SPECIFIC SITES FOR MODIFYING ANTIBODIES TO MAKE IMMUNOCONJUGATES

FIELD OF THE INVENTION

Due to the importance of antibodies for various therapeutic applications, there is a need for robust methods to selectively modify antibodies to introduce improved properties or additional functions. The invention provides specific sites for attaching moieties to antibodies for making modified antibodies, such as for use in preparation of antibody-drug conjugates (ADCs). The selective conjugation sites are located on constant regions of the antibody and thus are useful with various antibodies.

BACKGROUND

The value of methods for modifying antibodies is well known, and many methods for conjugation of antibodies to attach various "payload" moieties have been developed. Many of these methods rely upon the natural occurrence of specific reactive amino acid residues on the antibody, such as lysine and cysteine, which can be used to attach a payload. However, relying on the native amino acids is not always desirable, because the location and amount of payload attached depend on the number and position of those reactive amino acids: too many or too few such residues make it difficult to efficiently control loading of the payload onto the antibody. In addition, placement of the reactive amino acids may make it difficult to get complete conjugation, resulting in heterogeneous products during conjugation. Heterogeneity of a pharmaceutical active ingredient, for example, is typically undesirable because it compounds the unpredictability of administering a drug to a heterogeneous population of subjects: it is far preferable to administer a homogeneous product, and far more difficult to fully characterize and predict behavior of a heterogeneous one. Site-specific conjugation of a cytotoxic drug to an antibody through, for example, engineered cysteine residues results in homogenous immunoconjugates that exhibit improved therapeutic index (Junutula *et al.*, (2008) Nat Biotechnol. 26(8):925-932)).

Antibodies have been engineered to add certain residues like cysteine in specific positions where these residues can be used for conjugation (Lyons *et al.*, (1990) Protein Eng., 3:703-708), but the value of specific substitutions can vary with certain antibodies, as engineered cysteine might interfere with folding of the antibody and oxidation of the proper intra-molecular disulfide bonds (Voynov *et al.*, (2010) Bioconjug. Chem. 21(2):385-392).

Because engineered cysteines in antibodies expressed in mammalian cells are modified through disulfide bonds with glutathione (GSH) and/or cysteine during their biosynthesis (Chen *et al.* (2009) *mAbs* 1:6, 563-571), the modified cysteine(s) in the antibody drug conjugate

product as initially expressed is unreactive to thiol reactive reagents. Activation of the engineered cysteine(s) requires reduction of the GSH and/or cysteine adduct (which typically results in reduction of all inter-chain disulfide bonds of the antibody), followed by reoxidation and reformation of the native, inter-chain disulfide bonds prior to conjugation (Junutula *et al.*, (2008) Nat. Biotechnol. 26(8):925-32). Some of the sites where cysteine has been inserted interfere with the process of reoxidation and subsequently result in undesirable, non-homogeneous conjugation products. It is therefore important to identify sites on the antibody where the introduced cysteine does not interfere with the reoxidation process prior to the conjugation with a thiol reactive reagent such as maleimide or bromo-, chloro- or iodo-acetamide groups.

Conjugation of cysteine residues with bromo-acetamide, iodo-acetamide or chloro-acetamide results in the formation of a stable thioether linkage. (Alley *et al.*, (2008) Bioconjug Chem. 19(3):759-65). However, the chemistry is less efficient than maleimide conjugation chemistry. Since forming such thiol-maleimide linkages is a popular and highly efficient method to use when attaching a payload or linker to cysteine, there is a need to identify cysteine substitution sites on an antibody where maleimide linkages can be used. More importantly, site-specifically conjugated immunoconjugates can exhibit improved therapeutic index, thus there remains a need to identify specific privileged sites for cysteine substitution in antibodies that enables conjugation of payloads onto antibodies to form efficiently, and that provide conjugates having high stability. The instant invention provides such privileged cysteine substitution sites that give improved antibodies for conjugation purposes and immunoconjugates comprising such improved antibodies.

SUMMARY OF THE INVENTION

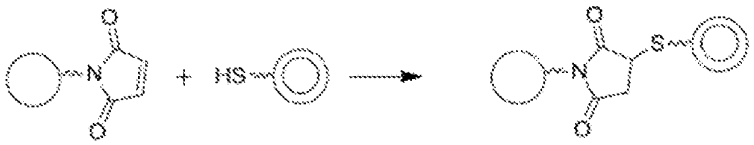
The invention provides specific sites in the constant region of an antibody or antibody fragment at which cysteine ("Cys") replacement of the native amino acid on a parental antibody or antibody fragment can be performed in order to provide a Cys residue for attachment of a chemical moiety (e.g., payload/drug moiety) to form an immunoconjugate with good efficiency and stability. The invention further provides engineered antibodies or antibody fragments having one or more Cys residues in one or more of these specific sites, as well as immunoconjugates made from such engineered antibodies or antibody fragments.

Methods for inserting Cys at specific locations of an antibody are known in the art, see, *e.g.*, WO 2011/005481. However, the current invention discloses specific sites in the constant region of antibodies or antibody fragments where replacing one or more native amino acids of a parental antibody or antibody fragment with Cys provides one or more of the following advantages: Good reactivity to promote efficient immunoconjugation; reduced propensity for

loss of payload when a Cys-maleimide conjugation linker is used; a reduced tendency to form undesired disulfide linkages, such as cross-linking between antibodies or the formation of non-native intramolecular disulfide bonds; and low hydrophobicity of the resulting ADC.

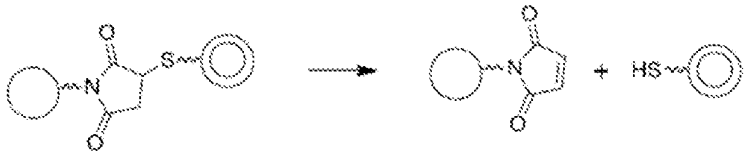
The specific privileged sites for Cys replacement of native amino acids in the constant region of a parental antibody or antibody fragment are selected to provide efficient conjugation while minimizing undesired effects. First, the specific sites for modification are selected so that replacing the native amino acid of a parental antibody or antibody fragment with Cys in one or more of the selected locations provides antibodies or antibody fragments that are readily conjugated on the new cysteine. The specific locations are selected to be sufficiently surface-accessible to allow the sulfhydryl of the cysteine residue to be reactive towards electrophiles in aqueous solutions. The identification of suitable sites for Cys replacement of native amino acids of a parental antibody or antibody fragment involved analyzing surface exposure of the native amino acids based on crystal structure data. Because the sites described herein are sufficiently accessible and reactive, they can be used to form immunoconjugates via chemistry that is well known in the art for modifying naturally-occurring cysteine residues. Conjugation of the replacement Cys residues thus uses conventional methods.

Selected modification sites can show a low propensity for reversal of conjugation when thiol-maleimide moieties are used in the conjugation. The thiol-maleimide conjugation reaction is often highly selective and extremely efficient, and may be used either to attach a payload to the thiol of a cysteine residue of a protein or as a linker elsewhere in the linkage between protein and payload. For example, a maleimide can be attached to a protein (*e.g.*, an antibody or antibody fragment), and a payload having an attached thiol can be added to the maleimide to form a conjugate:



Accordingly, in this conjugation step, the protein (*e.g.*, an antibody or antibody fragment) could be either the single circle or the double circle; the other would represent the payload. The immunoconjugate stability information here specifically relates to conjugation of the substituted cysteine by reaction with a maleimide group. In some embodiments, the thiol is from a cysteine on the protein (*e.g.*, an antibody or antibody fragment), so the double circle represents the protein and the single circle represents a payload.

While the thiol-maleimide reaction is often used for making conjugates, reversal of the conjugation step as depicted below can result in loss of payload or scrambling of payload with other thiol-containing species:



It has been reported that some sites for cysteine substitution provide more stable maleimide conjugates than others, presumably because the local chemical environment at certain points on the antibody surface around a new cysteine can promote the hydrolysis of the succinimide ring and hence prevent reversal of the thiol-maleimide linkage in the immunoconjugate (Shen *et al.* (2012), Nat. Biotechnol. 30(2):184-9). The identification of the advantageous sites for meeting this criterion involved inserting Cys in place of many of the native amino acids having suitable surface exposure, making immunoconjugates containing a thiol-maleimide linkage, and assessing stability of the immunoconjugate in order to eliminate sites where the stability of the conjugate was reduced by the local microenvironment around destabilizing sites. Because of this, the chemistry that can be used to attach linkers and payloads to the replacement Cys residues is not limited by the stability problems associated with the reversibility of thiol-maleimide conjugates that is discussed above. A number of methods can be used to form conjugates at cysteine, including maleimide conjugation. The sites for cysteine substitution described herein promote stability of the antibody conjugate product when using one of the most common conjugation methods, making these sites advantageous for antibody engineering, because the modified antibody can be used with the well-known and highly efficient maleimide conjugation methodology. Selection of sites based on this criterion is illustrated by data presented in Table 22 and Example 9.

,Selected sites can be positioned so as to minimize undesired disulfide formation that may interfere with formation of a homogeneous conjugate. When antibodies or antibody fragments containing engineered cysteines are produced in mammalian cells, the Cys residues are typically present as disulfides to a free Cys amino acid or to glutathione (Chen *et al*., (2009) mAbs 16, 353-571). To free the Cys residues for conjugation with thiol reactive groups, the antibody or antibody fragment needs to be reduced, breaking all of the disulfide bonds. The antibody or antibody fragment is then reoxidized under conditions that facilitate formation of the native disulfides that stabilize the antibody or antibody fragment. Upon reoxidation, cysteine residues that are too prominently exposed on the surface of the antibody or antibody fragment can form disulfides by reaction with Cys on another antibody or antibody fragment

("inter-antibody disulfides"), or by forming undesired intra-antibody disulfides. It has been found that cysteine residues placed in the specific sites described herein are suitably accessible to be available for efficient conjugation, but are sufficiently shielded or suitably positioned to reduce or eliminate formation of inter-antibody and intra-antibody disulfide bonds that would otherwise occur during the reduction / reoxidation procedures typically needed when expressing cys-modified antibodies. Similarly, after re-oxidation some sites were found to produce non-homogenous conjugation products that appear to be due to the location of the new Cys residue engineered into the protein, and the specific sites identified herein are ones where such heterogeneity is minimized.

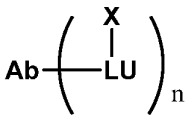
Conjugating drug payloads at sites where they are sequestered from solvent interactions and attachment can increase the hydrophobicity of the antibody upon drug attachment is preferred as reducing hydrophobicity of a protein drug is generally considered beneficial because it might reduce aggregation and clearance from circulation. Selecting attachment sites that result in minimal changes in hydrophobicity might be particularly beneficial when 4, 6 or 8 drugs are attached per antibody, or when particularly hydrophobic payloads are used.

Sites for Cys incorporation were evaluated using these and additional methods described in the Examples herein, leading to the selection of preferred sites for Cys incorporation for engineering antibodies or antibody fragments to introduce Cys as a site for conjugation, especially for making ADCs. Additional details regarding the selection of the specific sites for replacing a natural amino acid of an antibody with Cys are provided herein.

Cysteine substitution sites are located in the constant region of an antibody or antibody fragment, and are identified herein using standard numbering conventions. It is well known, however, that portions or fragments of antibodies can be used for many purposes instead of intact full-length antibodies, and also that antibodies can be modified in various ways that affect numbering of sites in the constant region even though they do not substantially affect the functioning of the constant region. For example, insertion of an S6 tag (a short peptide) into a loop region of an antibody has been shown to allow activity of the antibody to be retained, even though it would change the numbering of many sites in the antibody. Accordingly, while the preferred cysteine substitution sites described herein are identified by a standard numbering system based on intact antibody numbering, the invention includes the corresponding sites in antibody fragments or in antibodies containing other modifications, such as peptide tag insertion. The corresponding sites in those fragments or modified antibodies are thus preferred sites for cysteine substitution in fragments or modified antibodies, and references to the cysteine substitution sites by number include corresponding sites in modified antibodies or antibody fragments that retain the function of the relevant portion of the full-length antibody.

A corresponding site in an antibody fragment or modified antibody can readily be identified by aligning a segment of the antibody fragment or modified antibody with the full-length antibody to identify the site in the antibody fragment or modified antibody that matches one of the preferred cysteine substitution sites of the invention. Alignment may be based on a segment long enough to ensure that the segment matches the correct portion of the full-length antibody, such as a segment of at least 20 amino acid residues, or at least 50 residues, or at least 100 residues, or at least 150 residues. Alignment may also take into account other modifications that may have been engineered into the antibody fragment or modified antibody, thus differences in sequence due to engineered point mutations in the segment used for alignment, particularly for conservative substitutions, would be allowed. Thus, for example, an Fc domain can be excised from an antibody, and would contain amino acid residues that correspond to the cysteine substitution sites described herein, despite numbering differences: sites in the Fc domain corresponding to the cysteine substitution sites of the invention would also be expected to be advantageous sites for cysteine substation in the Fc domain, and are included in the scope of the invention.

In one embodiment, the invention provides an immunoconjugate of Formula (I):



wherein Ab represents an antibody or antibody fragment comprising at least one cysteine residue at one of the preferred cysteine substitution sites described herein;

LU is a linker unit as described herein;

X is a payload or drug moiety;

and n is an integer from 1 to 16.

Typically in compounds of Formula (I), LU is attached to a cysteine at one of the cysteine substitution sites described herein, X is a drug moiety such as an anticancer drug, and n is 2-8 when Ab is an antibody, or n can be 1-8 when Ab is an antibody fragment.

In an embodimentt, the invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region chosen from positions 121, 124, 152, 171, 174, 258, 292, 333, 360, and 375 of a heavy chain of said antibody or antibody fragment, and wherein said positions are numbered according to the EU system.

In ar embodiment, the invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region chosen from positions 107, 108, 142, 145, 159, 161, and 165 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the EU system, and wherein said light chain is human kappa light chain.

In an aspeembodimentt, the invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region chosen from positions 143, 147, 159, 163, and 168 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the Kabat system, and wherein said light chain is human lambda light chain.

In an embodiment, the invention provides a modified antibody or antibody fragment thereof comprising a substitution of one or more amino acids with cysteine at the positions described herein. The sites for cysteine substitution are in the constant regions of the antibody and are thus applicable to a variety of antibodies, and the sites are selected to provide stable and homogeneous conjugates. The modified antibody or fragment can have two or more cysteine substitutions, and these substitutions can be used in combination with other antibody modification and conjugation methods as described herein.

In an embodiment, the invention provides pharmaceutical compositions comprising the immunoconjugate disclosed above, and methods to use the immunoconjugates.

In an embodiment, the invention provides a nucleic acid encoding the modified antibody or antibody fragment described herein having at least one cysteine substitution at a site described herein. The invention further provides host cells comprising these nucleic acids and methods to use the nucleic acid or host cells to express and produce the antibodies or fragments described herein.

In an embodiment, the invention provides a method to select an amino acid of an antibody that is suitable for replacement by cysteine to provide a good site for conjugation, comprising:

(1) identifying amino acids in the constant region of the antibody that have a suitable surface exposure to provide a set of initial candidate sites;

(2) for each initial candidate site, expressing an antibody wherein the native amino acid at that site is replaced by cysteine;

(3) for each expressed antibody, determining whether the expressed protein is substantially homogeneous after reduction and reoxidation to provide a functional antibody having a free cysteine at the initial candidate site,

(4) for each expressed protein that is substantially homogeneous and functional, conjugating the cysteine at the initial candidate site with a maleimide moiety and determining whether the thiol-maleimide linkage is stable at that site;

(5) removing from the set of initial candidate sites those sites for which the expressed antibody is not substantially homogeneous and functional, and those wherein the thiol-maleimide linkage is destabilized, to provide a set of advantaged sites for cysteine substitution.

Optionally, the method further comprises a step of determining the melting temperature for the conjugate of each advantaged cysteine substitution site, and eliminating from the set any sites where cysteine substitution and conjugation causes the melting temperature to differ by 5°C or more from that of the native antibody.

In an embodiment, the invention provides a method to produce an immunoconjugate, which comprises attaching a Linker Unit (LU) or a Linker Unit-Payload combination (-LU-X) to a cysteine residue in an antibody or antibody fragment, wherein the cysteine is located at a cysteine substitution site selected from 121, 124, 152, 171, 174, 258, 292, 333, 360, and 375 of a heavy chain of said antibody or antibody fragment, and positions 107, 108, 142, 145, 159, 161, and 165 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the EU system.

Other aspects and embodiments of the invention are described in greater detail herein.

1. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region at a site selected from positions 121, 124, 152, 171, 174, 258, 292, 333, 334, 360, 375, and 392 of a heavy chain of said antibody or antibody fragment, and wherein said positions are numbered according to the EU system.

2. The immunoconjugate of embodiment 1, wherein the substitution of one or more amino acids with cysteine is selected from positions 121, 124, 152, 258, 334, 360, and 392.

3. The immunoconjugate of embodiments 1 or 2, wherein said antibody or antibody fragment comprises a sequence selected from the group consisting of SEQ ID NOs: 4, 5, 10, 17, 18, 29, 35, 42, 43, 48, 50, 54, 290, 291, 292, 293, 294, and 295.

4. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region at a site selected from positions 107, 108, 142, 145, 159, 161, and 165 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the EU system, and wherein said light chain is a human kappa light chain.

5. The immunoconjugate of embodiment 4, wherein the substitution of one or more amino acids with cysteine is selected from positions 145 or 165.

6. The immunoconjugate of embodiment 4, wherein said antibody or antibody fragment comprises a sequence selected from the group consisting of SEQ ID NOs: 61, 62, 69, 71, 75, 76, and 77.

7. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region at a site selected from positions 143, 147, 159, 163, and 168 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the Kabat system, and wherein said light chain is human a lambda light chain.

8. The immunoconjugate of embodiment 7, wherein said antibody or antibody fragment comprises a sequence selected from the group consisting of SEQ ID NOs: 92, 94, 96, 97, and 98.

9. The immunoconjugate of embodiment 1, 2, or 3, wherein said modified antibody or antibody fragment further comprises a substitution of one or more amino acids with cysteine on its constant region at a site selected from positions 107, 108, 142, 145,

159, 161, and 165 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the EU system, and wherein said light chain is a human kappa light chain.

10. The immunoconjugate of embodiment 1, 2, or 3, wherein said modified antibody or antibody fragment further comprises a substitution of one or more amino acids with cysteine on its constant region selected from positions 143, 147, 159, 163, and 168 of a light chain of said antibody or antibody fragment, wherein said light chain positions are numbered according to the Kabat system, and wherein said light chain is a human kappa light chain.

11. An immunoconjugate comprising a modified antibody or antibody fragment thereof wherein said modified antibody or antibody fragment comprises a combination of substitutions of two or more amino acids with cysteine on a constant region of a heavy chain at positions 152 and 375, or at positions 327 and 375, wherein said positions are numbered according to the EU system.

12. An immunoconjugate comprising a modified antibody or antibody fragment thereof wherein said modified antibody or antibody fragment comprises a combination of substitution of two or more amino acids with cysteine on its constant regions comprising position 107 of a light chain and 360 of a heavy chain, wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

13. An immunoconjugate comprising a modified antibody or antibody fragment thereof wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region selected from positions 117, 119, 121, 124, 139, 152, 153, 155, 157, 164, 169, 171, 174, 189, 205, 207, 246, 258, 269, 274, 286, 288, 290, 292, 293, 320, 322, 326, 333, 334, 335, 337, 344, 355, 360, 375, 382, 390, 392, 398, 400 and 422 of a heavy chain of said antibody or antibody fragment, and wherein said positions are numbered according to the EU system.

14. An immunoconjugate comprising a modified antibody or antibody fragment thereof wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region selected from positions 107,

108, 109, 114, 129, 142, 143, 145, 152, 154, 156, 159, 161, 165, 168, 169, 170, 182, 183, 197, 199, and 203 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the EU system, and wherein said light chain is a human kappa light chain.

15. An immunoconjugate comprising a modified antibody or antibody fragment thereof , wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine selected from positions 143, 145, 147, 156, 159, 163, and 168 on its constant region of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the Kabat system, and wherein said light chain is a human lambda light chain.

16. An immunoconjugate comprising a modified antibody or antibody fragment thereof , wherein said modified antibody or antibody fragment comprises a combination of substitution of two or more amino acids with cysteine on its constant regions wherein the combinations comprise substitutions at positions 375 of an antibody heavy chain and position 165 of an antibody light chain, or at position 334 of an antibody heavy chain at position 165 of an antibody light chain, and wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

17. The immunoconjugate of any of embodiments 11, 12, and 16 wherein the drug antibody ratio is about 4.

18. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment comprises a combination of substitution of three or more amino acids with cysteine on its constant regions wherein the combinations comprise substitutions selected from

    a. positions 375 and 392 of the an antibody heavy chain and position 165 of an antibody light chain,

    b. positions 334 and 375 of an antibody heavy chain and position 165 of an antibody light chain, and

    c. positions 334 and 392 of an antibody heavy chain and position 165 of an antibody light chain,

and wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

19. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment comprises a combination of substitution of three or more amino acids with cysteine on its constant regions wherein the combinations comprise substitutions selected from

    a. positions 152, 375 and 392 of the an antibody heavy chain,

    b. positions 152, 334 and 375 of an antibody heavy chain, and

    c. positions 152, 334 and 392 of an antibody heavy chain,

    and wherein said positions are numbered according to the EU system.

20. The immunoconjugates of embodiments 18 or 19 wherein the drug antibody ratio is about 6.

21. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment thereof comprises a combination of substitution of four or more amino acids with cysteine on its constant regions wherein the combinations comprise substitutions at positions 334, 375, and 392 of an antibody heavy chain and position 165 of an antibody light chain, or at positions 333, 375, and 392 of an antibody heavy chain and at position 165 of an antibody light chain, and wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

22. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment thereof comprises a combination of substitution of four or more amino acids with cysteine on its constant regions wherein the combinations comprise substitutions at positions 152, 334, 375, and 392 of an antibody heavy chain, or at positions 152, 333, 375, and 392 of an antibody heavy chain, and wherein said positions are numbered according to the EU system.

23. The immunoconjugates of embodiment 21 or 22 wherein the drug antibody ratio is about 8.

24. The immunoconjugate of any of embodiments 1-23 further comprising a drug moiety.

25. The immunoconjugate of embodiment 24, wherein a drug moiety is attached to the modified antibody or antibody fragment through the sulfur of said cysteine and an optional linker.

26. The immunoconjugate of embodiment 25, wherein said drug moiety is connected to said sulfur of said cysteine through a cleavable or non-cleavable linker.

27. The immunoconjugate of embodiment 25, wherein said drug moiety is connected to said sulfur of said cysteine through a non-cleavable linker.

28. The immunoconjugate of embodiment 25, wherein said immunoconjugate comprises a thiol-maleimide linkage.

29. The immunoconjugate of embodiment 25, wherein said immunoconjugate comprises a $–S-CH_2-C(=O)-$ linkage or a disulfide linkage.

30. The immunoconjugate of any of embodiments 25-29, wherein said drug moiety is a cytotoxic agent.

31. The immunoconjugate of embodiment 30, wherein said drug moiety is selected from the group consisting of taxanes, DNA-alkylating agents (e.g., CC-1065 analogs), anthracyclines, tubulysin analogs, duocarmycin analogs, auristatin E, auristatin F, and maytansinoids.

32. The immunoconjugate of any of embodiments 1-31, wherein said antibody is a monoclonal antibody.

33. The immunoconjugate of any of embodiments 1-31, wherein said antibody is a chimeric antibody.

34. The immunoconjugate of embodiment 31, wherein said antibody is a humanized or fully human antibody.

35. The immunoconjugate of embodiment 31, wherein said antibody is a bispecific or multi-specific antibody.

36. The immunoconjugate of any of embodiments 1-32, wherein said antibody or antibody fragment specifically binds to a cell surface marker characteristic of a tumor.

37. A pharmaceutical composition comprising the immunoconjugate of any of embodiments 1-36.

38. A modified antibody or antibody fragment thereof comprising a substitution of one or more amino acids with cysteine on its constant region selected from positions 117, 119, 121, 124, 139, 152, 153, 155, 157, 164, 169, 171, 174, 189, 205, 207, 246, 258, 269, 274, 286, 288, 290, 292, 293, 320, 322, 326, 333, 334, 335, 337, 344, 355, 360, 375, 382, 390, 392, 398, 400, and 422 of a heavy chain of said antibody or antibody fragment, and wherein said positions are numbered according to the EU system.

