqueous)/30-20% DMA (v/v). The conjugation reaction was allowed to proceed at ambient temperature for 3 h. Ab-DM1 conjugate was purified from excess unreacted or hydrolyzed reagent and excess DM1 using a G25 gel filtration column equilibrated in pH 7.4 phosphate buffer (aqueous). The conjugate was then dialyzed overnight into pH 7.4 phosphate buffer (aqueous) and then filtered through a 0.22 μm filter for final storage. The number of DM1 molecule per Ab molecule in the final conjugate was measured by determining absorbance of the conjugate at 252 and 280 nm and using known extinction coefficients for DM1 and antibody at these two wavelengths. Similarly, conjugates of antibody with DM4 thiol and SMCC can be prepared. These conjugates of antibody with DM1 or DM4 using SMCC linker contain thioether non-cleavable linker.

[148] The Ab-SMCC-DM1 conjugate made via the method described in this invention was characterized by MS analysis of deglycosylated conjugate (Figure 23). The conjugate made via the method described in this invention shows the desired MS spectrum containing the expected distribution of peaks with mass equal to Ab-(linker-DM1)$_n$.

Example 7. Conjugation of antibody with maytansinoid using heterobifunctional disulfide-containing linkers (SSNPB, SPP).

[149] Disulfide containing heterobifunctional linkers SSNPB (*N*-sulfosuccinimidyl-4-(5-nitro-2-pyridyldithio)butyrate) and SPP (*N*-succinimidyl-3-(2-pyridyldithio)propionate) can be used to prepare disulfide-linked antibody- maytansinoid conjugates by the method similar to that described for SPDB linker in Example 4. The structure of the disulfide-linked conjugate prepared using SPDB (Figure 19) is identical to that of the conjugate prepared with SSNPB (Figure 24). The MS of a disulfide-linked conjugate prepared using SPDB showed discrete peaks with mass values corresponding to different numbers of maytansinoid molecules attached to antibody.

Example 8. Conjugation of antibody with maytansinoid containing non-cleavable linkers with linear alkyl carbon chain.

[150] Conjugates containing non-cleavable linker with linear alkyl carbon chain were prepared using reaction mixture of maytansinoid and heterobifunctional linkers with linear alkyl carbon chain, similar to the method described for SMCC linker in example 6. For example, conjugates of a humanized antibody with DM1 were prepared using BMPS

(*N*-[β-maleimidopropyloxy]succinimide ester) or GMBS ((*N*-[γ-maleimidobutyryloxy]succinimide ester) linker as shown in Figure 26. The initial reaction mixture containing BMPS or GMBS (8 mM) and DM1 thiol (10.4 mM) in 60% DMA/40% (v/v) 200 mM succinate buffer, pH 5, showed complete reaction of maleimide moiety (based on decay of maleimide absorbance at 302-320 nm) when checked at 15 min. This reaction mixture was added, in two portions 30 min apart, to a humanized antibody solution at 2.5 mg/ml in 80% aqueous EPPS buffer, pH 8.1, containing 20% DMA (v/v) with the total linker added at 8 molar equivalents to antibody. The conjugate mixture was gel purified after 4 h and subjected to 2 rounds of dialysis. Conjugates with DM1/antibody ratio of 3.8 and 5.1 were prepared with 71-75% recovery, and high monomer % (96.2-97.6%). These conjugates prepared with GMBS or BMPS showed no unconjugated free drug by HISEP HPLC analysis. Similar conjugates containing non-cleavable linkers with linear alkyl chains can be prepared using AMAS (*N*-[β-maleimidoacetoxy]succinimide ester) or EMCS (*N*-[β-maleimidocaproyloxy]succinimide ester) or the sulfo-*N*-hydroxysuccinimide esters (sulfo-GMBS, sulfo-EMCS) as shown in Figure 25. Table 1 shows the monomer % for select conjugates prepared by the method described in this invention, which all showed high monomer % by size-exclusion chromatography analysis. For comparison, monomer % are also shown for conjugates prepared by the traditional two-step conjugation method (by the initial reaction of antibody with heterobifunctional linker followed by reaction with mayansinoid thiol).