39. A modified antibody or antibody fragment thereof comprising a substitution of one or more amino acids with cysteine on its constant region selected from positions 107, 108, 109, 114, 129, 142, 143, 145, 152, 154, 156, 159, 161, 165, 168, 169, 170, 182, 183, 197, 199, and 203 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the EU system, and wherein said light chain is human kappa light chain.

40. A modified antibody or antibody fragment thereof comprising a substitution of one or more amino acids with cysteine on its constant region selected from positions 143, 145, 147, 156, 159, 163,168 on its constant region of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the Kabat system, and wherein said light chain is human lambda light chain.

41. The modified antibody or antibody fragment of embodiment 38, wherein said substitution is at least one cysteine, selected from positions 121, 124, 152, 171, 174, 258, 292, 333, 360, and 375 of the heavy chain, and wherein said positions are numbered according to the EU system.

42. The modified antibody or antibody fragment of embodiment 39, wherein said substitution is two to six cysteines, wherein said cysteines are at positions selected from 121, 124, 152, 171, 174, 258, 292, 333, 360, and 375 of a heavy chain, and wherein said positions are numbered according to the EU system.

43. The modified antibody or antibody fragment of embodiment 39, wherein said substitution is at least one cysteine, selected from positions 107, 108, 142, 145, 159, 161, and 165 of a light chain, wherein said positions are numbered according to the EU system, and wherein said light chain is a human kappa light chain.

44. The modified antibody or antibody fragment of embodiment 40, wherein said substitution is two to six cysteines, wherein said cysteines are at positions selected from positions 107, 108, 142, 145, 159, 161, and 165 of a light chain, wherein said positions are numbered according to the EU system, and wherein said light chain is a human kappa light chain.

45. The modified antibody or antibody fragment of embodiment 40, wherein said substitution is at least one cysteine, selected from positions 143, 147, 159, 163, and 168 of a light chain, wherein said positions are numbered according to the Kabat system, and wherein said light chain is a human lambda light chain.

46. The modified antibody or antibody fragment of embodiment 40, wherein said substitution is two to six cysteines, wherein said cysteines are at positions selected from positions 143, 147, 159, 163, and 168 of a light chain, wherein said positions are numbered according to the Kabat system, and wherein said light chain is a human lambda light chain.

47. The modified antibody or antibody fragment of any of embodiment 11, 12, 14-22, 38-47 which is further attached to a drug moiety, and wherein said drug moiety is attached to the modified antibody or antibody fragment through the sulfur of said cysteine and an optional linker.

48. The modified antibody or antibody fragment of embodiment 47, wherein said drug moiety is attached to the sulfur of said cysteine through a Linker Unit

49. The modified antibody or antibody fragment of any of embodiment 38-48, further comprising at least one Pcl or unnatural amino acid substitution or a peptide tag for enzyme-mediated conjugation and/or combinations thereof.

50. A nucleic acid encoding the modified antibody or antibody fragment of any of embodiment 38-49.

51. A host cell comprising the nucleic acid of embodiment 50.

52. A method of producing a modified antibody or antibody fragment comprising incubating the host cell of embodiment 49 under suitable conditions for expressing the antibody or antibody fragment, and isolating said antibody or antibody fragment.

53. A method to select an amino acid of an antibody that is suitable for replacement by cysteine to provide a suitable site for conjugation, comprising

(1) identifying amino acids in the constant region of the antibody that have a suitable surface exposure to provide a set of initial candidate sites;

(2) for each initial candidate site, expressing an antibody wherein the native amino acid at that site is replaced by cysteine;

(3) for each expressed antibody, determining whether the expressed protein is substantially homogeneous after reduction and reoxidation to provide a functional antibody having a free cysteine at the initial candidate site,

(4) for each expressed protein that is substantially homogeneous and functional, conjugating the cysteine at the initial candidate site with a maleimide moiety and determining whether the thiol-maleimide linkage is destabilized at that site;

(5) removing from the set of initial candidate sites those sites for which the expressed antibody is not substantially homogeneous and functional, and those wherein the

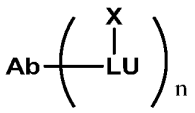
thiol-maleimide linkage is destabilized, to provide a set of advantaged sites for cysteine substitution.

54. The method of embodiment 53, further comprising a step of determining the melting temperature for the conjugate of each advantaged cysteine substitution site, and eliminating from the set any sites where cysteine substitution and conjugation causes the melting temperature to differ by 5°C or more from that of the parental antibody.

55. The method of embodiment 53 or 54, further comprising producing an antibody or antibody fragment containing cysteine at one or more of the substitution sites identified.

56. A method to produce an immunoconjugate, which comprises attaching a Linker Unit (LU) or a Linker Unit-Payload combination (-LU-X) to a cysteine residue in an antibody or antibody fragment, wherein the cysteine is located at a cysteine substitution site selected from 121, 124, 152, 171, 174, 258, 292, 333, 360, and 375 of a heavy chain of said antibody or antibody fragment, and positions 107, 108, 142, 145, 159, 161, and 165 of a light chain of said antibody or antibody fragment, wherein said positions are numbered according to the EU system.

57. The method of embodiment 56, wherein the immunoconjugate is of Formula (I):



wherein Ab represents an antibody or antibody fragment comprising at least one cysteine residue at one of the preferred cysteine substitution sites described herein;
LU is a linker unit as described herein;
X is a payload or drug moiety;
and n is an integer from 1 to 16.

Definitions

The term "amino acid" refers to canonical, synthetic, and unnatural amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the canonical amino acids. Canonical amino acids are proteinogenous amino acids encoded by the genetic code and include alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline serine, threonine, tryptophan, tyrosine, valine, as well as selenocysteine, pyrrolysine and its analog pyrroline-carboxy-lysine. Amino acid analogs refer to compounds that have the same basic chemical structure as a canonical amino acid, *i.e.*, an α-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, citrulline, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a canonical amino acid.

Amino acid mimetics refer to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a canonical amino acid. The term "unnatural amino acid", as used herein, is intended to represent amino acid structures that cannot be generated biosynthetically in any organism using unmodified or modified genes from any organism, whether the same or different. In addition, such "unnatural amino acids" typically require a modified tRNA and a modified tRNA synthetase (RS) for incorporation into a protein. This tRNA/RS pair preferentially incorporates the unnatural amino acid over canonical amino acids. Such orthogonal tRNA/RS pair is generated by a selection process as developed by Schultz *et al.* (see, *e.g.*, Liu *et al.*, (2010) *Annu. Rev. Biochem.* 79:413-444) or a similar procedure. The term "unnatural amino acid" does not include the natural occurring 22nd proteinogenic amino acid pyrrolysine (Pyl) as well as its demethylated analog pyrroline-carboxy-lysine (Pcl), because incorporation of both residues into proteins is mediated by the unmodified, naturally occurring pyrrolysyl-tRNA/tRNA synthetase pair and because Pyl and Pcl are generated biosynthetically (see, *e.g.*, Ou *et al.*, (2011) *Proc. Natl. Acad. Sci. USA*, 108:10437-10442; Cellitti *et al.*, (2011) *Nat. Chem. Biol.* 27;7(8):528-30). See also U.S. provisional application 61/76236, incorporated by reference, that sites specific amino acid residues in antibody light and heavy chains that can be substituted with Pcl.

The term "antibody" as used herein refers to a polypeptide of the immunoglobulin family that is capable of binding a corresponding antigen non-covalently, reversibly, and in a specific manner. For example, a naturally occurring IgG antibody is a tetramer comprising at least two heavy (H) chains (also referred to as "antibody heavy chain") and two light (L) chains (also referred to as "antibody light chain") inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy

chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hyper variability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (C1q) of the classical complement system.

The term "antibody" includes, but is not limited to, monoclonal antibodies, human antibodies, humanized antibodies, camelid antibodies, chimeric antibodies, and anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to antibodies of the invention). The antibodies can be of any isotype/class (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), or subclass (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2).

Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (V_L) and heavy (V_H) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (C_L) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention, the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The N-terminus is a variable region and at the C-terminus is a constant region; the CH3 and C_L domains actually comprise the carboxy-terminal domains of the heavy and light chain, respectively.

The term "antibody fragment" as used herein refers to either an antigen binding fragment of an antibody or a non-antigen binding fragment (*e.g.*, Fc) of an antibody. The term "antigen binding fragment", as used herein, refers to one or more portions of an antibody that retain the ability to specifically interact with (*e.g.*, by binding, steric hindrance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. Examples of binding fragments include, but are not limited to, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), Fab fragments, F(ab') fragments, a monovalent fragment consisting of the V_L, V_H, C_L and CH1 domains; a $F(ab)_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the V_H and CH1 domains; a Fv

fragment consisting of the V_L and V_H domains of a single arm of an antibody; a dAb fragment (Ward *et al.,* Nature 341:544-546, 1989), which consists of a V_H domain; and an isolated complementarity determining region (CDR), or other epitope-binding fragments of an antibody.

Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv ("scFv"); see, *e.g.*, Bird *et al., Science* 242:423-426, 1988; and Huston *et al., Proc. Natl. Acad. Sci.* 85:5879-5883, 1988). Such single chain antibodies are also intended to be encompassed within the term "antigen binding fragment." These antigen binding fragments are obtained using conventional techniques known to those of skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

Antigen binding fragments can also be incorporated into single domain antibodies, maxibodies, minibodies, nanobodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (see, *e.g.*, Hollinger and Hudson, Nature Biotechnology 23:1126-1136, 2005). Antigen binding fragments can be grafted into scaffolds based on polypeptides such as fibronectin type III (Fn3) (see U.S. Pat. No. 6,703,199, which describes fibronectin polypeptide monobodies).

Antigen binding fragments can be incorporated into single chain molecules comprising a pair of tandem Fv segments (V_H-CH1-V_H-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata *et al.*, *Protein Eng.* 8:1057-1062, 1995; and U.S. Pat. No. 5,641,870).

The term "monoclonal antibody" or "monoclonal antibody composition" as used herein refers to polypeptides, including antibodies and antibody fragments that have substantially identical amino acid sequence or are derived from the same genetic source. This term also includes preparations of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

The term "human antibody", as used herein, includes antibodies having variable regions in which both the framework and CDR regions are derived from sequences of human origin. Furthermore, if the antibody contains a constant region, the constant region also is derived from such human sequences, *e.g.*, human germline sequences, or mutated versions of human germline sequences or antibody containing consensus framework sequences derived from human framework sequences analysis, for example, as described in Knappik *et al.*, *J. Mol. Biol.* 296:57-86, 2000).

The human antibodies of the invention may include amino acid residues not encoded by human sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*, or a conservative substitution to promote stability or manufacturing).

The term "humanized" antibody, as used herein, refers to an antibody that retains the reactivity of a non-human antibody while being less immunogenic in humans. This can be achieved, for instance, by retaining the non-human CDR regions and replacing the remaining parts of the antibody with their human counterparts. See, e.g., Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984); Morrison and Oi, Adv. *Immunol.*, 44:65-92 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988); Padlan, *Molec. Immun.*, 28:489-498 (1991); Padlan, *Molec. Immun.*, 31(3):169-217 (1994).

The term "recognize" as used herein refers to an antibody or antigen binding fragment thereof that finds and interacts (*e.g.*, binds) with its epitope, whether that epitope is linear or conformational. The term "epitope" refers to a site on an antigen to which an antibody or antigen binding fragment of the invention specifically binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include techniques in the art, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance (see, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, G. E. Morris, Ed. (1996)).

The term "affinity" as used herein refers to the strength of interaction between antibody and antigen at single antigenic sites. Within each antigenic site, the variable region of the antibody "arm" interacts through weak non-covalent forces with antigen at numerous sites; the more interactions, the stronger the affinity.

The term "isolated antibody" refers to an antibody that is substantially free of other antibodies having different antigenic specificities. An isolated antibody that specifically binds to one antigen may, however, have cross-reactivity to other antigens. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

The term "conservatively modified variant" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially

identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

For polypeptide sequences, "conservatively modified variants" include individual substitutions, deletions or additions to a polypeptide sequence which result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention. The following eight groups contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, *e.g.*, Creighton, Proteins (1984)). In some embodiments, the term "conservative sequence modifications" are used to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence.

The term "optimized" as used herein refers to a nucleotide sequence has been altered to encode an amino acid sequence using codons that are preferred in the production cell or organism, generally a eukaryotic cell, for example, a yeast cell, a *Pichia* cell, a fungal cell, a *Trichoderma* cell, a Chinese Hamster Ovary cell (CHO) or a human cell. The optimized nucleotide sequence is engineered to retain completely or as much as possible the amino acid sequence originally encoded by the starting nucleotide sequence, which is also known as the "parental" sequence.

The terms "percent identical" or "percent identity," in the context of two or more nucleic acids or polypeptide sequences, refers to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, optionally

65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 30 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2:482c (1970), by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, *e.g.*, Brent *et al.*, Current Protocols in Molecular Biology, 2003).

Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402, 1977; and Altschul *et al.*, J. Mol. Biol. 215:403-410, 1990, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds

for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: The cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, (1989) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787, 1993). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

The percent identity between two amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller, Comput. Appl. Biosci. 4:11-17, 1988) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch, J. Mol. Biol. 48:444-453, 1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

Other than percentage of sequence identity noted above, another indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a

polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

The term "nucleic acid" is used herein interchangeably with the term "polynucleotide" and refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses silent variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, as detailed below, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, (1991) Nucleic Acid Res. 19:5081; Ohtsuka *et al.*, (1985) J. Biol. Chem. 260:2605-2608; and Rossolini *et al.*, (1994) Mol. Cell. Probes 8:91-98).

The term "operably linked" in the context of nucleic acids refers to a functional relationship between two or more polynucleotide (*e.g.*, DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, a promoter or enhancer sequence is operably linked to a coding sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, *i.e.*, they are cis-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

The terms "polypeptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to canonical amino acid polymers as well as

to non-canonical amino acid polymers. Unless otherwise indicated, a particular polypeptide sequence also implicitly encompasses conservatively modified variants thereof.

The term "immunoconjugate" or "antibody conjugate" as used herein refers to the linkage of an antibody or an antibody fragment thereof with another agent, such as a chemotherapeutic agent, a toxin, an immunotherapeutic agent, an imaging probe, a spectroscopic probe, and the like. The linkage can be through one or multiple covalent bonds, or non-covalent interactions, and can include chelation. Various linkers, many of which are known in the art, can be employed in order to form the immunoconjugate. Additionally, the immunoconjugate can be provided in the form of a fusion protein that may be expressed from a polynucleotide encoding the immunoconjugate. As used herein, "fusion protein" refers to proteins created through the joining of two or more genes or gene fragments which originally coded for separate proteins (including peptides and polypeptides). Fusion proteins may be created by joining at the N- or C-terminus, or by insertions of genes or gene fragments into permissible regions of one of the partner proteins. Translation of the fusion gene results in a single protein with functional properties derived from each of the original proteins.

The term "subject" includes human and non-human animals. Non-human animals include all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, and reptiles. Except when noted, the terms "patient" or "subject" are used herein interchangeably.

The term "cytotoxin", or "cytotoxic agent" as used herein, refer to any agent that is detrimental to the growth and proliferation of cells and may act to reduce, inhibit, or destroy a cell or malignancy.

The term "anti-cancer agent" as used herein refers to any agent that can be used to treat a cell proliferative disorder such as cancer, including but not limited to, cytotoxic agents, chemotherapeutic agents, radiotherapy and radiotherapeutic agents, targeted anti-cancer agents, and immunotherapeutic agents.

The term "drug moiety" or "payload" are used interchangeably and refers to a chemical moiety that is conjugated to the antibody or antibody fragment of the invention, and can include any moiety that is useful to attach to an antibody or antibody fragment. For example, a drug moiety or payload can be an anti-cancer agent, an anti-inflammatory agent, an antifungal agent, an antibacterial agent, an anti-parasitic agent, an anti-viral agent, an anesthetic agent. In certain embodiments a drug moiety is selected from a V-ATPase inhibitor, a HSP90 inhibitor, an IAP inhibitor, an mTor inhibitor, a microtubule stabilizer, a microtubule destabilizers, an auristatin, a dolastatin, a maytansinoid, a MetAP (methionine aminopeptidase), an inhibitor of nuclear export of proteins CRM1, a DPPIV inhibitor, an inhibitor of phosphoryl transfer reactions in

mitochondria, a protein synthesis inhibitor, a kinase inhibitor, a CDK2 inhibitor, a CDK9 inhibitor, a proteasome inhibitor, a kinesin inhibitor, an HDAC inhibitor, a DNA damaging agent, a DNA alkylating agent, a DNA intercalator, a DNA minor groove binder and a DHFR inhibitor. Suitable examples include auristatins such as MMAE and MMAF; calicheamycins such as gamma-calicheamycin; and maytansinoids such as DM1 and DM4. Methods for attaching each of these to a linker compatible with the antibodies and method of the invention are known in the art. See, *e.g.*, Singh *et al.*, (2009) Therapeutic Antibodies: Methods and Protocols, vol. 525, 445-457. In addition, a payload can be a biophysical probe, a fluorophore, a spin label, an infrared probe an affinity probe, a chelator, a spectroscopic probe, a radioactive probe, a lipid molecule, a polyethylene glycol, a polymer, a spin label, DNA, RNA, a protein, a peptide, a surface, an antibody, an antibody fragment, a nanoparticle, a quantum dot, a liposome, a PLGA particle, a saccharide or a polysaccharide, a reactive functional group, or a binding agent that can connect the conjugate to another moiety, surface, etc.

The term "drug antibody ratio" (also referred to as "DAR"), refers to the number or payload or drug moieties linked to an antibody of the immunoconjugate. For example a drug antibody of ratio of 2 means that average of two drug moieties bound to an each antibody in a sample of immunoconjugates. Some individual immunoconjugates will in a sample with a drug antibody ratio of two might have none or only one drug moiety linked; others immunoconjugates in that sample will have two, three, four, or even more moieties on individual antibody. But the average in the sample will be two. There are different methods known in the art for measuring drug antibody ratios of immunoconjugates.

In an embodiment of this invention, the DAR in a sample of immunoconjugates can be "homogenous". A "homogenous conjugation sample" is a sample with a narrow distribution of DAR. As an illustrative embodiment, in a homogenous conjugation sample having a DAR of 2, can contain within that sample antibodies that are not conjugated, and some antibodies having more than two moieties conjugated at about a DAR of two. "Most of the sample" means have at least over 70%, or at least over 80% or at least over 90% of the antibodies in the sample will be conjugated to two moieties.

As an illustrative embodiment, in a homogenous conjugation sample having a DAR of 4, can contain within that sample antibodies that have more or fewer than four moieties conjugated at about a DAR of four. "Most of the sample" means have at least over 70%, or at least over 80% or at least over 90% of the antibodies in the sample will be conjugated to four moieties.

As an illustrative embodiment, in a homogenous conjugation sample having a DAR of 6, can contain within that sample antibodies that are have more or fewer than six moieties

conjugated at about a DAR of six. "Most of the sample" means have at least over 70%, or at least over 80% or at least over 90% of the antibodies in the sample will be conjugated to six moieties.

As an illustrative embodiment, in a homogenous conjugation sample having a DAR of 8, can contain within that sample antibodies that has some antibodies having fewer or more than eight moieties conjugated at about a DAR of four. "Most of the sample" means have at least over 70%, or at least over 80% or at least over 90% of the antibodies in the sample will be conjugated to eight moieties.

An immunoconjugate having a "drug antibody ratio of about 2" refers to sample of immunoconjugates where in the drug antibody ratio can range from about 1.6-2.4 moieties/antibody, 1.8-2.3 moieties/antibody, or 1.9-2.1 moieties/antibody.

An immunoconjugate having a "drug antibody ratio of about 4" refers to sample of immunoconjugates where in the drug antibody ratio can range from about 3.6-4.4 moieties/antibody, 3.8-4.3 moieties/antibody, or 3.9-4.1 moieties/antibody.

An immunoconjugate having a "drug antibody ratio of about 6" refers to sample of immunoconjugates where in the drug antibody ratio can range from about 5.6-6.4 moieties/antibody, 5.8-6.3 moieties/antibody, or 5.9-6.1 moieties/antibody.

An immunoconjugate having a "drug antibody ratio of about 8" refers to sample of immunoconjugates where in the drug antibody ratio can range from about 7.6-84 moieties/antibody, 7.8-8.3 moieties/antibody, or 7.9-8.1 moieties/antibody.

"Tumor" refers to neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

The term "anti-tumor activity" means a reduction in the rate of tumor cell proliferation, viability, or metastatic activity. A possible way of showing anti-tumor activity is to show a decline in growth rate of abnormal cells that arises during therapy or tumor size stability or reduction. Such activity can be assessed using accepted *in vitro* or *in vivo* tumor models, including but not limited to xenograft models, allograft models, MMTV models, and other known models known in the art to investigate anti-tumor activity.

The term "malignancy" refers to a non-benign tumor or a cancer. As used herein, the term "cancer" includes a malignancy characterized by deregulated or uncontrolled cell growth. Exemplary cancers include: carcinomas, sarcomas, leukemias, and lymphomas.

The term "cancer" includes primary malignant tumors (*e.g.*, those whose cells have not migrated to sites in the subject's body other than the site of the original tumor) and secondary malignant tumors (*e.g.*, those arising from metastasis, the migration of tumor cells to secondary sites that are different from the site of the original tumor).

As used herein, the term "an optical isomer" or "a stereoisomer" refers to any of the various stereo isomeric configurations which may exist for a given compound of the present invention and includes geometric isomers. It is understood that a substituent may be attached at a chiral center of a carbon atom. The term "chiral" refers to molecules which have the property of non-superimposability on their mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner. Therefore, the invention includes enantiomers, diastereomers or racemates of the compound. "Enantiomers" are a pair of stereoisomers that are non- superimposable mirror images of each other. A 1:1 mixture of a pair of enantiomers is a "racemic" mixture. The term is used to designate a racemic mixture where appropriate. "Diastereoisomers" are stereoisomers that have at least two asymmetric atoms, but which are not mirror-images of each other. The absolute stereochemistry is specified according to the Cahn-lngold-Prelog R-S system. When a compound is a pure enantiomer the stereochemistry at each chiral carbon may be specified by either *R* or *S*. Resolved compounds whose absolute configuration is unknown can be designated (+) or (-) depending on the direction (dextro- or levorotatory) which they rotate plane polarized light at the wavelength of the sodium D line. Certain compounds described herein contain one or more asymmetric centers or axes and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (*R*)- or (*S*)-.

Depending on the choice of the starting materials and procedures, the compounds can be present in the form of one of the possible isomers or as mixtures thereof, for example as pure optical isomers, or as isomer mixtures, such as racemates and diastereoisomer mixtures, depending on the number of asymmetric carbon atoms. The present invention is meant to include all such possible isomers, including racemic mixtures, diasteriomeric mixtures and optically pure forms. Optically active (*R*)- and (*S*)-isomers may be prepared using chiral synthons or chiral reagents, or may be resolved using conventional techniques. If the compound contains a double bond, the substituent may be E or Z configuration. If the compound contains a disubstituted cycloalkyl, the cycloalkyl substituent may have a cis- or trans-configuration. All tautomeric forms are also intended to be included.

As used herein, the terms "salt" or "salts" refers to an acid addition or base addition salt of a compound of the invention. "Salts" include in particular "pharmaceutical acceptable salts". The term "pharmaceutically acceptable salts" refers to salts that retain the biological

effectiveness and properties of the compounds of this invention and, which typically are not biologically or otherwise undesirable. In many cases, the compounds of the present invention are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

Pharmaceutically acceptable acid addition salts can be formed with inorganic acids and organic acids, e.g., acetate, aspartate, benzoate, besylate, bromide/hydrobromide, bicarbonate/carbonate, bisulfate/sulfate, camphorsulfonate, chloride/hydrochloride, chlorotheophyllinate, citrate, ethandisulfonate, fumarate, gluceptate, gluconate, glucuronate, hippurate, hydroiodide/iodide, isethionate, lactate, lactobionate, laurylsulfate, malate, maleate, malonate, mandelate, mesylate, methylsulfate, naphthoate, napsylate, nicotinate, nitrate, octadecanoate, oleate, oxalate, palmitate, pamoate, phosphate/hydrogen phosphate/dihydrogen phosphate, polygalacturonate, propionate, stearate, succinate, sulfosalicylate, tartrate, tosylate and trifluoroacetate salts.

Inorganic acids from which salts can be derived include, for example, hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like.

Organic acids from which salts can be derived include, for example, acetic acid, propionic acid, glycolic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, toluenesulfonic acid, sulfosalicylic acid, and the like. Pharmaceutically acceptable base addition salts can be formed with inorganic and organic bases.

Inorganic bases from which salts can be derived include, for example, ammonium salts and metals from columns I to XII of the periodic table. In certain embodiments, the salts are derived from sodium, potassium, ammonium, calcium, magnesium, iron, silver, zinc, and copper; particularly suitable salts include ammonium, potassium, sodium, calcium and magnesium salts.

Organic bases from which salts can be derived include, for example, primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines, basic ion exchange resins, and the like. Certain organic amines include isopropylamine, benzathine, cholinate, diethanolamine, diethylamine, lysine, meglumine, piperazine and tromethamine.

The pharmaceutically acceptable salts of the present invention can be synthesized from a basic or acidic moiety, by conventional chemical methods. Generally, such salts can be prepared by reacting free acid forms of these compounds with a stoichiometric amount of the appropriate base (such as Na, Ca, Mg, or K hydroxide, carbonate, bicarbonate or the like), or by reacting free base forms of these compounds with a stoichiometric amount of the appropriate

acid. Such reactions are typically carried out in water or in an organic solvent, or in a mixture of the two. Generally, use of non-aqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile is desirable, where practicable. Lists of additional suitable salts can be found, e.g., in "Remington's Pharmaceutical Sciences", 20th ed., Mack Publishing Company, Easton, Pa., (1985); and in "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, Weinheim, Germany, 2002).

Any formula given herein is also intended to represent unlabeled forms as well as isotopically labeled forms of the compounds. Isotopically labeled compounds have structures depicted by the formulas given herein except that one or more atoms are replaced by an atom having a selected atomic mass or mass number. Examples of isotopes that can be incorporated into compounds of the invention include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, and chlorine, such as ^{2}H, ^{3}H, ^{11}C, ^{13}C, ^{14}C, ^{15}N, ^{18}F ^{31}P, ^{32}P, ^{35}S, ^{36}Cl, ^{125}I respectively. The invention includes various isotopically labeled compounds as defined herein, for example those into which radioactive isotopes, such as ^{3}H and ^{14}C, or those into which non-radioactive isotopes, such as ^{2}H and ^{13}C are present. Such isotopically labeled compounds are useful in metabolic studies (with ^{14}C), reaction kinetic studies (with, for example ^{2}H or ^{3}H), detection or imaging techniques, such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT) including drug or substrate tissue distribution assays, or in radioactive treatment of patients. In particular, an ^{18}F or labeled compound may be particularly desirable for PET or SPECT studies. Isotopically-labeled compounds of formula (I) can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described in the accompanying Examples and Preparations using an appropriate isotopically-labeled reagents in place of the non-labeled reagent previously employed.

Further, substitution with heavier isotopes, particularly deuterium (i.e., ^{2}H or D) may afford certain therapeutic advantages resulting from greater metabolic stability, for example increased *in vivo* half-life or reduced dosage requirements or an improvement in therapeutic index. It is understood that deuterium in this context is regarded as a substituent of a compound of the formula (I). The concentration of such a heavier isotope, specifically deuterium, may be defined by the isotopic enrichment factor. The term "isotopic enrichment factor" as used herein means the ratio between the isotopic abundance and the natural abundance of a specified isotope. If a substituent in a compound of this invention is denoted deuterium, such compound has an isotopic enrichment factor for each designated deuterium atom of at least 3500 (52.5% deuterium incorporation at each designated deuterium atom), at least 4000 (60% deuterium incorporation), at least 4500 (67.5% deuterium incorporation), at least 5000 (75% deuterium incorporation), at least 5500 (82.5% deuterium incorporation), at least 6000 (90% deuterium

incorporation), at least 6333.3 (95% deuterium incorporation), at least 6466.7 (97% deuterium incorporation), at least 6600 (99% deuterium incorporation), or at least 6633.3 (99.5% deuterium incorporation).

As used herein, the term "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drug stabilizers, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, and the like and combinations thereof, as would be known to those skilled in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289- 1329). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

The term "a therapeutically effective amount" of a compound of the present invention refers to an amount of the compound of the present invention that will elicit the biological or medical response of a subject, for example, reduction or inhibition of an enzyme or a protein activity, or ameliorate symptoms, alleviate conditions, slow or delay disease progression, or prevent a disease, etc. In one non-limiting embodiment, the term “a therapeutically effective amount” refers to the amount of a compound of the present invention that, when administered to a subject, is effective to at least partially alleviate, inhibit, prevent and/or ameliorate a condition, or a disorder or a disease, or at least partially inhibit activity of a targeted enzyme or receptor.

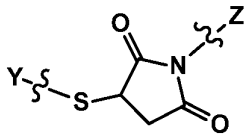
As used herein, the term “inhibit”, "inhibition" or “inhibiting” refers to the reduction or suppression of a given condition, symptom, or disorder, or disease, or a significant decrease in the baseline activity of a biological activity or process.

As used herein, the term “treat”, “treating" or "treatment" of any disease or disorder refers in one embodiment, to ameliorating the disease or disorder (i.e., slowing or arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment “treat”, "treating" or "treatment" refers to alleviating or ameliorating at least one physical parameter including those which may not be discernible by the patient. In yet another embodiment, “treat”, "treating" or "treatment" refers to modulating the disease or disorder, either physically, (*e.g.*, stabilization of a discernible symptom), physiologically, (*e.g.*, stabilization of a physical parameter), or both. In yet another embodiment, “treat”, "treating" or "treatment" refers to preventing or delaying the onset or development or progression of the disease or disorder.

As used herein, a subject is “in need of” a treatment if such subject would benefit biologically, medically or in quality of life from such treatment.

As used herein, the term "a," "an," "the" and similar terms used in the context of the present invention (especially in the context of the claims) are to be construed to cover both the singular and plural unless otherwise indicated herein or clearly contradicted by the context.

The term "thiol-maleimide" as used herein describes a group formed by reaction of a thiol with maleimide, having this general formula



,

where Y and Z are groups to be connected via the thiol-maleimide linkage and can be linker units, and can be attached to antibodies or payloads. In some instances, Y is an engineered antibody according to the invention, and the sulfur atom shown in the formula is from a cysteine at one of the substitution sites described herein; while Z represents a linker unit connected to a payload.

"Linker Unit" (LU) as used herein refers to a covalent chemical connection between two moieties, such as an antibody and a payload. Each LU can be comprised of one or more components described herein as L_1, L_2, L_3, L_4, L_5 and L_6. The linker unit can be selected to provide suitable spacing between the connected moieties, or to provide certain physicochemical properties, or to allow cleavage of the linker unit under certain conditions.

"Cleavable" as used herein refers to a linker or linker unit (LU) that connects two moieties by covalent connections, but breaks down to sever the covalent connection between the moieties under physiological conditions. Cleavage may be enzymatic or non-enzymatic, but generally releases a payload from an antibody without degrading the antibody.

"Non-cleavable" as used herein refers to a linker or linker unit (LU) that is not susceptible to breaking down under physiological conditions. While the linker may be modified physiologically, it keeps the payload connected to the antibody until the antibody is substantially degraded, *i.e.*, the antibody degradation precedes cleavage of the linker *in vivo*.

"Cyclooctyne" as used herein refers to an 8-membered ring containing a carbon-carbon triple bond (acetylene). The ring is optionally fused to one or two phenyl rings, which may be substituted with 1-4 C_{1-4} alkyl, C_{1-4} alkoxy, halo, hydroxyl, COOH, $COOL_1$, $-C(O)NH-L_1$, $O-L_1$, or similar groups, and which may contain N, O or S as a ring member. In preferred embodiments, cyclooctyne can be a C_8 hydrocarbon ring, particularly an isolated ring that is

saturated aside from the triple bond, and may be substituted with F or Hydroxy, and may be linked to a linker or LU via –O-, –C(O), C(O)NH, or C(O)O.

"Cyclooctene" as used herein refers to an 8-membered ring containing at least one double bond, especially a trans-double bond. The ring is optionally fused to one or two phenyl rings, which may be substituted with 1-4 C_{1-4} alkyl, C_{1-4} alkoxy, halo, hydroxyl, COOH, $COOL_1$, -C(O)NH-L_1, O-L_1, or similar groups, and which may contain N, O or S as a ring member. In preferred embodiments, cyclooctene can be an isolated C_8 hydrocarbon ring that is saturated aside from the trans double bond and is optionally substituted with F or Hydroxy, and may be linked to a linker or LU via –O-, –C(O), C(O)NH, or C(O)O.

All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.* "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Surface accessibility plot of amino acid residues in human IgG1 heavy chain (A) and kappa light chain (B). Surface accessibility was calculated using Surface Racer 5.0 and is expressed as Angstrom square [$Å^2$].

FIG. 2. Location of selected 92 TAG mutations in the structure of a human IgG1 with a kappa light chain. Selected residues for TAG mutations are shown in black on only one of the two heavy chains and for one of the two kappa light chains (1HZH.pdb). Structures are shown using PyMOL, an open-source molecular modeling package (The PyMOL Molecular Graphics System, Version 1.5.0. Schrödinger. LLC).

FIG. 3. The amino acid sequence alignment of the heavy chain constant regions of trastuzumab and antibody 14090. Residues mutated to Cys in the trastuzumab antibody and in antibody 14090 are underlined. Amino acid residues in heavy chain are numbered by Eu numbering system (Edelman et al., 1969).

FIG. 4. Amino acid sequence alignment of constant regions of trastuzumab, human IgG1, IgG2, IgG3 and IgG4.

FIG. 5. The amino acid sequence alignment of the constant regions of human kappa and lambda light chains. A. Residues mutated to Cys in the kappa light chain of trastuzumab and in the lambda light chain of antibody 14090 are underlined. B. Residues selected for Cys

mutations are shown in a PyMOL structure model of a human lambda light chain (Protein Structure Databank entry 3G6D.pdb)

FIG. 6. Analysis of trastuzumab Cys antibodies by non-reducing SDS-PAGE.

FIG. 7. Size exclusion chromatography of the trastuzumab LC-S156C mutant antibody (dashed line) and wild-type trastuzumab (solid line).

FIG. 8. Analysis of wild-type trastuzumab (A) and the trastuzumab LC-E158C mutant antibody (B) by reverse phase high pressure liquid chromatography (RP-HPLC).

FIG. 9. MS analysis of trastuzumab LC-R108C mutant antibody after Protein A purification (intact MS).

FIG. 10. Structure of MC-MMAF.

FIG. 11. Analysis of conjugation mixtures of trastuzumab Cys antibodies with MC-MMAF by RP-HPLC. RP-HPLC traces of the conjugation mixtures are shown as dashed lines. RP-HPLC traces of unmodified antibodies are shown as solid lines. A. LC-R108C-MMAF, B. HC-360C-MMAF, C. LC-S156C-MMAF, and D. HC-S275C-MMAF ADC.

FIG. 12. Analysis of conjugation mixtures of trastuzumab Cys antibodies with MC-MMAF by RP-HPLC. RP-HPLC traces of the conjugation mixtures are shown as dashed lines. RP-HPLC traces of unmodified antibodies are shown as solid lines. A. HC-S134C-MMAF, and B. HC-S136C-MMAF ADC.

FIG. 13. Analysis of trastuzumab Cys-MMAF ADCs by analytical size-exclusion chromatography (AnSEC). Trastuzumab HC-K290C-MMAF ADC (short dashed line), trastuzumab LC-R142C-MMAF ADC (dashed line), and trastuzumab LC-L154C-MMAF ADC (dotted line) are compared to unmodified wild-type trastuzumab (solid line).

FIG. 14. Thermal melting curve of unmodified wild-type trastuzumab and trastuzumab HC-T335C-MMAF, trastuzumab HC-S337C-MMAF and trastuzumab HC-K360C-MMAF ADCs.

FIG. 15. Cell proliferation assays for trastuzumab LC-S159C-MMAF with A. HCC1954, B. MDA-MB231 clone 16 and C. MDA-MB231 clone 40 cells.

FIG. 16. IC_{50} of trastuzumab Cys-MMAF ADCs in MDA-MB231 clone 16 cell proliferation assay.

FIG. 17. Cell proliferation assays for Antibody 14090 HC-S375C-MMAF ADC with A. CMK11-5 and B. Jurkat cells.

FIG. 18. Pharmacokinetics study of trastuzumab LC-Cys-MMAF ADCs displaying no significant drug lost. A. Wild-type unconjugated trastuzumab, B. LC-K107C-MMAF, C. LC-R108C-MMAF, D. LC-L154C-MMAF, and E. LC-S159C-MMAF ADC.

FIG. 19. Pharmacokinetics study of trastuzumab HC-Cys-MMAF ADCs displaying no significant drug lost. A. HC-K121C-MMAD, B. HC-L174C-MMAF, C. HC-E258C-MMAF, and D. HC-R292C-MMAF ADC.

FIG. 20. Pharmacokinetics study of trastuzumab Cys-MMAF ADCs displaying significant drug lost. A. LC-T129C-MMAF, B. LC-E143C-MMAF, C. HC-K246C-MMAF, and D. HC-R344C-MMAF ADC.

FIG. 21. Pharmacokinetics study of two trastuzumab Cys-MMAF ADCs displaying fast clearance *in vivo*. A. HC-T335C-MMAF and B. HC-S337C-MMAF ADC.

FIG. 22. *In vivo* efficacy studies of trastuzumab Cys-MMAF ADCs in MDA-MB231 clone 16 xenograft mouse model.

FIG. 23: Retention times of trastuzumab Pcl MMAF DAR 2 ADCs as measured by Hydrophobic Interaction Chromotography. ABA-MMAF is attached at a Pcl residue substituted for the indicated HC or LC residue. A) HC conjugated ADCs. B) LC conjugated ADCs. The retention time of unconjugated wild-type antibody is indicated (WT).

FIG. 24. Location of selected payload sites in the structure of a human IgG1 with a kappa light chain. Selected residues are shown in black on only one of the two heavy chains and for one of the two kappa light chains (1HZH.pdb). Three rotations of the structure are shown using PyMOL, an open-source molecular modeling package (The PyMOL Molecular Graphics System, Version 1.5.0. Schrödinger, LLC).

FIG. 25. Pharmacokinetics study of trastuzumab and antibody 14090 Cys-MMAF ADCs with DAR 4, 6 and 8 prepared with antibodies with 2, 3 or 4 Cys mutations. DAR 4 trastuzumab ADCs: HC-E258C-LC-S159C-MMAF (A), HC-S375C-LC-S159C-MMAF (B), HC-E258C-LC-E165C-MMAF (C), HC-S375C-LC-E165C-MMAF (D), HC-E152C-LC-R142C-MMAF (E), HC-P171C-LC-R142C-MMAF, and HC-E152C-LC-S159C-MMAF (G); DAR 4 antibody 14090 ADCs: HC-S375C-LC-A143C-MMAF (H), HC-K360C-LC-V159C-MMAF (I), and HC-S375C-LC-V159C-MMAF (J); K. DAR 6 trastuzumab ADCs HC-K334C-S375C-LC-E165C-MMAF and HC-K334C-K392C-LC-E165C-MMAF; L. DAR 8 trastuzumab ADCs HC-K334C-K360C-S375C-LC-E165C-MMAF, HC-K334C-K360C-K392C-LC-E165C-MMAF and HC-K334C-S375C-K392C-LC-E165C-MMAF. Antibody

14090 is mouse cross-reactive and therefore is cleared more rapidly that then the trastuzumab ADCs which do not bind to any mouse antigens.

DETAILED DESCRIPTION

The present invention provides methods of site-specific labeling of antibodies or antibody fragments by replacing one or more amino acids of a parental antibody or antibody fragment at specific positions with cysteine amino acids ("Cys"), such that the engineered antibodies or antibody fragments are capable of conjugation to various agents (*e.g.*, cytotoxic agents). The present invention also provides immunoconjugates that are produced by using the methods described herein.

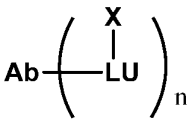
When a cysteine is engineered into a parental antibody or antibody fragment, the modified antibody or antibody fragment is first recovered from the expression medium with cysteine or glutathione (GSH) attached at the engineered cysteine site(s) via a disulfide linkage (Chen *et al.*, (2009) mAbs 16, 353-571). The attached cysteine or GSH is then removed in a reduction step, which also reduces all native inter-chain disulfide bonds of the parental antibody or antibody fragment. In a second step these disulfide bonds are re-oxidized before conjugation occurs. The present disclosure shows that when cysteine is engineered at certain sites, the re-oxidation step does not proceed well, presumably due to formation of the incorrect disulfide bonds. Accordingly, the present invention provides unique sets of sites on the antibody heavy chain constant region and antibody light chain constant region, respectively, where Cys substitution as described herein produces modified antibodies or antibody fragments that perform well in the re-oxidation process, and also produce stable and well behaved immunoconjugates.

The site-specific antibody labeling according to the present invention can be achieved with a variety of chemically accessible labeling reagents, such as anti-cancer agents, fluorophores, peptides, sugars, detergents, polyethylene glycols, immune potentiators, radio-imaging probes, prodrugs, and other molecules.

Accordingly, the present invention provides methods of preparation of homogeneous immunoconjugates with a defined drug-to-antibody ratio for use in cancer therapy and other indications as well as imaging reagents. The present invention also provides immunoconjugates

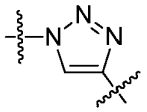
prepared thereby, as well as pharmaceutical compositions comprising these immunoconjugates. The methods of the instant invention can be used in combination with other conjugation methods known in the art.

The following enumerated embodiments represent certain aspects and variations of the invention:

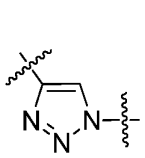
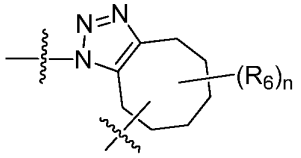
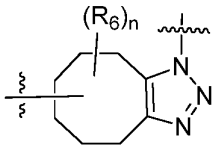


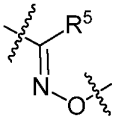
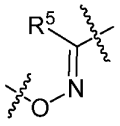
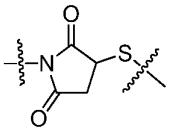
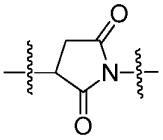
wherein Ab represents an antibody or antibody fragment comprising at least one cysteine residue at one of the preferred cysteine substitution sites described herein;
LU is a linker unit as described herein;
X is a payload or drug moiety;
and n is an integer from 1 to 16. In these embodiments, n is preferably about 2, about 4, about 6, or about 8. LU is typically a group of formula $-L_1-L_2-L_3-L_4-L_5-L_6-$, wherein L_1, L_2, L_3, L_4, L_5 and L_6 are independently selected from $-A_1-$, $-A_1X^2-$ and $-X^2-$;
wherein:

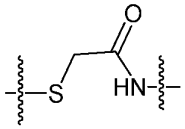
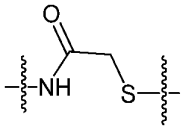
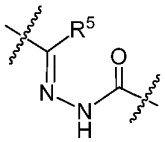
A_1 is $-C(=O)NH-$, $-C(=O)NH(CH_2)_n-$, $-C(=O)NH(C(R^4)_2)_n-$, $-(O(CH_2)_n)_m-$
, $-(O(C(R^4)_2)_n)_m-$,$-((CH_2)_nO)_m-$, $-((C(R^4)_2)_nO)_m-$, $-((CH_2)_nO)_m(CH_2)_n-$
, $-((C(R^4)_2)_nO)_mC(R^4)_2)_n-$, $-(CH_2)_nC(=O)NH-$, $-(C(R^4)_2)_nC(=O)NH-$
, $-(CH_2)_nNHC(=O)-$, $-(C(R^4)_2)_nNHC(=O)-$, $-NHC(=O)(CH_2)_n-$, $-NHC(=O)(C(R^4)_2)_n-$
, $-C(=O)NH(CH_2)_nS-$, $-C(=O)NH(C(R^4)_2)_nS-$, $-S(CH_2)_nC(=O)NH-$
, $-S(C(R^4)_2)_nC(=O)NH-$, $-C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n-$
, $-C(=O)NH(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n-$, $-C(=O)(CH_2)_n-$, $-C(=O)(C(R^4)_2)_n-$
, $-(CH_2)_nC(=O)-$, $-(C(R^4)_2)_nC(=O)-$, $-(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$
, $-(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n-$, $-(CH_2)_nNHC(=O)(CH_2)_n-$
, $-(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n-$, $-(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n-$
, $-(C(R^4)_2)_nNH((C(R^4)_2)_nO)_m(C(R^4)_2)_n-$, $-(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$,
or $-(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n-$;

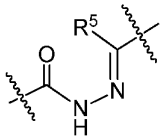
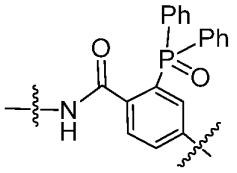
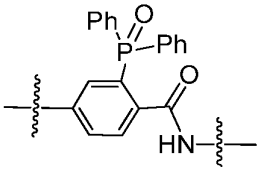


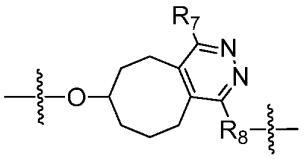
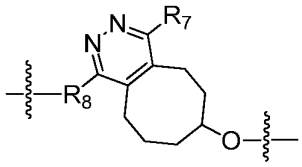
each X^2 is independently selected from a bond, R^8, ,

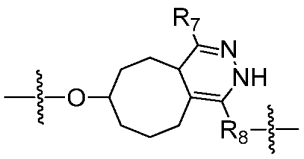
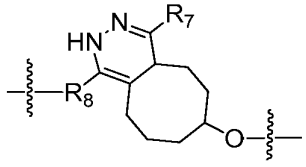

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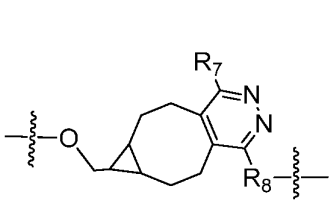
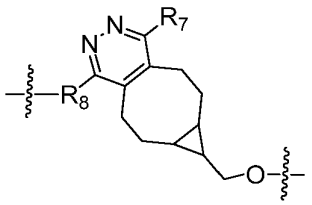

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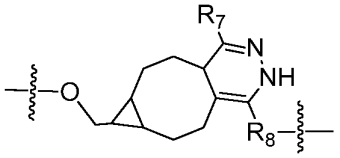
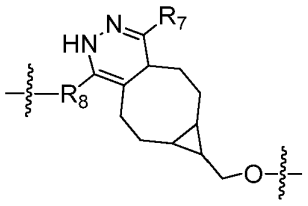

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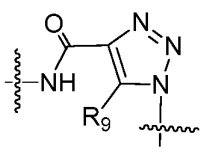
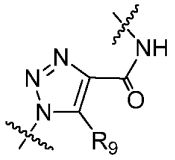
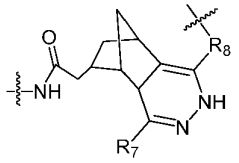

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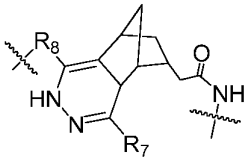
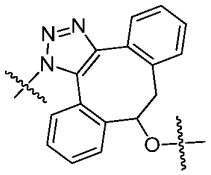
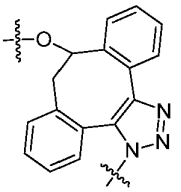

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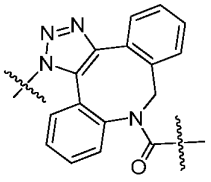
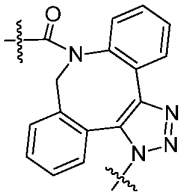

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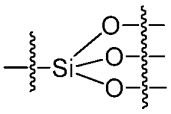


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-S-, -Si(OH)$_2$O-,



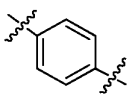
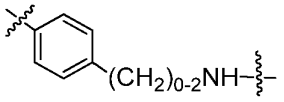
, -CHR^4(CH$_2$)$_n$C(=O)NH-, -CHR^4(CH$_2$)$_n$NHC(=O)-, -C(=O)NH- and -NHC(=O)-;

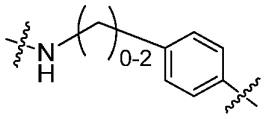
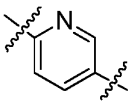
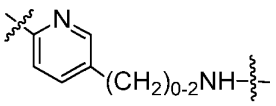
each R^4 is independently selected from H, C$_{1-4}$alkyl, side chains of known amino acids, -C(=O)OH and -OH,

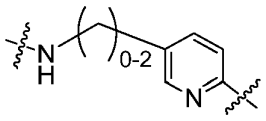
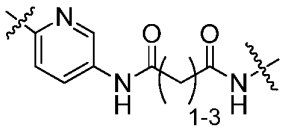
each R^5 is independently selected from H, C_{1-4}alkyl, phenyl or C_{1-4}alkyl substituted with 1 to 3 –OH groups;

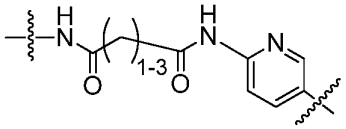
each R^6 is independently selected from H, fluoro, benzyloxy substituted with -C(=O)OH, benzyl substituted with –C(=O)OH, C_{1-4}alkoxy substituted with -C(=O)OH and C_{1-4}alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C_{1-4}alkyl, phenyl, pyrimidine and pyridine;

R^8 is independently selected from  ,  ,

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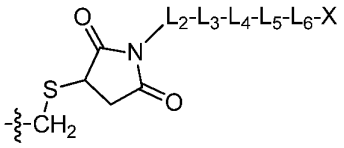
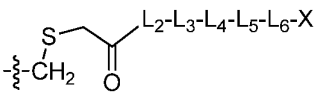
,

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9.