Table 1. Monomer % for select conjugates made by the method described in this application versus by traditional two-step conjugation methods

Conjugate	D/A	Conjugation method	% Monomer
Ab-PEG_4-Mal-DM1	6.6	this invention	99.0
Ab-PEG_4-Mal-DM1	6.8	two-step	98.0
Ab-Sulfo-Mal-DM1	3.6	this invention	99.0
Ab-Sulfo-Mal-DM1	4.0	two-step	96.7
Ab-SMCC-DM1	4.0	this invention	98.6
Ab-SMCC-DM1	3.8	two-step	97.0
Ab-PEG_4-Mal-DM4	6.2	this invention	96.9
Ab-PEG_4-Mal-DM4	6.1	two-step	84.5
Ab-SPDB-DM4	4.1	this invention	99.4
Ab-SPDB-DM4	3.9	two-step, one-pot	95.7

CLAIMS

1. A process for preparing a purified conjugate in a solution, wherein the conjugate comprises a maytansinoid molecule linked to an antibody, the process comprising the steps of:

(a) contacting a maytansinoid molecule comprising a thiol group with a bifunctional linker reagent to covalently attach the linker to the maytansinoid molecule, wherein an excess of maytansinoid molecule relative to the bifunctional linker reagent is used, and thereby prepare an unpurified first mixture comprising the maytansinoid molecule having linkers bound thereto,

(b) conjugating an antibody to the maytansinoid molecule having linkers bound thereto by reacting the unpurified first mixture with the antibody in a solution having a pH from about 4 to about 9 to prepare a second mixture, and

(c) subjecting the second mixture to tangential flow filtration, dialysis, gel filtration, adsorptive chromatography, selective precipitation or a combination thereof to thereby prepare the purified conjugate.

2. The process of claim 1, wherein the second mixture in step (b) is substantially free of undesired cross-linked, hydrolyzed species formed due to intra-molecular or inter-molecular reactions.

3. The process of claim 1, wherein the maytansinoid molecule is DM1.

4. The process of claim 1, wherein the maytansinoid molecule is DM4.

5. The process of claim 1, wherein the antibody is a monoclonal antibody.

6. The process of claim 1, wherein the antibody is a human or a humanized monoclonal antibody.

7. The process of claim 1, wherein the antibody is MY9, anti-B4, C242, or an antibody that binds to an antigen selected from the group consisting of EpCAM, CD2, CD3, CD4, CD5, CD6, CD11, CD19, CD20, CD22, CD26, CD30, CD33, CD37, CD38, CD40, CD44, CD56, CD79, CD105, CD138, EphA receptors, EphB receptors, EGFR, EGFRvIII, HER2, HER3, mesothelin, cripto, $alpha_v beta_3$, $alpha_v beta_5$, and $alpha_v beta_6$ integrin.

8. The process of claim 6, wherein the human or the humanized antibody is huMy9-6, huB4, huC242, huN901, DS6, CNTO 95, B-B4, trastuzumab, pertuzumab, bivatuzumab, sibrotuzumab, rituximab, or a human or humanized antibody that binds to an antigen selected from the group consisting of EphA2 receptor, CD38, and IGF-IR.

9. The process of claim 1, wherein the linker is a cleavable or a non cleavable linker.

10. The process of claim 1, wherein the pH of the solution in step (b) is from about 5 to about 8.7.

11. The process of claim 1, wherein the pH of the solution in step (b) is from about 6.5 to about 8.5.

12. The process of claim 1, wherein the process further comprises the step of quenching the excess maytansinoid in the unpurified first mixture with a quenching reagent between steps (a) and (b).

13. The process of claim 12, wherein the quenching reagent is selected from the group consisting of 4-maleimidobutyric acid, 3-maleimidopropionic acid, N-ethylmaleimide, iodoacetamide, and iodoacetamidopropionic acid.