In some of these embodiments, the immunoconjugate comprises a group of the formula

 or 

IIA IIB

wherein the sulfur atom is the sulfur of a cysteine residue in a modified antibody or antibody fragment and is located at one of the substitution sites identified herein.

In any of the foregoing embodiments, the cysteine substitution site may be a position that corresponds to one of the sites identified by a position number, even though the position of the site in the sequence has been changed by a modification or truncation of the full-length antibody. Corresponding sites can be readily identified by alignment of an antibody or fragment with a full-length antibody.

1. Site-Specific Cysteine Engineered Antibodies

Site-specific Labeling

The antibodies (*e.g.*, a parent antibody, optionally containing one or more non-canonical amino acids) of the present invention are numbered according to the EU numbering system as set forth in Edelman *et al.*, (1969) Proc. Natl. Acad. USA 63:78-85, except that the lambda light chain is numbered according to the Kabat numbering system as set forth in Kabat *et al.*, (1991) Fifth Edition. NIH Publication No. 91-3242. Human IgG1 constant region is used as a representative throughout the application. However, the invention is not limited to human IgG1; corresponding amino acid positions can be readily deduced by sequence alignment. For example, FIG. 4 shows sequence alignment of human IgG1, IgG2, IgG3 and IgG4 heavy chain constant regions, so that an identified Cys engineering site in the IgG1 constant region can be readily identified for IgG2, IgG3, and IgG4 as shown in FIG. 4. For the light chain constant region, IgG1, IgG2, IgG3 and IgG4 are the same. Table 1 below lists the amino acid positions in the constant region of the heavy chain of an antibody that can be replaced by a cysteine. Table 2 lists the amino acid positions in the constant region of the kappa light chain of an antibody that can be replaced by a cysteine. Table 3 lists the amino acid positions in the constant region of the lambda light chain of an antibody that can be replaced by a cysteine.

Table 1. Identified cysteine substitution sites in the heavy chain constant region of human IgG1 (Sites numbered according to EU numbering system).

EU number	Residue	Surface accessibility [Å^2]	Selected HC Cys	SEQ ID NO.
117	SER	128.0	HC-S117C	2
119	SER	79.1	HC-S119C	3
121	LYS	135.9	HC-K121C	4
124	SER	40.2	HC-S124C	5
132	SER	34.4	HC-S132C	6

EU number	Residue	Surface accessibility [Å^2]	Selected HC Cys	SEQ ID NO.
134	SER	123.3	HC-S134C	7
136	SER	182.9	HC-S136C	8
139	THR	32.9	HC-T139C	9
152	GLU	52.1	HC-E152C	10
153	PRO	89.1	HC-P153C	11
155	THR	69.0	HC-T155C	12
157	SER	39.0	HC-S157C	13
164	THR	125.4	HC-T164C	14
165	SER	183.2	HC-S165C	15
169	THR	60.0	HC-T169C	16
171	PRO	33.3	HC-P171C	17
174	LEU	68.1	HC-L174C	18
176	SER	161.9	HC-S176C	19
177	SER	68.1	HC-S177C	20
189	PRO	86.4	HC-P189C	21
191	SER	126.8	HC-S191C	22
195	THR	111.3	HC-T195C	23
197	THR	89.8	HC-T197C	24
205	LYS	217.1	HC-K205C	25
207	SER	50.0	HC-S207C	26
212	ASP	97.0	HC-D212C	27
246	LYS	55.1	HC-K246C	28
258	GLU	42.1	HC-E258C	29
269	GLU	189.2	HC-E269C	30
274	LYS	137.8	HC-K274C	31
286	ASN	119.4	HC-N286C	32
288	LYS	181.8	HC-K288C	33
290	LYS	177.0	HC-K290C	34
292	ARG	251.5	HC-R292C	35
293	GLU	83.3	HC-E293C	36
294	GLN	73.5	HC-E294C	37
320	LYS	55.0	HC-K320C	38
322	LYS	78.3	HC-K322C	39
326	LYS	212.7	HC-K326C	40
330	ALA	96.3	HC-A330C	41
333	GLU	84.7	HC-E333C	42
334	LYS	49.6	HC-K334C	43
335	THR	70.1	HC-T335C	44
337	SER	15.1	HC-S337C	45
344	ARG	98.2	HC-R344C	46
355	ARG	249.4	HC-R355C	47

EU number	Residue	Surface accessibility [Å^2]	Selected HC Cys	SEQ ID NO.
360	LYS	113.9	HC-K360C	48
362	GLN	40.8	HC-Q362C	49
375	SER	28.9	HC-S375C	50
382	GLU	21.8	HC-E382C	51
389	ASN	189.5	HC-N389C	52
390	ASN	36.4	HC-N390C	53
392	LYS	81.8	HC-K392C	54
393	THR	35.8	HC-T393C	55
398	LEU	110.9	HC-L398C	56
400	SER	81.3	HC-S400C	57
413	ASP	79.6	HC-D413C	58
415	SER	69.0	HC-S415C	59
422	VAL	80.8	HC-V422C	60

Table 2. Identified cysteine substitution sites in the kappa light chain constant region of human IgG1 (Sites numbered according to EU numbering system).

EU number	Residue	Surface accessibility [Å^2]	Selected LC Cys	SEQ ID NO.
107	LYS	90	LC-K107C	61
108	ARG	49	LC-R108C	62
109	THR	148	LC-T109C	63
112	ALA	50	LC-A112C	64
114	SER	39	LC-S114C	65
122	ASP	90	LC-D122C	66
123	GLU	51	LC-E123C	67
129	THR	41	LC-T129C	68
142	ARG	55	LC-R142C	69
143	GLU	117	LC-E143C	70
145	LYS	160	LC-K145C	71
152	ASN	157	LC-N152C	72
154	LEU	117	LC-L154C	73
156	SER	122	LC-S156C	74
159	SER	22	LC-S159C	75
161	GLU	66	LC-E161C	76
165	GLU	74	LC-E165C	77
168	SER	170	LC-S168C	78
169	LYS	241	LC-K169C	79
170	ASP	48	LC-D170C	80
182	SER	59	LC-S182C	81

EU number	Residue	Surface accessibility [Å^2]	Selected LC Cys	SEQ ID NO.
183	LYS	131	LC-K183C	82
188	LYS	201	LC-K188C	83
190	LYS	167	LC-K190C	84
191	VAL	58	LC-V191C	85
197	THR	38	LC-T197C	86
199	GLN	127	LC-Q199C	87
203	SER	110	LC-S203C	88
206	THR	70	LC-T206C	89

Table 3. Identified cysteine substitution sites on the lambda light chain of human IgG1.

Kabat number	Residue	Surface accessibility [Å^2]	Selected LC Cys	SEQ ID NO.
143	ALA	82	LC-A143C	92
145	THR	106	LC-T145C	93
147	ALA	14	LC-A147C	94
156	LYS	233	LC-K156C	95
159	VAL	28	LC-V159C	96
163	THR	157	LC-T163C	97
168	SER	166	LC-S168C	98

Because of the high sequence homology of constant regions of IgG1, IgG2, IgG3 and IgG4 antibodies, findings of the invention are not limited to any specific antibodies or antibody fragments.

In one embodiment, the present invention provides immunoconjugates comprising a modified antibody or an antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises a substitution of one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acids on its heavy chain constant region chosen from positions identified in Table 1. In a specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region chosen from positions 121, 124, 152, 171, 174, 258, 292, 333, 334, 360, 375, and 392 of the heavy chain. For example, an immunoconjugate of the invention comprises a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of two amino acids with cysteine on its constant region chosen from positions 121 and 124, 121 and 152, 121 and 171, 121 and 174, 121 and 258, 121 and 292, 121 and 333, 121

and 334, 121 and 360, 121 and 375, 121 and 392, 124 and 152, 124 and 171, 124 and 174, 124 and 258, 124 and 292, 124 and 333, 124 and 334, 124 and 360, 124 and 375, 124 and 392,152 and 171, 152 and 174, 152 and 258, 152 and 292, 152 and 333, 152 and 334, 152 and 360, 152 and 375, 152 and 392, 171 and 174, 171 and 258, 171 and 292, 171 and 333, 171 and 360, 171 and 375, 174 and 258, 174 and 292, 174 and 333, 174 and 334, 174 and 360, 174 and 375, 174 and 392, 258 and 292, 258 and 333, 258 and 334, 258 and 360, 258 and 375, 258 and 392, 292 and 333, 292 and 334, 292 and 360, 292 and 375, 292 and 392, 333 and 334, 333 and 360, 333 and 375, 333 and 392; 334 and 360, 334 and 375, 334 and 392, 360 and 375, 360 and 392, or 375 and 392 of the heavy chain.

In another embodiment, an immunoconjugate of the invention comprises a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of three amino acids with cysteine on its constant region chosen from positions 121, 124 and 152; 121, 124 and 171; 121, 124 and 174; 121, 124 and 258; 121, 124 and 292; 121, 124 and 333; 121, 124 and 334; 121, 124 and 360; 121, 124 and 375; 121, 124 and 392; 121, 152 and 171; 121, 152 and 174; 121, 152 and 258; 121, 152 and 292; 121, 152 and 333; 121, 152 and 334; 121, 152 and 360; 121, 152 and 375; 121, 152 and 392; 121, 171 and 174; 121, 171 and 258; 121, 171 and 292; 121, 171 and 333; 121, 171 and 334; 121, 171 and 360; 121, 171 and 375; 121, 171 and 392; 121, 174 and 258, 121, 174 and 292; 121, 174 and 333; 121, 174 and 334; 121, 174 and 360; 121, 174 and 375; 121, 174 and 392; 121, 258 and 292; 121, 258 and 333; 121, 258 and 334; 121, 258 and 360; 121, 258 and 375; 121, 258 and 392; 121, 292 and 333; 121, 292 and 334; 121, 292 and 360; 121, 292 and 375; 121, 292 and 392; 121, 333 and 334; 121, 333 and 360; 121, 333 and 375; 121, 333 and 392; 121, 334 and 360; 121, 334 and 375; 121, 334 and 392; 121, 360 and 375; 121, 360 and 392; 121, 375 and 392; 124, 152 and 171; 124, 152 and 174; 124, 152 and 258; 124, 152 and 292; 124, 152 and 333; 124, 152 and 334; 124, 152 and 360; 124, 152 and 375; 124, 152 and 392; 124, 171 and 174; 124, 171 and 258; 124, 171 and 292; 124, 171 and 333; 124, 171 and 334; 124, 171 and 360; 124, 171 and 375; 124, 171 and 392; 124, 174 and 258; 124, 174 and 292; 124, 174 and 333; 124, 174 and 334; 124, 174 and 360; 124, 174 and 375; 124, 174 and 392; 124, 258 and 292; 124, 258 and 333; 124, 258 and 334; 124, 258 and 360; 124, 258 and 375; 124, 258 and 392; 124, 292 and 333; 124, 292 and 334; 124, 292 and 360; 124, 292 and 375; 124, 292 and 392; 124, 333 and 360; 124, 333 and 334; 124, 333 and 375; 124, 333 and 392; 124, 334 and 360; 124, 334 and 375; 124, 334 and 392; 124, 360 and 375; 124, 360 and 392; 124, 375 and 392; 152, 171 and 174; 152, 171 and 258; 152, 171 and 292; 152, 171 and 333; 152, 171 and 334; 152, 171 and 360; 152, 171 and 375; 152, 171 and 392; 152, 174 and 258; 152, 174 and 292; 152, 174 and 333; 152, 174 and 334; 152, 174 and 360; 152, 174 and 375; 152, 174 and 392; 152, 258 and 292; 152, 258 and 333; 152, 258 and 334; 152, 258

and 360; 152, 258 and 375; 152, 258 and 392; 152, 292 and 333; 152, 292 and 334; 152, 292 and 360; 152, 292 and 375; 152, 292 and 392; 152, 333 and 334; 152, 333 and 360; 152, 333 and 375; 152, 333 and 392; 152, 334 and 360; 152, 334 and 375; 152, 334 and 392; 152, 360 and 375; 152, 360 and 392; 152, 375 and 392; 171, 174 and 258; 171, 174 and 292; 171, 174 and 333; 171, 174 and 334; 171, 174 and 360; 171, 174 and 375; 171, 174 and 392; 171, 258 and 292; 171, 258 and 292; 171, 258 and 333; 171, 258 and 334; 171, 258 and 360; 171, 258 and 375; 171, 258 and 392; 171, 292 and 333; 171, 292 and 334; 171, 292 and 360; 171, 292 and 375; 171, 292 and 392; 171, 333 and 334; 171, 333 and 360; 171, 333 and 375; 171, 333 and 392; 171, 334 and 360; 171, 334 and 392; 171, 360 and 375; 171, 360 and 392; 171, 375 and 392; 174, 258 and 292; 174, 258 and 333; 174, 258 and 334; 174, 258 and 360; 174, 258 and 375; 174, 258 and 392; 174, 292 and 333; 174, 292 and 334; 174, 292 and 360; 174, 292 and 375; 174, 292 and 392; 174, 333 and 334; 174, 333 and 360; 174, 333 and 375; 174, 333 and 392; 174, 334 and 360; 174, 334 and 375; 174, 334 and 392; 174, 360 and 375; 174, 360 and 392; 174, 375 and 392; 258, 292 and 333; 258, 292 and 334; 258, 292 and 360; 258, 292 and 375; 258, 292 and 392; 258, 333 and 360; 258, 333 and 375; 258, 333 and 392; 258, 334 and 360; 258, 334 and 375; 258, 334 and 392; 258, 360 and 375; 258, 360 and 392; 258, 375 and 392; 292, 333 and 334; 292, 333 and 360; 292, 333 and 375; 292, 333 and 392; 292, 334 and 360; 292, 334 and 375; 292, 334 and 392; 292, 360 and 375; 292, 360 and 392; 292, 375 and 392; 333, 334 and 360; 333, 334 and 375; 333, 334 and 392; 333, 360 and 375, 333, 360 and 392; 333, 375 and 392; 334, 360 and 375; 334, 360 and 392; or 360, 375 and 392 of the heavy chain.

In an embodiment, an immunoconjugate of the invention comprises a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of four amino acids with cysteine on its constant region chosen from positions 152, 333, 375 and 392; or 152, 334, 375 and 392 of the heavy chain.

In a specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises SEQ ID NO: 2, 3, 9, 11, 12, 13, 14, 16, 21, 25, 26, 28, 30, 31, 32, 33, 34, 36, 38, 39, 40, 43, 44, 45, 46, 47, 51, 53, 54, 56, 57, or 60. In another specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or an antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises SEQ ID NO: 6, 7, 8, 15, 19, 20, 22, 23, 24, 27, 36, 37, 41, 49, 52, 55, 58, or 59.

In another embodiment, the present invention provides immunoconjugates comprising a modified antibody or an antibody fragment thereof, and a drug moiety, wherein said modified

antibody or antibody fragment thereof comprises a substitution of one or more amino acids (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) on its light chain constant region chosen from positions identified in Table 2. In a specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region chosen from positions 107, 108, 142, 145, 159, 161, and 165 of the light chain, wherein said light chain is human kappa light chain. For example, an immunoconjugate of the invention comprises a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of two amino acids with cysteine on its constant region chosen from positions 107 and 108; 107 and 142; 107 and 145; 107 and 159; 107 and 161; 107 and 165; 108 and 142; 108 and 145; 108 and 159; 108 and 161; 108 and 165; 142 and 145; 142 and 159; 142 and 161; 142 and 165; 145 and 159; 145 and 161; 145 and 165; 159 and 161; 159 and 165; 161 and 165 of the light chain, wherein said light chain is human kappa light chain. In another embodiment, an immunoconjugate of the invention comprises a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of three amino acids with cysteine on its constant region chosen from positions 107, 108 and 142; 107, 108 and 145; 107, 108 and 159; 107, 108 and 161; 107, 108 and 165; 107, 142 and 145; 107, 142 and 159; 107, 142 and 161; 107, 142 and 165; 107, 145 and 159; 107, 145 and 161; 107, 145 and 165; 107, 159 and 161; 107, 159 and 165; 107, 161 and 165; 108, 142 and 145; 108, 142 and 159; 108, 142 and 161; 108, 142 and 165; 108, 145 and 159; 108, 145 and 161; 108, 145 and 165; 108, 159 and 161; 108, 159 and 165; 108, 161 and 165; 142, 145 and 159; 142, 145 and 161; 142, 145 and 165; 142, 159 and 161; 142, 159 and 165; 142, 161 and 165; 145, 159 and 161; 145, 159 and 165; 145, 161 and 165; or 159, 161 and 165 of the light chain, wherein said light chain is human kappa light chain.

In a specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises SEQ ID NO: 63, 65, 68, 70, 72, 73, 74, 78, 79, 80, 81, 82, 83, 86, 87, or 88. In another specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises SEQ ID NO: 64, 66, 67, 84, 85, or 89 63, 64, 65, 66, 67, 68, 70, 72, 73, 74, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, or 89.

In another embodiment, the present invention provides immunoconjugates comprising a modified antibody or an antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises a substitution of one or more amino acids on

its light chain constant region chosen from positions identified in Table 3. In a specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region chosen from positions 143, 147, 159, 163, and 168 of the light chain, wherein said light chain positions are numbered according to the Kabat system, and wherein said light chain is human lambda light chain. For example, an immunoconjugate of the invention comprises a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of two amino acids with cysteine on its constant region chosen from positions 143 and 147; 143 and 159; 143 and 163; 143 and 168; 147 and 159; 147 and 163; 147 and 168; 159 and 163; 159 and 168; or 163 and 168 of the light chain, wherein said light chain positions are numbered according to the Kabat system, and wherein said light chain is human lambda light chain. In another embodiment, an immunoconjugate of the invention comprises a modified antibody or antibody fragment thereof and a drug moiety, wherein said modified antibody or antibody fragment comprises a substitution of three amino acids with cysteine on its constant region chosen from positions 143, 147 and 159; 143, 147 and 163; 143, 147 and 168; 143, 159 and 163; 143, 159 and 168; 143, 163 and 168; 147, 159 and 163; 147, 159 and 168; 147, 163 and 168; or 159, 163 and 168 of the light chain, wherein said light chain positions are numbered according to the Kabat system, and wherein said light chain is human lambda light chain.

In an embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises SEQ ID NO: 92, 94, 96, 97 or 98. In another specific embodiment, the present invention provides an immunoconjugate comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment thereof comprises SEQ ID NO: 93 or 95.

In an embodiment, the immunoconjugate can have an DAR of about 2 or about 4. In an embodiment, the present invention provides immunoconjugates comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment comprises a Cys substitution of one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acids on its heavy chain constant region chosen from positions identified in Table 1, and a Cys substitution of one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acids on its light chain constant region chosen from positions identified in Table 2 or Table 3. In one embodiment, the present invention provides immunoconjugates comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment comprises a Cys substitution of one or more amino acids in its heavy chain constant region

chosen from positions 121, 124, 152, 171, 174, 258, 292, 333, 334, 360, 375 and 392; and a Cys substitution of one or more amino acids in its light chain constant region chosen from positions 107, 108, 142, 145, 159, 161, and 165, wherein said light chain is human kappa light chain. In an embodiment, a modified antibody or antibody fragment according to the present invention may comprise a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 142 of a human

kappa light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 108 of a human

kappa light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 107 of a human kappa light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 107 of a human

kappa light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 108 of a human kappa light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 142 of a human kappa light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 145 of a human kappa light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 159 of a human kappa light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 161 of a human kappa light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain.In a embodiment , a modified antibody or antibody fragment according to the present invention comprises a Cys substitution on position 375 and on position 392 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain. In an embodiment a modified antibody or antibody fragment according to the present invention may comprise a Cys substitution on position 334 and on position 375 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain. In another example, a modified antibody or antibody fragment according to the present invention may comprise a Cys substitution on position 334 and on position 392 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain. In an embodiment, an immunoconjugates of those combinations can have a DAR of about 4 or about 6.

In an embodiment , a modified antibody or antibody fragment according to the present invention may comprise a Cys substitution on position 334, on position 375 and on position 392 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain. In an embodiment, a modified antibody or antibody fragment according to the present invention may comprise a Cys substitution on position 333, on position 375 and on position 392 of a heavy chain, and a Cys substitution on position 165 of a human kappa light chain. In an embodiment, those combinations can have a DAR of about 4, 6, or 8.

In an embodiment, the present invention provides immunoconjugates comprising a modified antibody or antibody fragment thereof, and a drug moiety, wherein said modified antibody or antibody fragment comprises a Cys substitution of one or more amino acids in its heavy chain constant region chosen from positions 121, 124, 152, 171, 174, 258, 292, 333, 334 360, 375 and 392; and a Cys substitution of one or more amino acids in its light chain constant region chosen from positions 143, 147, 159, 163, and 168, wherein said light chain is human

lambda light chain. For example, a modified antibody or antibody fragment according to the present invention may comprise a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 121 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 124 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 152 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 171 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 174 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or

a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 258 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 292 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 333 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 334 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 360 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or

a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 375 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 143 of a human lambda light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 147 of a human lambda light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 159 of a human lambda light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 163 of a human lambda light chain; or a Cys substitution on position 392 of a heavy chain, and a Cys substitution on position 168 of a human lambda light chain;

In an embodiment of the invention, the amino acid substitution described herein is cysteine comprising a thiol group. In some aspects of the invention, the thiol group is utilized for chemical conjugation, and is attached to a linker unit (LU) and/or drug moiety. In some embodiments, the immunoconjugates of the invention comprise a drug moiety selected from the group consisting of a V-ATPase inhibitor, a HSP90 inhibitor, an IAP inhibitor, an mTor inhibitor, a microtubule stabilizer, a microtubule destabilizers, an auristatin, a dolastatin, a maytansinoid, a MetAP (methionine aminopeptidase), an inhibitor of nuclear export of proteins CRM1, a DPPIV inhibitor, proteasome inhibitors, an inhibitors of phosphoryl transfer reactions in mitochondria, a protein synthesis inhibitor, a kinase inhibitor, a CDK2 inhibitor, a CDK9 inhibitor, an kinesin inhibitor, an HDAC inhibitor, a DNA damaging agent, a DNA alkylating agent, a DNA intercalator, a DNA minor groove binder and a DHFR inhibitor. In some embodiments, the immunoconjugates of the invention comprise a drug moiety that is an anti-cancer agent. The modified antibody or antibody fragments of the present invention can be any formats known in the art, such as a monoclonal, chimeric, humanized, fully human, bispecific, or multispecific antibody or antibody fragment thereof.

According to the present invention, the modified antibody heavy chain and/or light chain (or antibody fragment thereof) may contain 1, 2, 3, 4, 5, 6, 7, 8, or more cysteine substitutions in its constant regions. In one embodiment, the modified antibodies or antibody fragments contain 2, 4, 6, 8, or more cysteine substitutions in its constant regions. In some embodiments, the modified antibody, antibody fragment or immunoconjugate thereof comprises 2 or 4 Cys substitutions.

In one embodiment, the parental antibody (antibody without cysteine substitution) is an IgG, IgM, IgE, or IgA antibody. In a specific embodiment, the parental antibody is an IgG1 antibody. In another specific embodiment, the parental antibody is an IgG2, IgG3, or IgG4 antibody.

The present invention also provides modified antibodies or antibody fragments thereof comprising a substitution of one or more amino acids on its heavy chain constant region chosen from positions identified in Table 1. In some embodiments, the present invention provides modified antibodies or antibody fragments thereof comprising a substitution of one or more amino acids on its light chain constant region chosen from positions identified in Table 2 or Table 3.

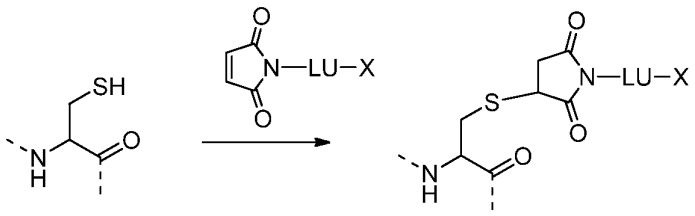
In certain embodiments, the modified antibodies or antibody fragments provided herein are labeled using the methods of the invention in combination with other conjugation methods known in the art including, but not limited to, chemoselective conjugation through lysine, histidine, tyrosine, formyl-glycine, pyrrolysine, pyrroline-carboxy-lysine, unnatural amino acids, and protein tags for enzyme-mediated conjugation (e.g., S6 tags).

2. Conjugation Chemistry

The conjugated antibody or antibody fragment thereof provided herein is produced by post-translational modification of at least one cysteine residue that was incorporated into the antibody or antibody fragment thereof as described above by site-specific labeling methods. The conjugated antibody or antibody fragment can be prepared by methods known in the art for conjugation of a payload of interest to cysteine residues that occur naturally in proteins, and by methods described for conjugation to proteins engineered to contain an additional cysteine residue substituted for another amino acid of a natural protein sequence.

In certain embodiments the modified antibodies or antibody fragment thereof provided herein are conjugated using known methods wherein the incorporated cysteine (cys) is conjugated to a maleimide derivative as Scheme Ia below. Modified antibodies of the invention that undergo this type of conjugation contain a thiol-maleimide linkage.

Scheme Ia. Conjugation via thiol-maleimide adduct formation.



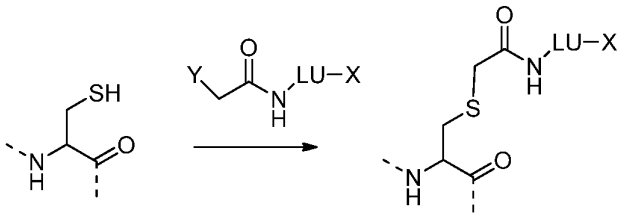
where:

LU is a Linker Unit (LU), and

X is a payload or drug moiety.

In other embodiments, the Cys incorporated into the modified antibodies or antibody fragment is conjugated by reaction with an alpha-halo carbonyl compound such as a chloro-, bromo-, or iodo-acetamide as shown in Scheme Ib below. It is understood that other leaving groups besides halogen, such as tosylate, triflate and other alkyl or aryl sulfonates, can be used as the leaving group Y. While Scheme Ib depicts reaction of a Cys thiol with an alpha-halo acetamide, the method includes any alkylation of a sulfur of an incorporated Cys with a group of the formula Y-CHR-C(=O)-, where R is H or C_{1-4} alkyl, Y is a leaving group (typically Cl, Br, or I, and optionally an alkylsulfonate or arylsulfonate); it is not limited to amides.

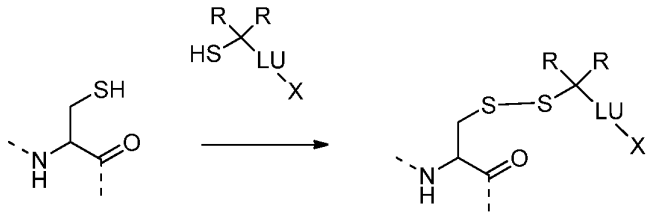
Scheme Ib. Conjugation via reaction with an alpha-halo carbonyl compound.



Y is a leaving group (Cl, Br, I, OTs, OTf, and the like)
LU is a linker unit
X is a payload or drug moiety

Alternatively, the Cys incorporated into the modified antibodies or antibody fragment can be conjugated by reaction with an external thiol under conditions that induce formation of a disulfide bond between the external thiol and the sulfur atom of the incorporated cysteine residue as shown in Scheme Ic below. In these examples, R can be H; however, compounds where one or both R groups represent an alkyl group, e.g., Methyl, have been found to increase the stability of the disulfide.

Scheme Ic. Conjugation via disulfide formation.



each R is independently H or C_{1-4} alkyl
LU is a linker unit
X is a payload or drug moiety

By way of example only, such post-translational modifications are illustrated in Schemes (Ia)-(Ic) above, where the starting structure represents a cysteine incorporated into a light chain or heavy chain of an antibody at one of the specific sites identified herein. Methods for performing each of these conjugation methods are well known in the art. An antibody can be modified by these methods in its light chains, or its heavy chains, or in both light and heavy chains. An antibody in which each light chain or each heavy chain has been modified to contain a single incorporated cysteine will generally contain two conjugation sites, since an antibody typically contains two light and two heavy chains.

Upon conjugation, the modified antibodies of the invention typically contain 1-12, frequently 2-8, and preferably 2, 4 or 6 –LU-X (Linker Unit-Payload) moieties. In some embodiments, an antibody light or heavy chain is modified to incorporate two new Cys residues at two of the specific sites identified herein for Cys substitutions (or alternatively one Cys is incorporated in the light chain and one in the heavy chain), so the tetrameric antibody ultimately contains four conjugation sites. Similarly the antibody can be modified by replacement of 3 or 4 of its native amino acids with Cys at the specific sites identified herein, in light chain or heavy chain or a combination thereof, resulting in 6 or 8 conjugation sites in the tetrameric antibody.

X in these conjugates represents a payload, which can be any chemical moiety that is useful to attach to an antibody. In some embodiments, X is a drug moiety selected from a cytotoxin, an anti-cancer agent, an anti-inflammatory agent, an antifungal agent, an antibacterial agent, an anti-parasitic agent, an anti-viral agent, an immune potentiator, and an anesthetic agent or any other therapeutic, or biologically active moiety or drug moiety. In other embodiments, X is a label such as a biophysical probe, a fluorophore, an affinity probe, a spectroscopic probe, a radioactive probe, a spin label, or a quantum dot. In other embodiments, X is a chemical moiety that modifies the antibody's physicochemical properties such as a lipid molecule, a polyethylene glycol, a polymer, a polysaccharide, a liposome, or a chelator. In other embodiments, X is a functional or detectable biomolecule such as a nucleic acid, a ribonucleic acid, a protein, a peptide (e.g., an enzyme or receptor), a sugar or polysaccharide, an antibody, or an antibody fragment. In other embodiments, X is an anchoring moiety such as a nanoparticle, a PLGA particle, or a surface, or any binding moiety for specifically binding the conjugate to another moiety, such as a histidine tag, poly-G, biotin, avidin, streptavidin, and the like. In other embodiments, X is a reactive functional group that can be used to attach the antibody conjugate to another chemical moiety, such as a drug moiety, a label, another antibody, another chemical moiety, or a surface.

The Linker Unit (LU) can be any suitable chemical moiety that covalently attaches the thiol-reactive group (e.g., maleimide, alpha-halo carbonyl, vinyl carbonyl (e.g., acrylate or acrylamide), vinyl sulfone, vinylpyridine, or thiol) to a payload. Many suitable LUs are known

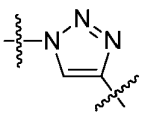
in the art. For example, LU can be comprised of one, two, three, four, five, six, or more than six linkers referred to herein as L_1, L_2, L_3, L_4, L_5 and L_6. In certain embodiments the LU comprises a linker selected from a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker or any combination thereof, and the LU optionally contains a self-immolative spacer.

In some embodiments, LU is a group of the formula $-L_1-L_2-L_3-L_4-$ or $-L_1-L-L_3-L_4-L_5-L_6-$. Linking groups L_1, L_2, L_3, L_4, L_5 and L_6 for use in LU include alkylene groups $-(CH_2)_n-$ (where n is 1-20, typically 1-10 or 1-6), ethylene glycol units $(-CH_2CH_2O-)_n$ (where n is 1-20, typically 1-10 or 1-6), amides –C(=O)-NH- or –NH-C(=O)-, esters –C(=O)-O- or –O-C(=O)-, rings having two available points of attachment such as divalent phenyl, C_{3-8} cycloalkyl or C_{4-8} heterocyclyl groups, amino acids –NH-CHR*-C=O- or –C(=O)-CHR*-NH-, where R* is the side chain of a known amino acid (frequently one of the canonical amino acids, but also including e.g. norvaline, norleucine, homoserine, homocysteine, phenylglycine, citrulline, and other named alpha-amino acids), polypeptides of known amino acids (e.g., dipeptides, tripeptides, tetrapeptides, etc.), thiol-maleimide linkages (from addition of –SH to maleimide), $-S-CR_2-$ and other thiol ethers such as $-S-CR_2-C(=O)-$ or $-C(=O)-CR_2-S-$, where R is as defined above for Scheme Ic, $-CH_2-C(=O)-$, and disulfides (-S-S-), as well as combinations of any of these with other linkers described below, e.g., a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker or a linker that comprises a self-immolative spacer.

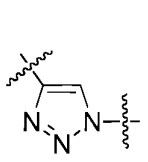
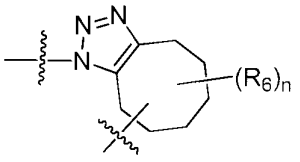
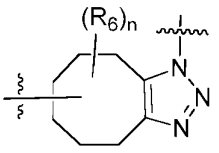
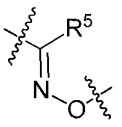
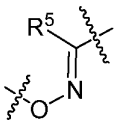
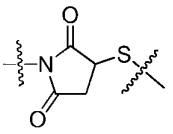
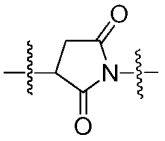
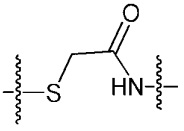
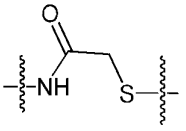
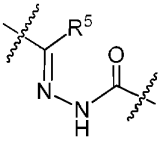
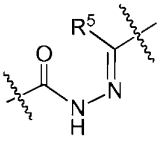
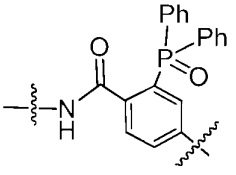
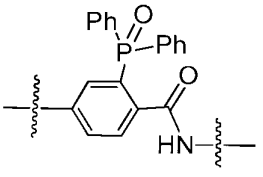
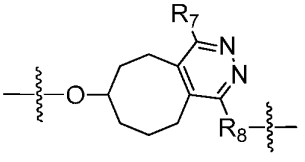
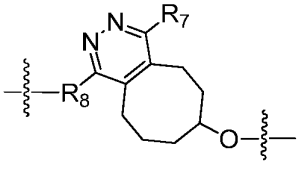
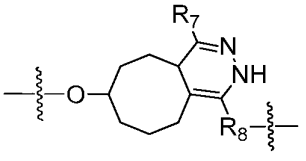
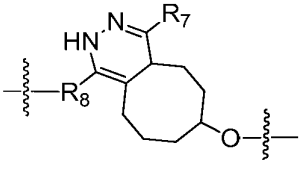
In some embodiments when LU is $-L_1-L-L_3-L_4-L_5-L_6-$, L_1, L_2, L_3, L_4, L_5 and L_6 can be selected from:

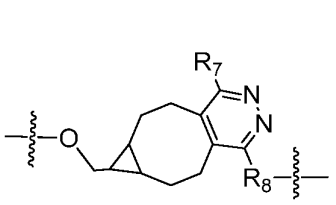
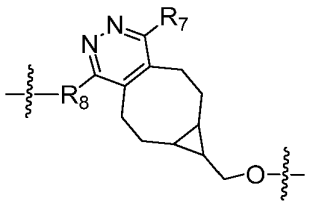
$-A_1-$, $-A_1X^2-$ and $-X^2-$; wherein:

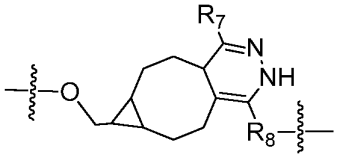
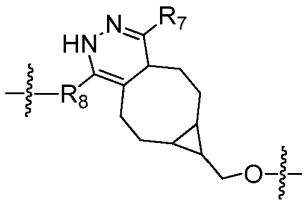
A_1 is $-C(=O)NH-$, $-C(=O)NH(CH_2)_n-$, $-C(=O)NH(C(R^4)_2)_n-$, $-(O(CH_2)_n)_m-$
, $-(O(C(R^4)_2)_n)_m-$,$-((CH_2)_nO)_m-$, $-((C(R^4)_2)_nO)_m-$, $-((CH_2)_nO)_m(CH_2)_n-$
, $-((C(R^4)_2)_nO)_mC(R^4)_2)_n-$, $-(CH_2)_nC(=O)NH-$, $-(C(R^4)_2)_nC(=O)NH-$
, $-(CH_2)_nNHC(=O)-$, $-(C(R^4)_2)_nNHC(=O)-$, $-NHC(=O)(CH_2)_n-$, $-NHC(=O)(C(R^4)_2)_n-$
, $-C(=O)NH(CH_2)_nS-$, $-C(=O)NH(C(R^4)_2)_nS-$, $-S(CH_2)_nC(=O)NH-$
, $-S(C(R^4)_2)_nC(=O)NH-$, $-C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n-$
, $-C(=O)NH(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n-$, $-C(=O)(CH_2)_n-$, $-C(=O)(C(R^4)_2)_n-$
, $-(CH_2)_nC(=O)-$, $-(C(R^4)_2)_nC(=O)-$, $-(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$
, $-(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n-$, $-(CH_2)_nNHC(=O)(CH_2)_n-$
, $-(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n-$, $-(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n-$
, $-(C(R^4)_2)_nNH((C(R^4)_2)_nO)_m(C(R^4)_2)_n-$, $-(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$,
or $-(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n-$;

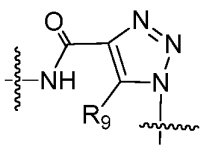
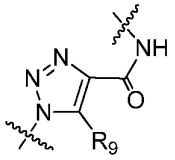
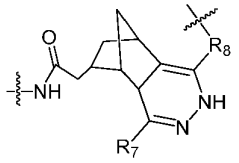


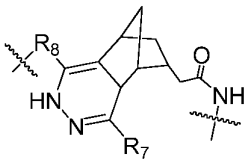
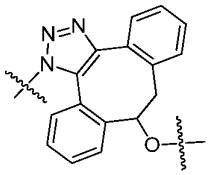
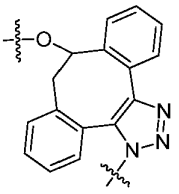
each X^2 is independently selected from a bond, R^8,

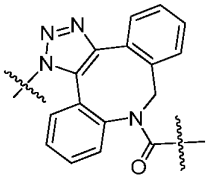
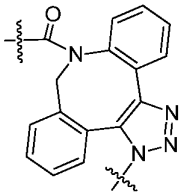

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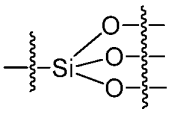


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-S-, -Si(OH)$_2$O-,



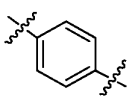
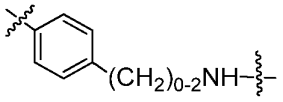
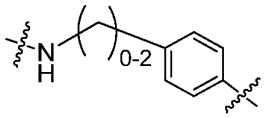
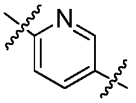
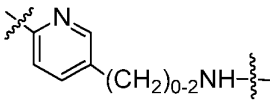
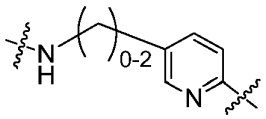
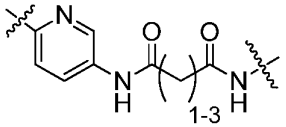
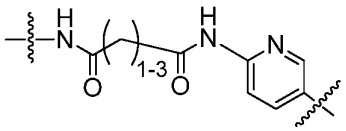
, -CHR^4(CH$_2$)$_n$C(=O)NH-, -CHR^4(CH$_2$)$_n$NHC(=O)-, -C(=O)NH- and -NHC(=O)-;

each R^4 is independently selected from H, C$_{1-4}$alkyl, side chains of known amino acids, -C(=O)OH and -OH,

each R^5 is independently selected from H, C_{1-4}alkyl, phenyl or C_{1-4}alkyl substituted with 1 to 3 –OH groups;

each R^6 is independently selected from H, fluoro, benzyloxy substituted with -C(=O)OH, benzyl substituted with –C(=O)OH, C_{1-4}alkoxy substituted with -C(=O)OH and C_{1-4}alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C_{1-4}alkyl, phenyl, pyrimidine and pyridine;

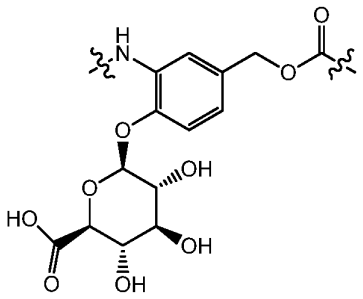
R^8 is independently selected from  ,  ,  ,  ,  ,  ,  , and  ,

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

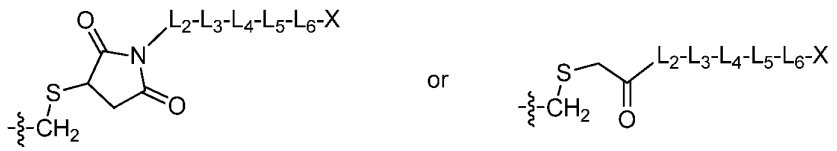
each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9.

In some embodiments, at least one of L_1, L_2, L_3, L_4, L_5 and L_6 is a stable, or non-cleavable, linker. In some embodiments, at least one of L_1, L_2, L_3, L_4, L_5 and L_6 is a cleavable linker, which may be chemically cleavable (hydrazones, disulfides) or enzymatically cleavable. In some embodiments, the enzymatically cleavable linker is one readily cleaved by a peptidase: The Val-Cit linker (valine-citrulline), a dipeptide of two known amino acids, is one such linker. In other embodiments, the enzymatically cleavable linker is one that is triggered by activity of a glucuronidase:



is an example of such a linker, which also comprises a self-immolative spacer that falls apart spontaneously under physiological conditions once glucuronidase cleaves the glycosidic linkage.

In some embodiments, the immunoconjugate of the invention comprises a modified cysteine residue of the formula IIA or IIB:



IIA or IIB

wherein $-CH_2$-S- represents the side chain of Cys incorporated at one of the selected Cys substitution sites described herein, and L_2 – L_6 and X represent linking groups and payloads, respectively, as further described herein. In some embodiments of IIA, L_2 is a bond. In some embodiments of IIB, L_2 is NH or O. In some embodiments of both IIA and IIB, L_3 is selected from $(CH_2)_{1-10}$ and $(CH_2CH_2O)_{1-6}$. L_4, L_5 and L_6 are additional optional linkers selected from those described herein. In certain embodiments, L_6 can be a carbonyl (C=O) or a linker that comprises a self-immolative spacer.

In certain embodiments the Linker Unit (LU) is $-L_1-L_2-L_3-L_4-$, wherein:

L_1 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

L_2 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

L_3 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker, and

L_4 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker or a linker that comprises a self-immolative spacer.

In certain embodiments the Linker Unit (LU) is $-L_1-L_2-L_3-L_4-$, wherein

L_1 is a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

L_2 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

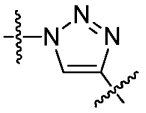
L_3 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker, and

L_4 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker or a linker that comprises a self-immolative spacer.

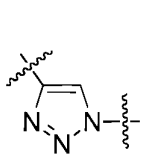
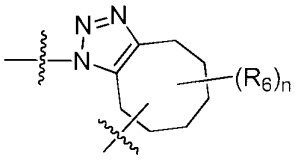
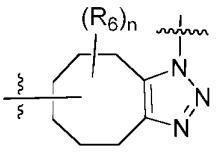
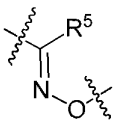
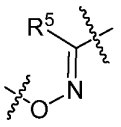
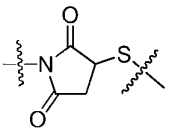
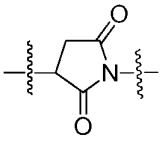
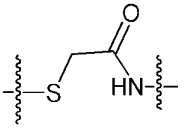
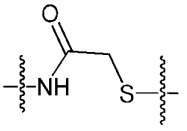
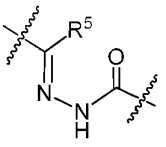
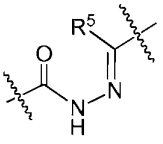
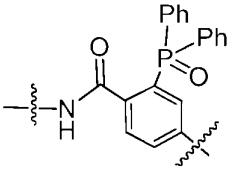
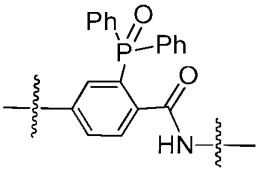
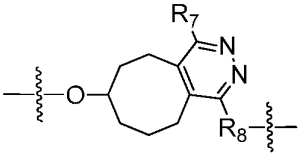
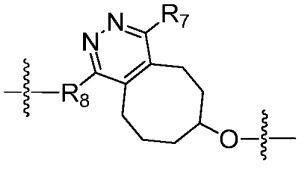
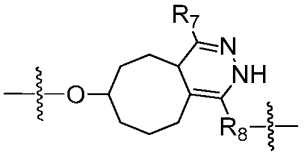
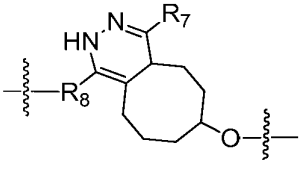
In some of the embodiments of LU at least one of L_1, L_2, L_3, L_4, L_5 and L_6 is a cleavable linker, and LU is considered cleavable. Similarly, in some of the embodiments of LU at least one of L_1, L_2, L_3, L_4, L_5 and L_6 is a non-cleavable linker. In certain of these embodiments, each linker of LU is non-cleavable, and LU is considered non-cleavable.

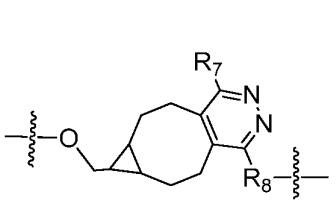
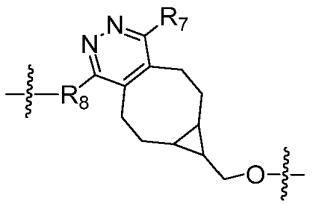
In some of the foregoing embodiments wherein LU is $-L_1-L_2-L_3-L_4-$, at least one of L_1, L_2, L_3 and L_4 is a linker selected from $-A_1-$, $-A_1X^2-$ and $-X^2-$; wherein:

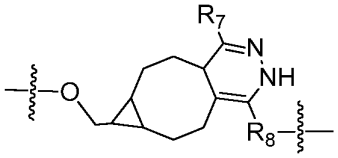
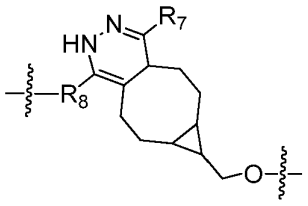
A_1 is $-C(=O)NH-$, $-C(=O)NH(CH_2)_n-$, $-C(=O)NH(C(R^4)_2)_n-$, $-(O(CH_2)_n)_m-$, $-(O(C(R^4)_2)_n)_m-$,$-((CH_2)_nO)_m-$, $-((C(R^4)_2)_nO)_m-$, $-((CH_2)_nO)_m(CH_2)_n-$, $-(((C(R^4)_2)_nO)_mC(R^4)_2)_n-$, $-(CH_2)_nC(=O)NH-$, $-(C(R^4)_2)_nC(=O)NH-$, $-(CH_2)_nNHC(=O)-$, $-(C(R^4)_2)_nNHC(=O)-$, $-NHC(=O)(CH_2)_n-$, $-NHC(=O)(C(R^4)_2)_n-$, $-C(=O)NH(CH_2)_nS-$, $-C(=O)NH(C(R^4)_2)_nS-$, $-S(CH_2)_nC(=O)NH-$, $-S(C(R^4)_2)_nC(=O)NH-$, $-C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n-$, $-C(=O)NH(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n-$, $-C(=O)(CH_2)_n-$, $-C(=O)(C(R^4)_2)_n-$, $-(CH_2)_nC(=O)-$, $-(C(R^4)_2)_nC(=O)-$, $-(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$, $-(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n-$, $-(CH_2)_nNHC(=O)(CH_2)_n-$, $-(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n-$, $-(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n-$, $-(C(R^4)_2)_nNH((C(R^4)_2)_nO)_m(C(R^4)_2)_n-$, $-(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$, or $-(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n-$;

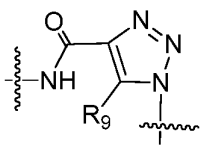
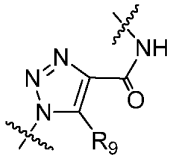
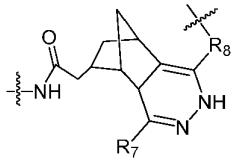


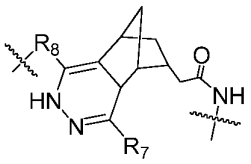
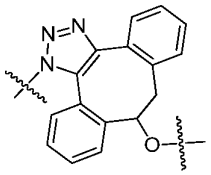
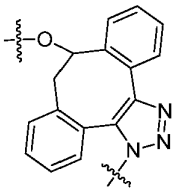
each X^2 is independently selected from a bond, R^8,

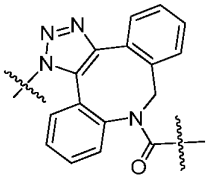
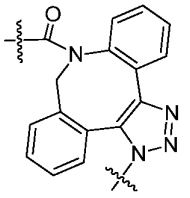

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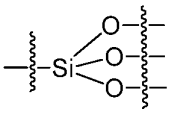


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-S-, -Si(OH)$_2$O-,



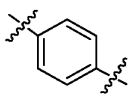
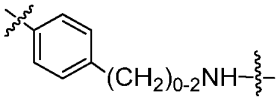
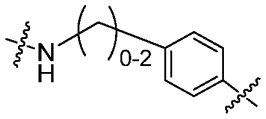
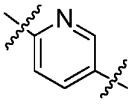
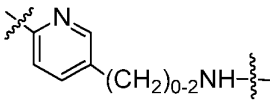
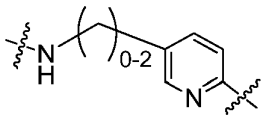
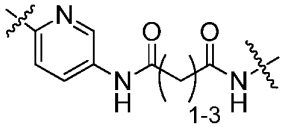
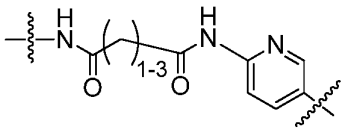
, -CHR^4(CH$_2$)$_n$C(=O)NH-, -CHR^4(CH$_2$)$_n$NHC(=O)-, -C(=O)NH- and -NHC(=O)-;

each R^4 is independently selected from H, C$_{1-4}$alkyl, side chains of known amino acids, -C(=O)OH and -OH,

each R^5 is independently selected from H, C_{1-4}alkyl, phenyl or C_{1-4}alkyl substituted with 1 to 3 –OH groups;

each R^6 is independently selected from H, fluoro, benzyloxy substituted with -C(=O)OH, benzyl substituted with –C(=O)OH, C_{1-4}alkoxy substituted with -C(=O)OH and C_{1-4}alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C_{1-4}alkyl, phenyl, pyrimidine and pyridine;

R^8 is independently selected  ,  ,  ,  ,  ,  ,  , and  ;

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

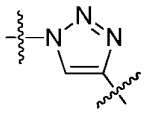
each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9.

In these embodiments, the other linkers of LU are independently selected from a bond, -A^1- , -A_1X^2- , -X^2- , a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker and a linker that comprises a self-immolative spacer.

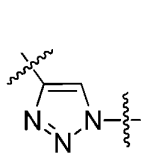
In certain embodiments the Linker Unit (LU) is –L_1–L_2–L_3–L_4–, wherein L_1 is a bond, -A_1-, -A_1X^2- or -X^2-; where:

A_1 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_n$-, -$(O(CH_2)_n)_m$-, -$(O(C(R^4)_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((C(R^4)_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(((C(R^4)_2)_nO)_mC(R^4)_2)_n$-, -$(CH_2)_nC(=O)NH$-, -$(C(R^4)_2)_nC(=O)NH$-, -$(CH_2)_nNHC(=O)$-, -$(C(R^4)_2)_nNHC(=O)$-, -NHC(=O)$(CH_2)_n$-, -NHC(=O)$(C(R^4)_2)_n$-, -

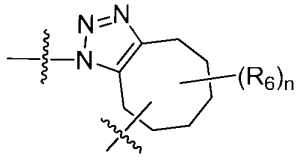
$C(=O)NH(CH_2)_nS$-, -$C(=O)NH(C(R^4)_2)_nS$-, -$S(CH_2)_nC(=O)NH$-, -$S(C(R^4)_2)_nC(=O)NH$-, -$C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n$-, -$C(=O)NH(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$C(=O)(CH_2)_n$-, -$C(=O)(C(R^4)_2)_n$-, -$(CH_2)_nC(=O)$-, -$(C(R^4)_2)_nC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_nNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$-, -$(C(R^4)_2)_nNH((C(R^4)_2)_nO)_m(C(R^4)_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, or -$(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-;



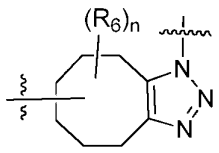
each X^2 is independently selected from a bond, R^8



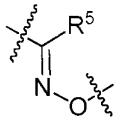
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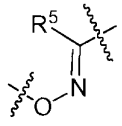
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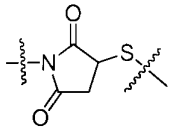
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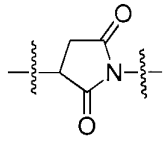
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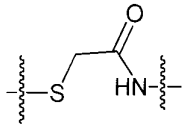
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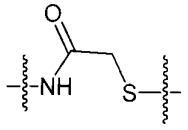
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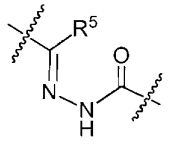
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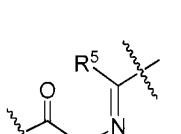
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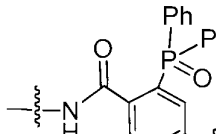
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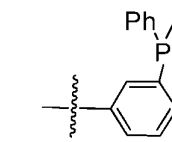
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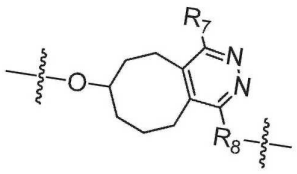
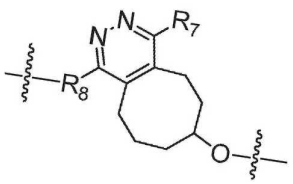
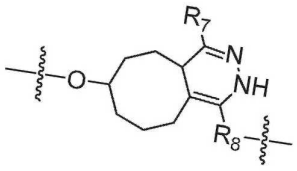
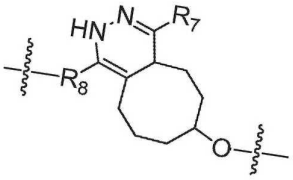
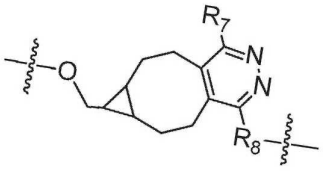
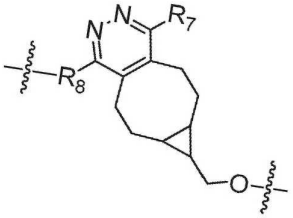
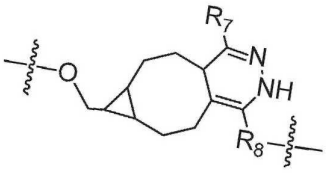
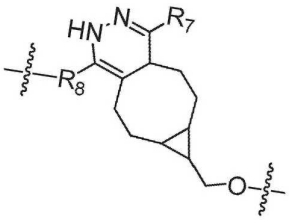
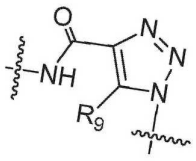
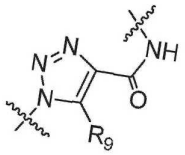
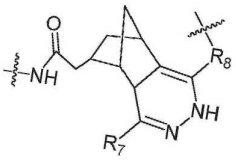
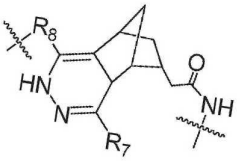
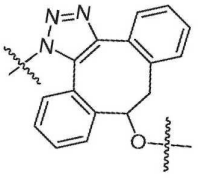
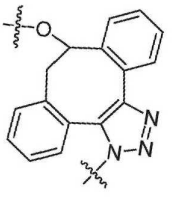


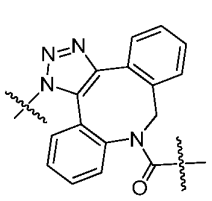
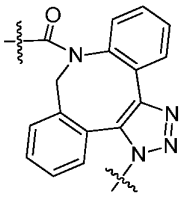
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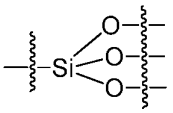
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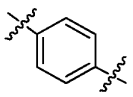
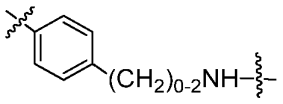
, -CHR^4(CH$_2$)$_n$C(=O)NH-, -CHR^4(CH$_2$)$_n$NHC(=O)-, -C(=O)NH- and -NHC(=O)-;

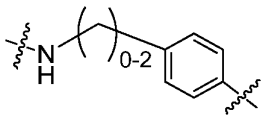
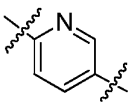
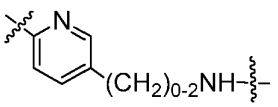
each R^4 is independently selected from H, C$_{1-4}$alkyl, side chains of known amino acids, -C(=O)OH and -OH,

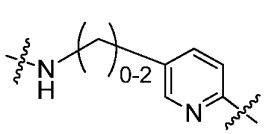
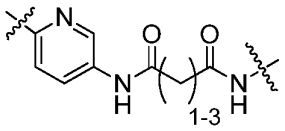
each R^5 is independently selected from H, C$_{1-4}$alkyl, phenyl or C$_{1-4}$alkyl substituted with 1 to 3 –OH groups;

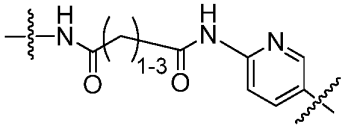
each R^6 is independently selected from H, fluoro, benzyloxy substituted with –C(=O)OH, benzyl substituted with –C(=O)OH, C$_{1-4}$alkoxy substituted with –C(=O)OH and C$_{1-4}$alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C$_{1-4}$alkyl, phenyl, pyrimidine and pyridine;

R^8 is independently selected from , ,

, , ,

, , and



;

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9;

L_2 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

L_3 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker, and

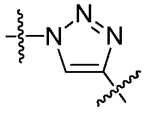
L_4 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker or a linker that comprises a self-immolative spacer.

In certain embodiments, L_1 is C(=O)-CH_2CH_2-NH-C(=O)-CH_2CH_2-S- , so LU is –C(=O)-CH_2CH_2-NH-C(=O)-CH_2CH_2-S-L_2-L_3-L_4-.

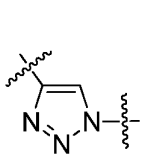
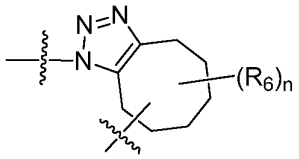
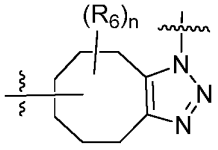
In certain embodiments the Linker Unit (LU) is $–L_1–L_2–L_3–L_4–$, wherein

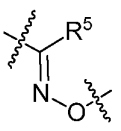
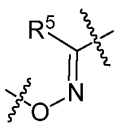
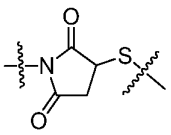
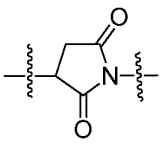
L_1 is a bond, -A_1-, -A_1X^2- or -X^2-; where:

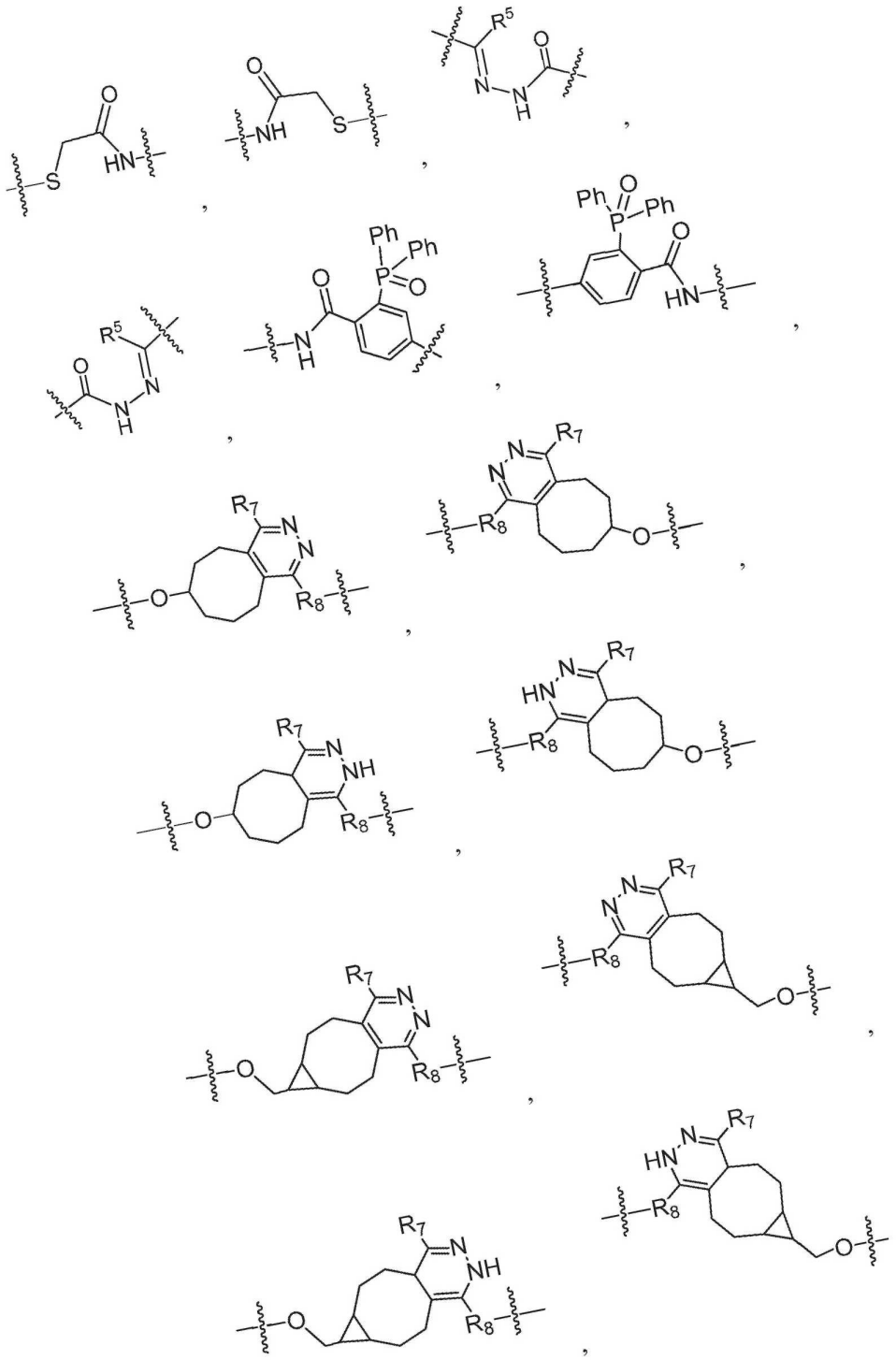
A_1 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(CH_2)_n$C(=O)NH-, -$(CH_2)_n$NHC(=O)-, -NHC(=O)$(CH_2)_n$-, -C(=O)NH$(CH_2)_n$S-, -S$(CH_2)_n$C(=O)NH-, -C(=O)NH$(CH_2)_n$NHC(=O)$(CH_2)_n$-, -C(=O)$(CH_2)_n$-, -$(CH_2)_n$C(=O)-, -$(CH_2)_n(O(CH_2)_n)_m$NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NH$((CH_2)_nO)_m(CH_2)_n$-, or -$(O(CH_2)_n)_m$NHC(=O)$(CH_2)_n$-;

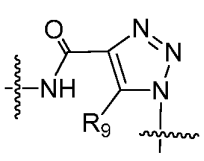
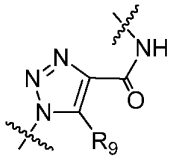
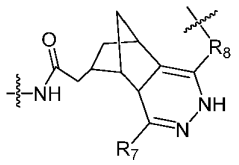


each X^2 is independently selected from a bond, R^8 ,

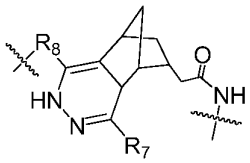
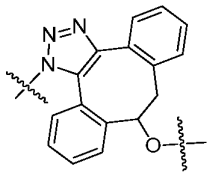
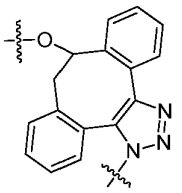

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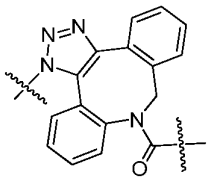
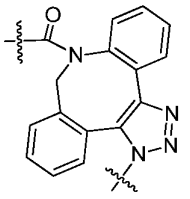


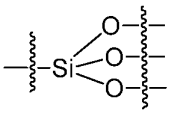
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, -S-, -Si(OH)$_2$O-,



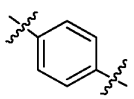
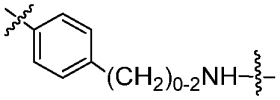
, -CHR^4(CH$_2$)$_n$C(=O)NH-, -CHR^4(CH$_2$)$_n$NHC(=O)-, -C(=O)NH- and -NHC(=O)-;

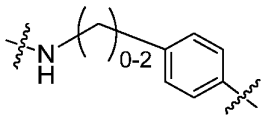
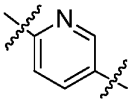
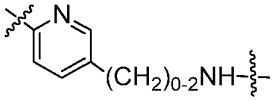
each R^4 is independently selected from H, C$_{1-4}$alkyl, side chains of known amino acids, -C(=O)OH and -OH,

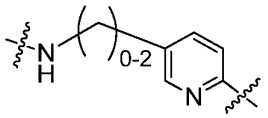
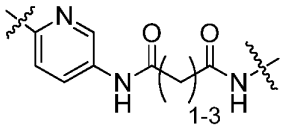
each R^5 is independently selected from H, C$_{1-4}$alkyl, phenyl or C$_{1-4}$alkyl substituted with 1 to 3 –OH groups;

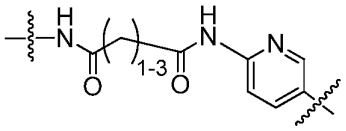
each R^6 is independently selected from H, fluoro, benzyloxy substituted with –C(=O)OH, benzyl substituted with –C(=O)OH, C$_{1-4}$alkoxy substituted with –C(=O)OH and C$_{1-4}$alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C$_{1-4}$alkyl, phenyl, pyrimidine and pyridine;

R^8 is independently selected  ,  ,

 ,  ,  ,

 ,  , and

 ;

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9;

L_2 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

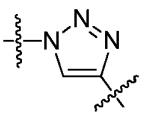
L_3 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

L_4 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker or a linker that comprises a self-immolative spacer.

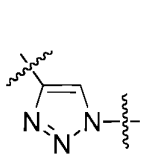
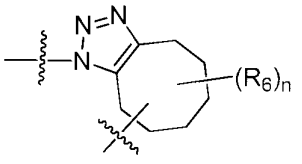
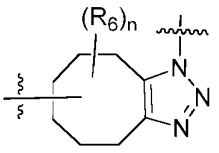
In certain embodiments the Linker Unit (LU) is $-L_1-L_2-L_3-L_4-$, wherein

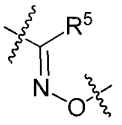
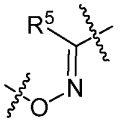
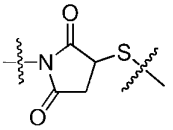
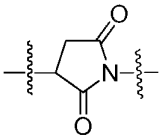
L_1 is a bond, $-A_1-$, $-A_1X^2-$ or $-X^2-$; where:

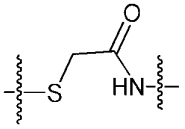
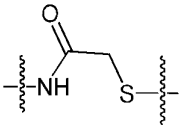
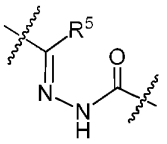
A_1 is $-C(=O)NH-$, $-C(=O)NH(CH_2)_n-$, $-C(=O)NH(CH_2)_nS-$, $-(O(CH_2)_n)_m-$, $-((CH_2)_nO)_m(CH_2)_n-$, $-NHC(=O)(CH_2)_n-$, $-C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n-$, $-(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n-$ or $-(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$;

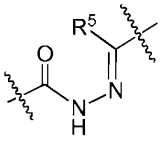
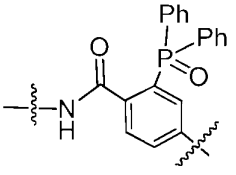
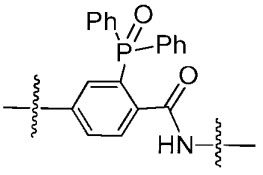


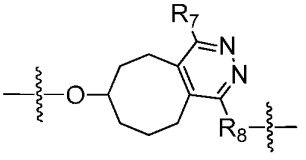
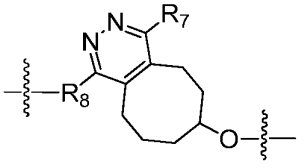
each X^2 is independently selected from a bond, R^8 ,

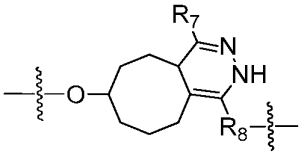
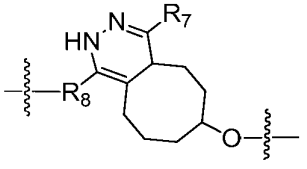

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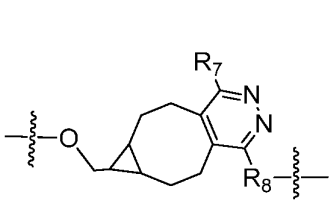
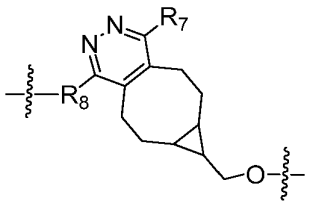

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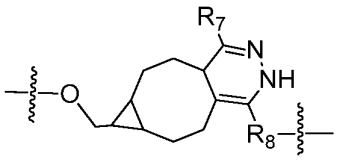
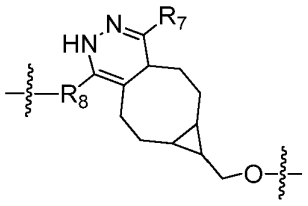

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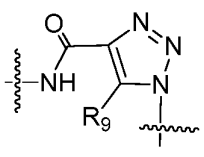
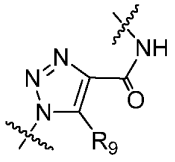
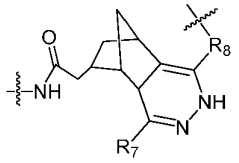

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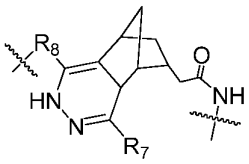
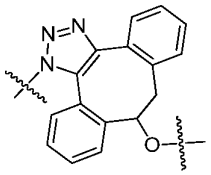
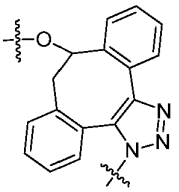

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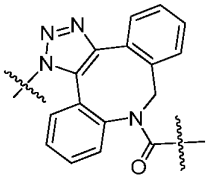
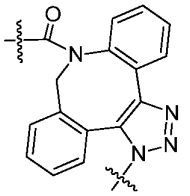

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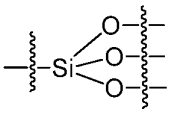


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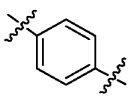
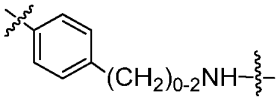
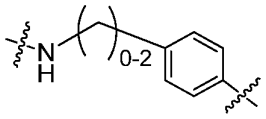
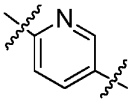
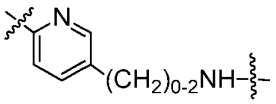
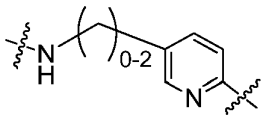
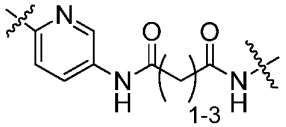
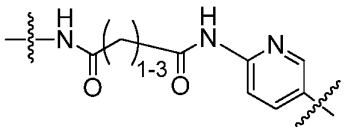
, -CHR^4(CH$_2$)$_n$C(=O)NH-, -CHR^4(CH$_2$)$_n$NHC(=O)-, -C(=O)NH- and -NHC(=O)-;

each R^4 is independently selected from H, C$_{1-4}$alkyl, side chains of known amino acids, -C(=O)OH and -OH,

each R^5 is independently selected from H, C_{1-4}alkyl, phenyl or C_{1-4}alkyl substituted with 1 to 3 –OH groups;

each R^6 is independently selected from H, fluoro, benzyloxy substituted with –C(=O)OH, benzyl substituted with –C(=O)OH, C_{1-4}alkoxy substituted with –C(=O)OH and C_{1-4}alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C_{1-4}alkyl, phenyl, pyrimidine and pyridine;

R^8 is independently selected  ,  ,  ,  ,  ,  ,  , and  ;

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9;

L_2 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker;

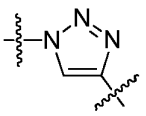
L_3 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker or a photo-cleavable linker, and

L_4 is a bond, a non-enzymatically cleavable linker, a non-cleavable linker, an enzymatically cleavable linker, a photo-stable linker, a photo-cleavable linker or a linker that comprises a self-immolative spacer.

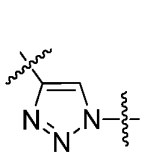
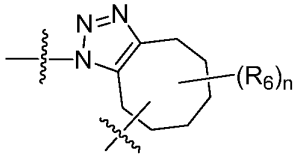
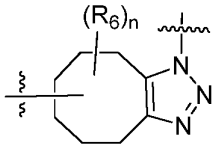
In certain embodiments the Linker Unit (LU) is $–L_1–L_2–L_3–L_4–$, wherein

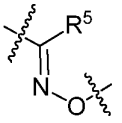
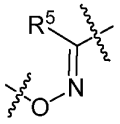
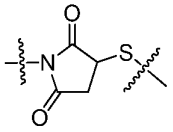
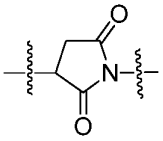
L_1 is a bond, $-A_1-$, $-A_1X^2-$ or $-X^2-$; where:

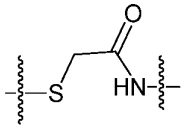
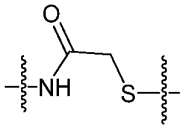
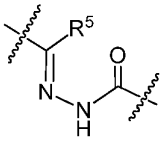
A_1 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -C(=O)NH$(CH_2)_n$S-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -NHC(=O)$(CH_2)_n$-, -C(=O)NH$(CH_2)_n$NHC(=O)$(CH_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$- or-$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-;

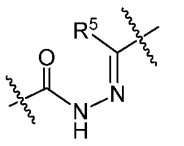
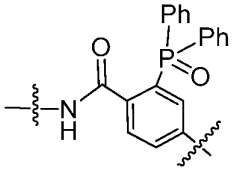
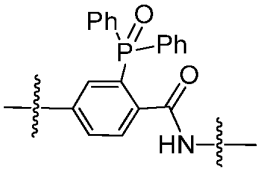


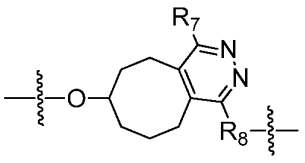
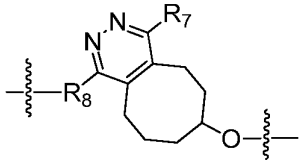
each X^2 is independently selected from a bond, R^8 ,

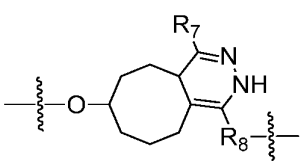
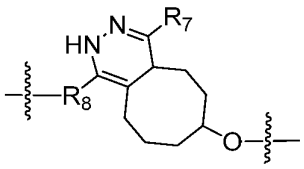

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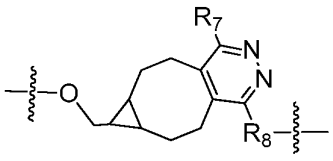
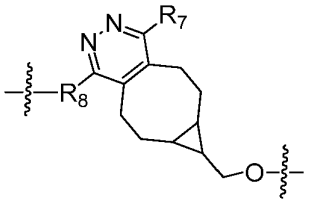

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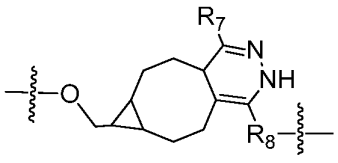
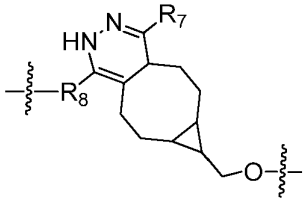

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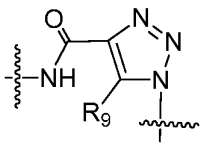
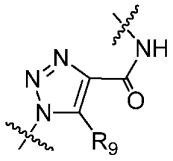
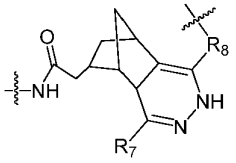

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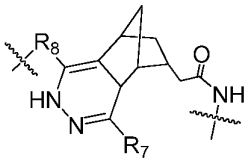
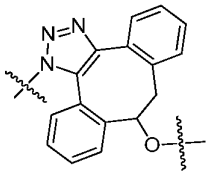
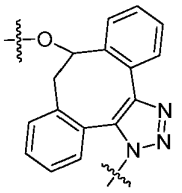

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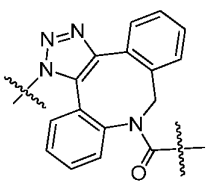
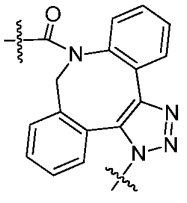


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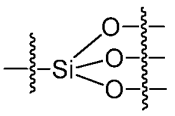


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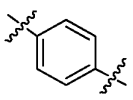
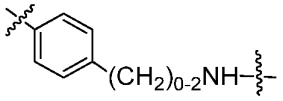
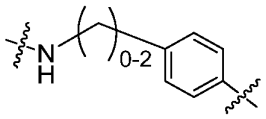
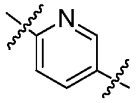
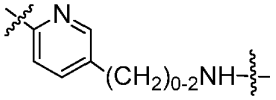
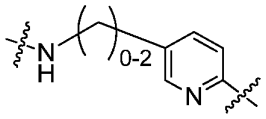
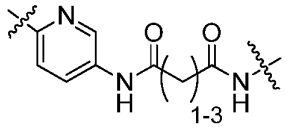
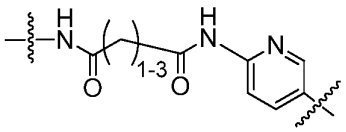
, $-CHR^4(CH_2)_nC(=O)NH-$, $-CHR^4(CH_2)_nNHC(=O)-$, -C(=O)NH- and -NHC(=O)-;

each R^4 is independently selected from H, C_{1-4}alkyl, side chains of known amino acids, -C(=O)OH and -OH,

each R^5 is independently selected from H, C_{1-4}alkyl, phenyl or C_{1-4}alkyl substituted with 1 to 3 –OH groups;

each R^6 is independently selected from H, fluoro, benzyloxy substituted with –C(=O)OH, benzyl substituted with –C(=O)OH, C_{1-4}alkoxy substituted with –C(=O)OH and C_{1-4}alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C_{1-4}alkyl, phenyl, pyrimidine and pyridine;

R^8 is independently selected from , , , , , , , and ;

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9;

L_2 is a bond, a non-enzymatically cleavable linker or a non-cleavable linker;

L_3 is a bond, a non-enzymatically cleavable linker or a non-cleavable linker;

L_4 is a bond, an enzymatically cleavable linker or a linker that comprises a self-immolative spacer.

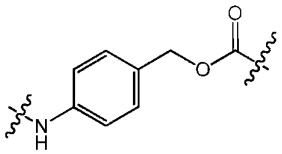
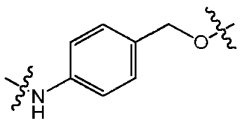
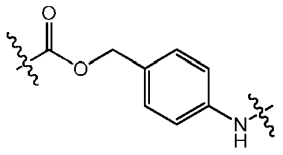
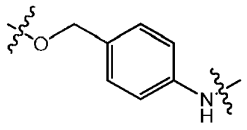
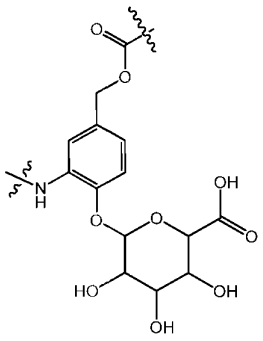
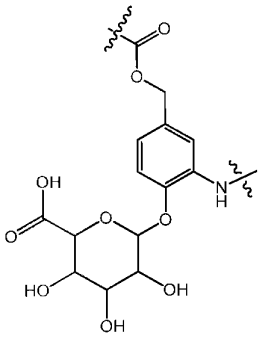
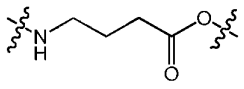
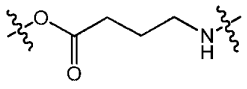
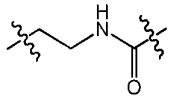
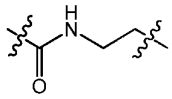
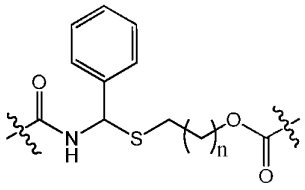
In certain embodiments the Linker Unit (LU) is $-L_1-L_2-L_3-L_4-$, wherein

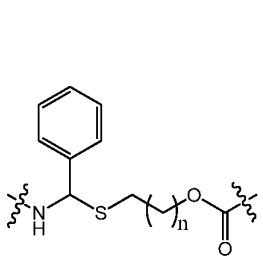
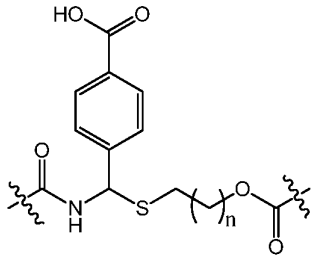
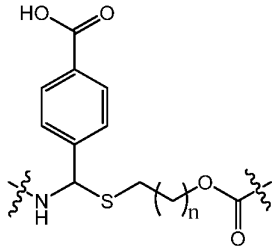
L_1 is a bond, $-A_1-$, $-A_1X^2-$ or $-X^2-$;

L_2 is a bond, $-A_2-$, or $-A_2X^2-$;

L_3 is a bond, $-A_3-$, or $-A_3X^2-$;

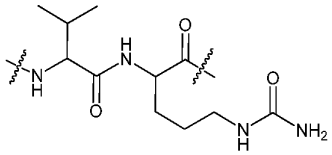
L_4 is a bond, $-A_4-$, $-A_4X^2-$,


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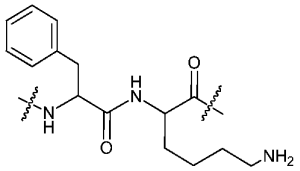

,

, or

;

A_1 is -C(=O)NH-, -NHC(=O)-, -C(=O)NH$(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_n$-, -$(O(CH_2)_n)_m$-, -$(O(C(R^4)_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((C(R^4)_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(((C(R^4)_2)_nO)_mC(R^4)_2)_n$-, -$(CH_2)_nC(=O)NH$-, -$(C(R^4)_2)_nC(=O)NH$-, -$(CH_2)_nNHC(=O)$-, -$(C(R^4)_2)_nNHC(=O)$-, -NHC(=O)$(CH_2)_n$-, -NHC(=O)$(C(R^4)_2)_n$-, -C(=O)NH$(CH_2)_nS$-, -C(=O)NH$(C(R^4)_2)_nS$-, -$S(CH_2)_nC(=O)NH$-, -$S(C(R^4)_2)_nC(=O)NH$-, -C(=O)NH$(CH_2)_nNHC(=O)(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -C(=O)$(CH_2)_n$-, -C(=O)$(C(R^4)_2)_n$-, -$(CH_2)_nC(=O)$-, -$(C(R^4)_2)_nC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_nNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$-, -$(C(R^4)_2)_nNH((C(R^4)_2)_nO)_m(C(R^4)_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, or -$(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-;

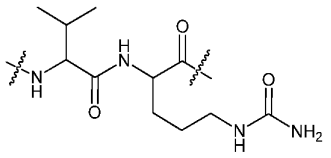
A_2 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_n$-, -$(O(CH_2)_n)_m$-, -$(O(C(R^4)_2)_n)_m$-,-$((CH_2)_nO)_m$-, -$((C(R^4)_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$((C(R^4)_2)_nO)_mC(R^4)_2)_n$-, -$(CH_2)_nC(=O)NH$-, -$(C(R^4)_2)_nC(=O)NR^4$-, -$(CH_2)_nNHC(=O)$-, -$(C(R^4)_2)_nNHC(=O)$-, -NHC(=O)$(CH_2)_n$-, -NHC(=O)$(C(R^4)_2)_n$-, -C(=O)NH$(CH_2)_nS$-, -C(=O)NH$(C(R^4)_2)_nS$-, -$S(CH_2)_nC(=O)NH$-, -$S(C(R^4)_2)_nC(=O)NH$-, -$(CH_2)_nS$-, -$(C(R^4)_2)_nS$-, -$S(CH_2)_n$-, -$S(C(R^4)_2)_n$-, -$(CH_2)_nNH$-, -$(C(R^4)_2)_nNH$-, -C(=O)NH$(CH_2)_nNHC(=O)(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -C(=O)$(CH_2)_n$-, -C(=O)$(C(R^4)_2)_n$-, -$(CH_2)_nC(=O)$-, -$(C(R^4)_2)_nC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_n(O(CH_2)_n)_mOC(=O)NH(CH_2)_n$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mOC(=O)NH(C(R^4)_2)_n$-, -$(CH_2)_nNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$-, -$(C(R^4)_2)_nNH((C(R^4)_2)_nO)_m(C(R^4)_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -



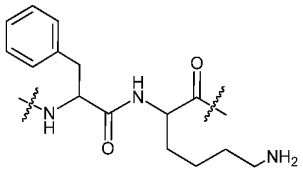
$(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-, or;



A_3 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_n$-, -O$(CH_2)_n)_m$-, -O$(C(R^4)_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((C(R^4)_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(((C(R^4)_2)_nO)_mC(R^4)_2)_n$-, -$(CH_2)_nC(=O)NH$-, -$(C(R^4)_2)_nC(=O)NH$-, -$(CH_2)_nNHC(=O)$-, -$(C(R^4)_2)_nNHC(=O)$-, -$NHC(=O)(CH_2)_n$-, -$NHC(=O)(C(R^4)_2)_n$-, -C(=O)NH$(CH_2)_nS$-, -C(=O)NH$(C(R^4)_2)_nS$-, -$S(CH_2)_nC(=O)NH$-, -$S(C(R^4)_2)_nC(=O)NH$-, -$(CH_2)_nS$-, -$(C(R^4)_2)_nS$-, -$S(CH_2)_n$-, -$S(C(R^4)_2)_n$-, -C(=O)NH$(CH_2)_nNHC(=O)(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$C(=O)(CH_2)_n$-, -$C(=O)(C(R^4)_2)_n$-, -$(CH_2)_nC(=O)$-, -$(C(R^4)_2)_nC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-,-$(CH_2)_n(O(CH_2)_n)_mOC(=O)NH(CH_2)_n$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mOC(=O)NH(C(R^4)_2)_n$-, -$(CH_2)_n(O(CH_2)_n)_mOC(=O)$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mOC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mC(=O)$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mC(=O)$-, -$(CH_2)_nNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-,



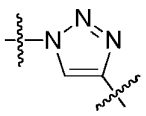
or



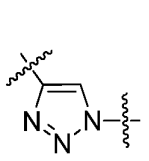
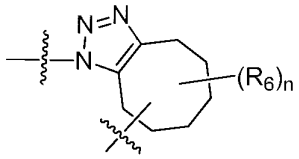
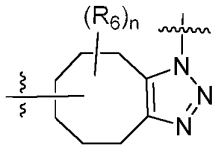
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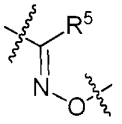
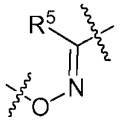
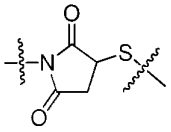
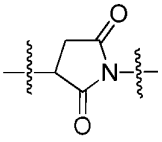
A_4 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_n$-, -O$(CH_2)_n)_m$-, -O$(C(R^4)_2)_n)_m$-,-$((CH_2)_nO)_m$-, -$((C(R^4)_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(((C(R^4)_2)_nO)_mC(R^4)_2)_n$-, -$(CH_2)_nC(=O)NH$-, -$(C(R^4)_2)_nC(=O)NH$-, -$(CH_2)_nNHC(=O)$-, -$(C(R^4)_2)_nNHC(=O)$-, -$NHC(=O)(CH_2)_n$-, -$NHC(=O)(C(R^4)_2)_n$-, -C(=O)NH$(CH_2)_nS$-, -C(=O)NH$(C(R^4)_2)_nS$-, -$S(CH_2)_nC(=O)NH$-, -$S(C(R^4)_2)_nC(=O)NH$-, -C(=O)NH$(CH_2)_nNHC(=O)(CH_2)_n$-, -C(=O)NH$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$C(=O)(CH_2)_n$-, -$C(=O)(C(R^4)_2)_n$-, -$(CH_2)_nC(=O)$-, -$(C(R^4)_2)_nC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_n(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_nNHC(=O)(CH_2)_n$-, -$(C(R^4)_2)_nNHC(=O)(C(R^4)_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$-, -

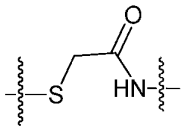
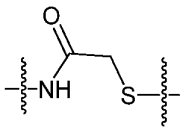
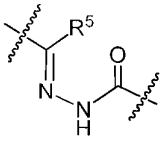
$(C(R^4)_2)_nNH((C(R^4)_2)_nO)_m(C(R^4)_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, or -$(O(C(R^4)_2)_n)_mNHC(=O)(C(R^4)_2)_n$-;

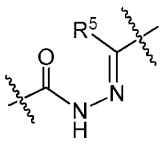
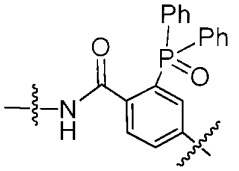
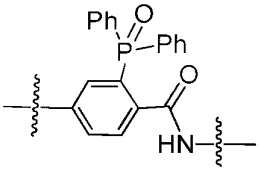


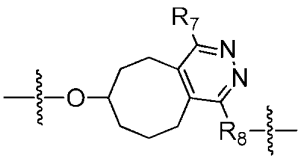
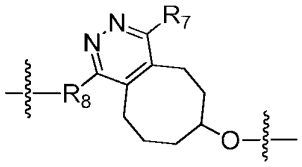
each X^2 is independently selected from a bond, R^8

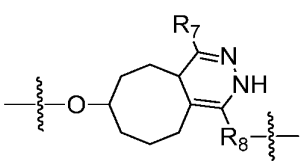
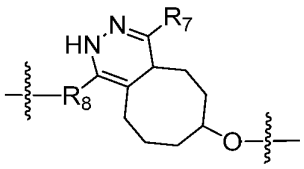

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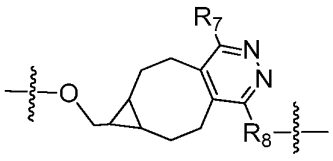
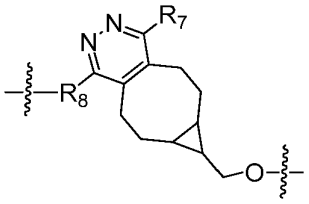

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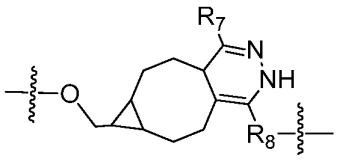
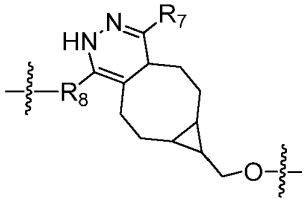

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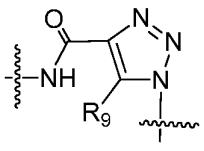
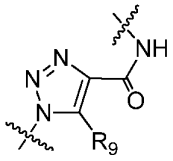
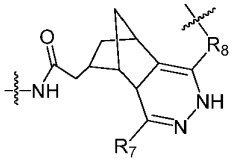

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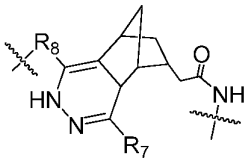
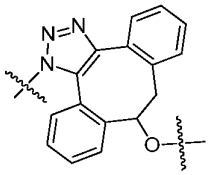
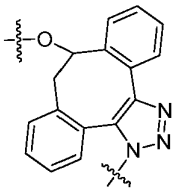

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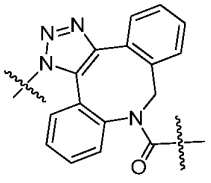
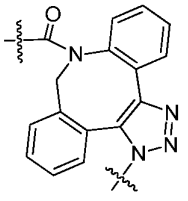


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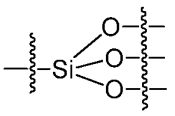


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, -S-, -Si(OH)$_2$O-,



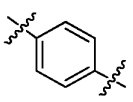
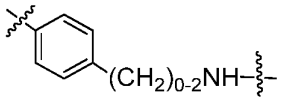
, $-CHR^4(CH_2)_nC(=O)NH-$, $-CHR^4(CH_2)_nNHC(=O)-$, -C(=O)NH- and -NHC(=O)-;

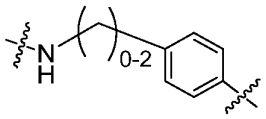
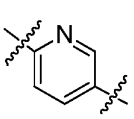
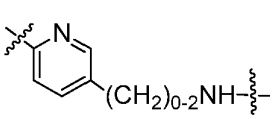
each R^4 is independently selected from H, C_{1-4}alkyl, side chains of known amino acids, -C(=O)OH and -OH,

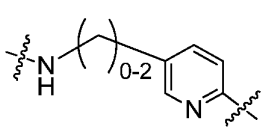
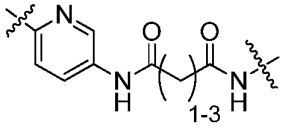
each R^5 is independently selected from H, C_{1-4}alkyl, phenyl or C_{1-4}alkyl substituted with 1 to 3 –OH groups;

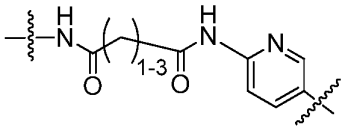
each R^6 is independently selected from H, fluoro, benzyloxy substituted with –C(=O)OH, benzyl substituted with –C(=O)OH, C_{1-4}alkoxy substituted with –C(=O)OH and C_{1-4}alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C_{1-4}alkyl, phenyl, pyrimidine and pyridine;

R^8 is independently selected from , ,

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;

R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

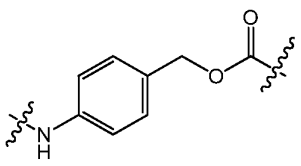
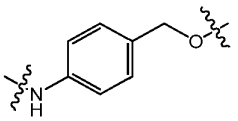
each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9.

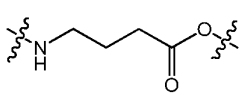
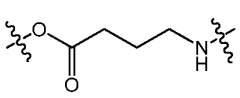
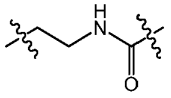
In certain embodiments the Linker Unit (LU) is $–L_1–L_2–L_3–L_4–$, wherein

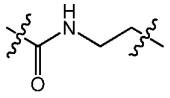
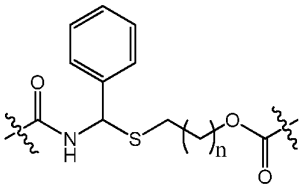
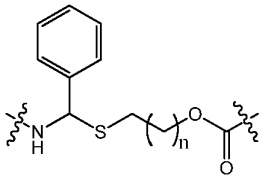
L_1 is a bond, $-A_1-$, $-A_1X^2-$ or $-X^2-$;

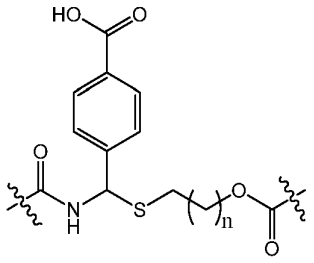
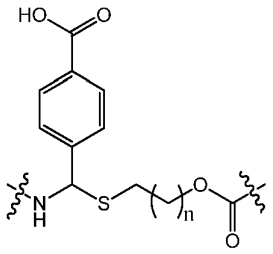
L_2 is a bond, $-A_2-$, or $-A_2X^2-$;

L_3 is a bond, -A_3-, or -A_3X^2-;

L_4 is a bond, -A_4-, -A_4X^2-,  ,  ,

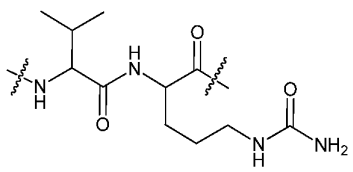
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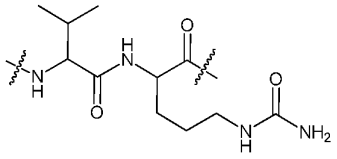
 , or  ;

A_1 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(CH_2)_n$C(=O)NH-, -NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NHC(=O)-, -C(=O)NH$(CH_2)_n$S-, -S$(CH_2)_n$C(=O)NH-, -C(=O)NH$(CH_2)_n$NHC(=O)$(CH_2)_n$-, -C(=O)$(CH_2)_n$-, -$(CH_2)_n$C(=O)-, -$(CH_2)_n(O(CH_2)_n)_m$NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NH$((CH_2)_nO)_m(CH_2)_n$- or -$(O(CH_2)_n)_m$NHC(=O)$(CH_2)_n$-;

A_2 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(CH_2)_n$C(=O)NH-, -NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NHC(=O)-, -C(=O)NH$(CH_2)_n$S-, -S$(CH_2)_n$C(=O)NH-, -C(=O)NH$(CH_2)_n$NHC(=O)$(CH_2)_n$-, -C(=O)$(CH_2)_n$-, -$(CH_2)_n$C(=O)-, -$(CH_2)_n(O(CH_2)_n)_m$NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NHC(=O)$(CH_2)_n$-, -$(CH_2)_n$NH$((CH_2)_nO)_m(CH_2)_n$-, -$(O(CH_2)_n)_m$NHC(=O)$(CH_2)_n$- or

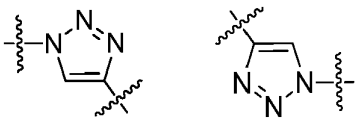
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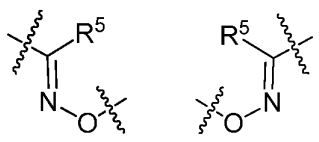
A_3 is -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(CH_2)_nC(=O)NH$-, -NHC(=O)$(CH_2)_n$-, -$(CH_2)_nNHC(=O)$-, -C(=O)NH$(CH_2)_nS$-, -$S(CH_2)_nC(=O)NH$-, -C(=O)NH$(CH_2)_nNHC(=O)(CH_2)_n$-, -C(=O)$(CH_2)_n$-, -$(CH_2)_nC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(CH_2)_nNHC(=O)(CH_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$- or

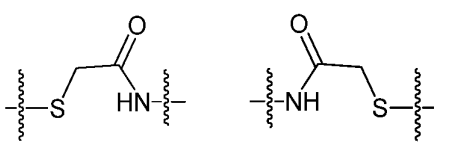


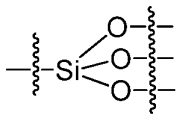
;

A_4 -C(=O)NH-, -C(=O)NH$(CH_2)_n$-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$(CH_2)_nC(=O)NH$-, -NHC(=O)$(CH_2)_n$-, -$(CH_2)_nNHC(=O)$-, -C(=O)NH$(CH_2)_nS$-, -$S(CH_2)_nC(=O)NH$-, -C(=O)NH$(CH_2)_nNHC(=O)(CH_2)_n$-, -C(=O)$(CH_2)_n$-, -$(CH_2)_nC(=O)$-, -$(CH_2)_n(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-, -$(CH_2)_nNHC(=O)(CH_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$- or -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-;

each X^2 is independently selected from a bond,  , , ,

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S-, -$Si(OH)_2O$-, , -$CHR^4(CH_2)_nC(=O)NH$-, -$CHR^4(CH_2)_nNHC(=O)$-, -C(=O)NH- and -NHC(=O)-;

each R^4 is independently selected from H, C_{1-4}alkyl, side chains of known amino acids, -C(=O)OH and -OH,

each R^5 is independently selected from H, C_{1-4}alkyl, phenyl or C_{1-4}alkyl substituted with 1 to 3 – OH groups;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

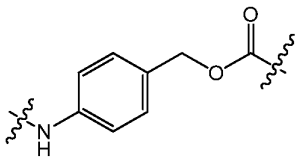
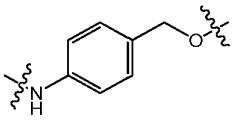
each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9.

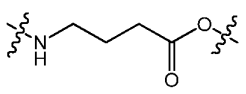
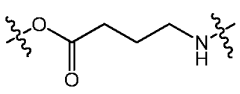
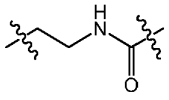
In certain embodiments the Linker Unit (LU) is $–L_1–L_2–L_3–L_4–$, wherein

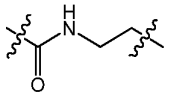
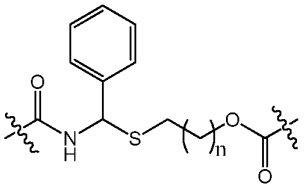
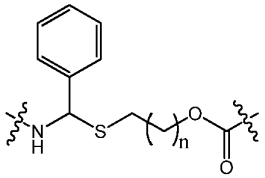
L_1 is a bond, $-A_1-$, $-A_1X^2-$ or $-X^2-$;

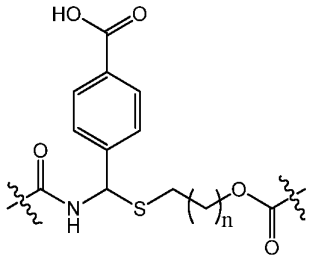
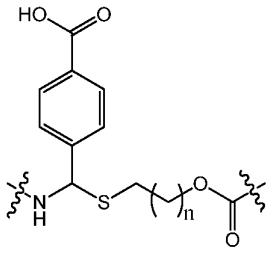
L_2 is a bond, $-A_2-$, or $-A_2X^2-$;

L_3 is a bond, $-A_3-$, or $-A_3X^2-$;

L_4 is a bond, $-A_4-$, $-A_4X^2-$,  ,  ,

 ,  ,  ,

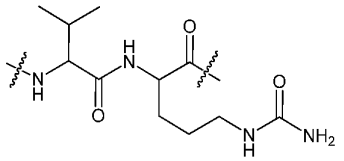
 ,  ,  ,

  , or ;

A_1 is $-C(=O)NH-$, $-C(=O)NH(CH_2)_n-$, $-C(=O)NH(CH_2)_nS-$, $-(O(CH_2)_n)_m-$, $-((CH_2)_nO)_m(CH_2)_n-$, $-NHC(=O)(CH_2)_n-$, $-(CH_2)_nNHC(=O)-$, $-C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n-$, $-(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n-$ or $-(O(CH_2)_n)_mNHC(=O)(CH_2)_n-$;

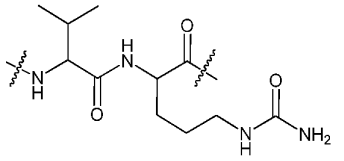
A_2 is $-C(=O)NH-$, $-C(=O)NH(CH_2)_n-$, $-C(=O)NH(CH_2)_nS-$, $-(O(CH_2)_n)_m-$,$-((CH_2)_nO)_m(CH_2)_n-$, $-NHC(=O)(CH_2)_n-$, $-(CH_2)_nNHC(=O)-$, $-C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n-$, -

$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$- or



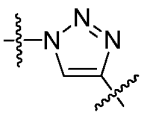
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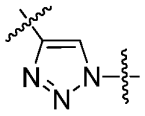
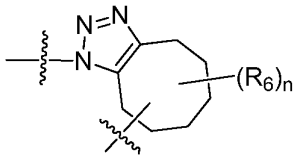
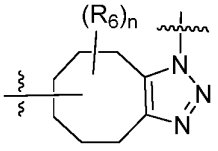
A_3 is -C(=O)NH-, -$C(=O)NH(CH_2)_n$-, -$C(=O)NH(CH_2)_nS$-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$NHC(=O)(CH_2)_n$-, -$(CH_2)_nNHC(=O)$-, -$C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$-, -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$- or

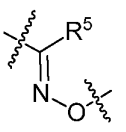
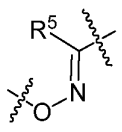
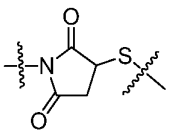
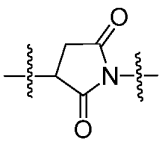


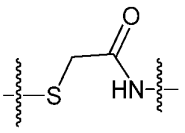
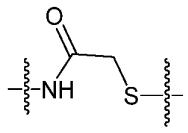
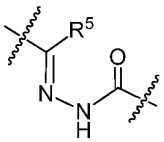
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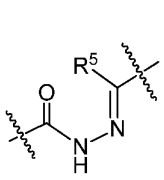
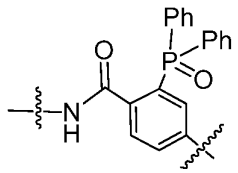
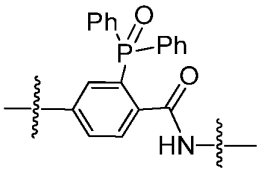
A_4 is -C(=O)NH-, -$C(=O)NH(CH_2)_n$-, -$C(=O)NH(CH_2)_nS$-, -$(O(CH_2)_n)_m$-, -$((CH_2)_nO)_m(CH_2)_n$-, -$NHC(=O)(CH_2)_n$-, -$(CH_2)_nNHC(=O)$-, -$C(=O)NH(CH_2)_nNHC(=O)(CH_2)_n$-, -$(CH_2)_nNH((CH_2)_nO)_m(CH_2)_n$- or -$(O(CH_2)_n)_mNHC(=O)(CH_2)_n$-;

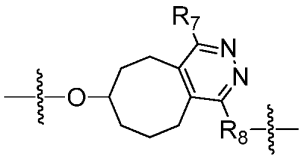
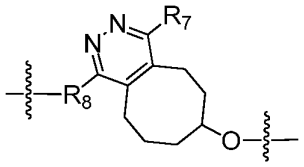
each X^2 is independently selected from a bond, R^8 ,

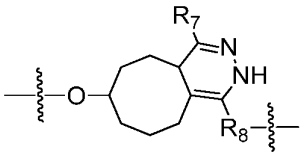
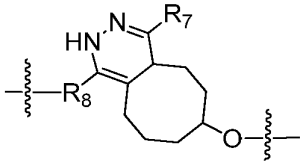
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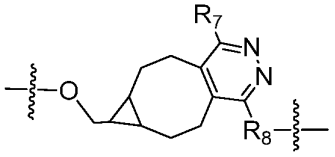
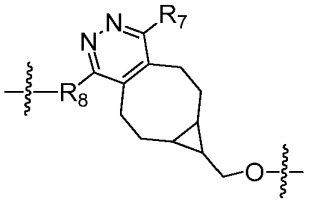
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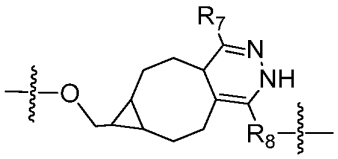
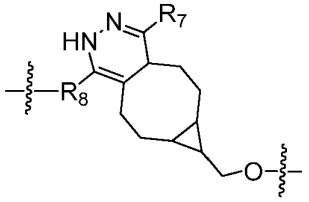
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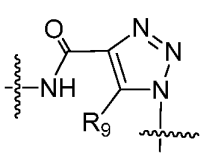
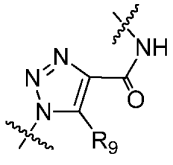
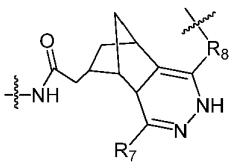




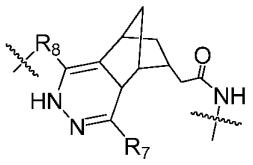
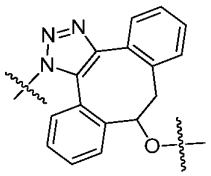
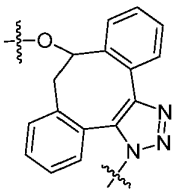



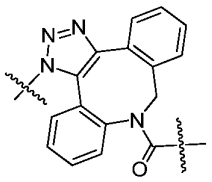
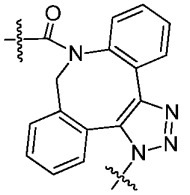



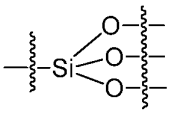






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, -S-, -Si(OH)$_2$O-,

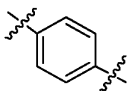
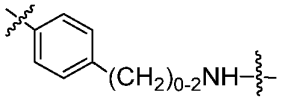

, -CHR^4(CH$_2$)$_n$C(=O)NH-, -CHR^4(CH$_2$)$_n$NHC(=O)-, -C(=O)NH- and -NHC(=O)-;

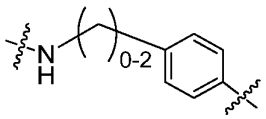
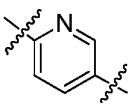
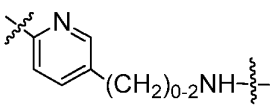
each R^4 is independently selected from H, C$_{1-4}$alkyl, side chains of known amino acids, -C(=O)OH and -OH,

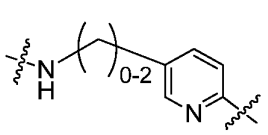
each R^5 is independently selected from H, C$_{1-4}$alkyl, phenyl or C$_{1-4}$alkyl substituted with 1 to 3 –OH groups;

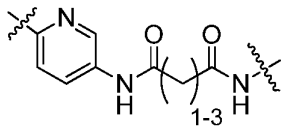
each R^6 is independently selected from H, fluoro, benzyloxy substituted with –C(=O)OH, benzyl substituted with –C(=O)OH, C$_{1-4}$alkoxy substituted with –C(=O)OH and C$_{1-4}$alkyl substituted with –C(=O)OH;

R^7 is independently selected from H, C$_{1-4}$alkyl, phenyl, pyrimidine and pyridine;

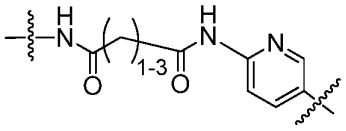
R^8 is independently selected from 
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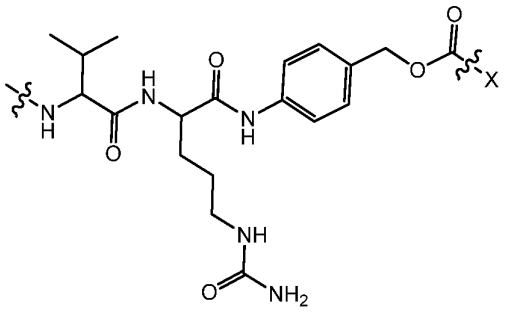
R^9 is independently selected from H and C_{1-6}haloalkyl;

each n is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9, and

each m is independently selected from 1, 2, 3, 4, 5, 6, 7, 8 and 9.

In one embodiment, L_1 is $-(CH_2)_{1-10}$-C(=O)- , e.g., $-(CH_2)_5$-C(=O)-; and L_2, L_3 and L_4 each represent a bond.

In certain embodiments LU comprises a val-cit linker of this formula, wherein X represents a payload, typically a drug moiety such as one having anticancer activity:



.

When L_4-L_5-L_6 is a val-cit linker as shown above, L_3 is preferably $-(CH_2)_{2-6}$-C(=O)-.

In certain embodiments the X group is a maytansinoid such as DM1 or DM4, or a dolastatin analog or derivative such as dolastatin 10 or 15 and auristatins MMAF or MMAE, or a calicheamicin such as N-acetyl-γ-calicheamicin, or a label or dye such as rhodamine or tetramethylrhodamine.

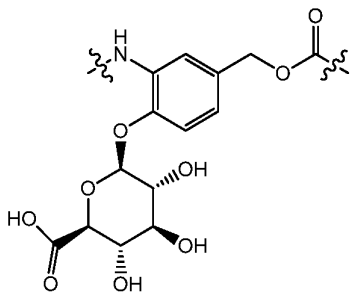
As used herein, a "linker" is any chemical moiety that is capable of connecting an antibody or a fragment thereof to an X group (payload) to form an immunoconjugate. Linkers can be susceptible to cleavage, such as, acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage, at conditions under which the compound or the antibody remains active. Alternatively, linkers can

be substantially resistant to cleavage. A linker may or may not include a self-immolative spacer.

Non-limiting examples of the non-enzymatically cleavable linkers as used herein to conjugate an X^1 group to the modified antibodies or antibody fragment thereof provided herein include, acid-labile linkers, linkers containing a disulfide moiety, linkers containing a triazole moiety, linkers containing a hydrazone moiety, linkers containing a thioether moiety, linkers containing a diazo moiety, linkers containing an oxime moiety, linkers containing an amide moiety and linkers containing an acetamide moiety.

Non-limiting examples of the enzymatically cleavable linkers as used herein to conjugate an X group to the modified antibodies or antibody fragment thereof provided herein include, but are not limited to, linkers that are cleaved by a protease, linkers that are cleaved by an amidase, and linkers that are cleaved by -glucuronidase or another glycosidase.

In certain embodiments, such enzyme cleavable linkers are linkers which are cleaved by cathepsin, including cathepsin Z, cathepsin B, cathepsin H and cathepsin C. In certain embodiments the enzymatically cleavable linker is a dipeptide cleaved by cathepsin, including dipeptides cleaved by cathepsin Z, cathepsin B, cathepsin H or cathepsin C. In certain embodiments the enzymatically cleavable linker is a cathepsin B-cleavable peptide linker. In certain embodiments the enzymatically cleavable linker is a cathepsin B-cleavable dipeptide linker. In certain embodiments the enzymatically cleavable dipeptide linker is valine-citrulline or phenylalanine-lysine. Other non-limiting examples of the enzymatically cleavable linkers as used herein conjugate an X group to the modified antibodies or antibody fragment thereof provided herein include, but are not limited to, linkers which are cleaved



by -glucuronidase, e.g., .

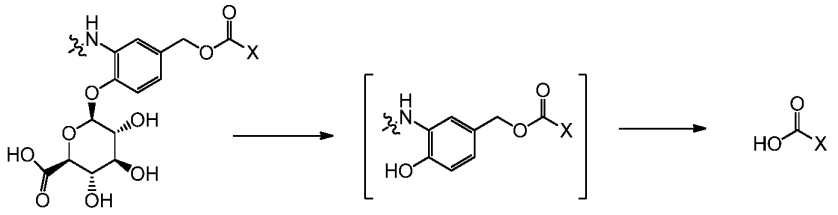
See Ducry *et al*, Bioconjugate Chem, (2010) vol. 21(1), 5-13.

"Self-immolative spacers" are bifunctional chemical moieties covalently linked at one terminus to a first chemical moiety and at the other terminus to a second chemical moiety, thereby forming a stable tripartate molecule. A linker can comprise a self-immolative spacer

bonded to a third chemical moiety that is cleavable from the spacer either chemically or enzymatically. Upon cleavage of a bond between the self-immolative spacer and the first chemical moiety or the third chemical moiety, self-immolative spacers undergo rapid and spontaneous intramolecular reactions and thereby separate from the second chemical moiety. These intramolecular reactions generally involve electronic rearrangements such as 1,4, or 1,6, or 1,8 elimination reactions or cyclizations to form highly favored five- or six-membered rings. In certain embodiments of the present invention, the first or third moiety is an enzyme cleavable group, and this cleavage results from an enzymatic reaction, while in other embodiments the first or third moiety is an acid labile group and this cleavage occurs due to a change in pH. As applied to the present invention, the second moiety is the "Payload" group as defined herein. In certain embodiments, cleavage of the first or third moiety from the self-immolative spacer results from cleavage by a proteolytic enzyme, while in other embodiments it results from cleaved by a hydrolase. In certain embodiments, cleavage of the first or third moiety from the self-immolative spacer results from cleavage by a cathepsin enzyme or a glucuronidase.

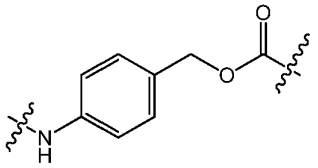
In certain embodiments, the enzyme cleavable linker is a peptide linker and the self-immolative spacer is covalently linked at one of its ends to the peptide linker and covalently linked at its other end to a drug moiety. This tripartite molecule is stable and pharmacologically inactive in the absence of an enzyme, but which is enzymatically cleavable by enzyme at a bond covalently linking the spacer moiety and the peptide moiety. The peptide moiety is cleaved from the tripartate molecule which initiates the self-immolating character of the spacer moiety, resulting in spontaneous cleavage of the bond covalently linking the spacer moiety to the drug moiety, to thereby effect release of the drug in pharmacologically active form.

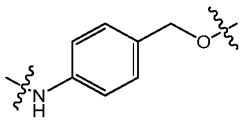
In other embodiments, a linker comprises a self-immolative spacer that connects to the peptide, either directly or indirectly at one end, and to a payload at the other end; and the spacer is attached to a third moiety that can be cleaved from the spacer enzymatically, such as by a glucuronidase. Upon cleavage of the third moiety, the spacer degrades or rearranges in a way that causes the payload to be released. An example of a linker with this type of self-immolative spacer is this glucuronidase-cleavable linker, where hydrolysis of the acetal catalyzed by glucoronidase releases a phenolic compound that spontaneously decomposes under physiological conditions:

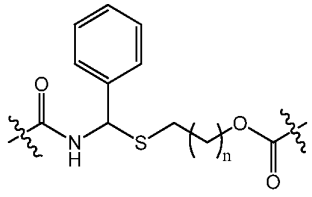
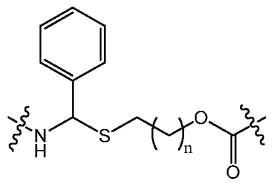


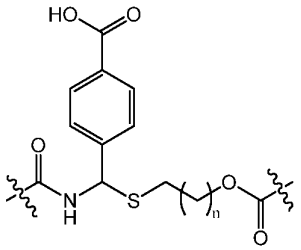
Non-limiting examples of the self-immolative spacer optionally used in the conjugation of an X^1 group to the modified antibodies or antibody fragment thereof provided herein include, but are not limited to, moieties which include a benzyl carbonyl moiety, a benzyl ether moiety, a 4-aminobutyrate moiety, a hemithioaminal moiety or a N-acylhemithioaminal moiety.

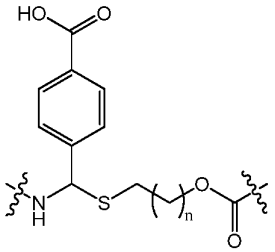
Other examples of self-immolative spacers include, but are not limited to, p-aminobenzyloxycarbonyl groups, aromatic compounds that are electronically similar to the p-aminobenzyloxycarbonyl group, such as 2-aminoimidazol-5-methanol derivatives and ortho or para-aminobenzylacetals. In certain embodiments, self-immolative spacers used herein which undergo cyclization upon amide bond hydrolysis, include substituted and unsubstituted 4-aminobutyric acid amides and 2-aminophenylpropionic acid amides.

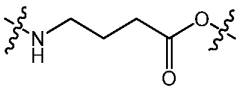
In certain embodiments, the self-immolative spacer is 

or , while in other embodiments the self-immolative spacer is

, where n is 1 or 2. In other embodiments the self-immolative spacer is , where n is 1 or 2. In other embodiments the self-

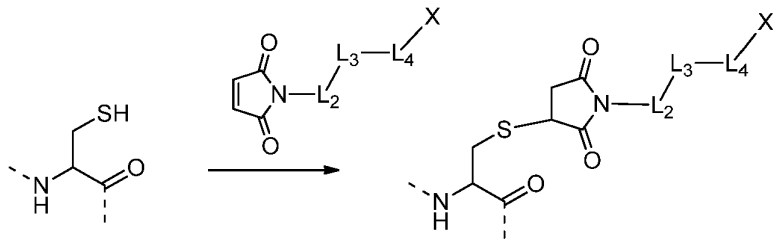
immolative spacer is  , where n is 1 or 2. In other embodiments

the self-immolative spacer is  , where n is 1 or 2. In other

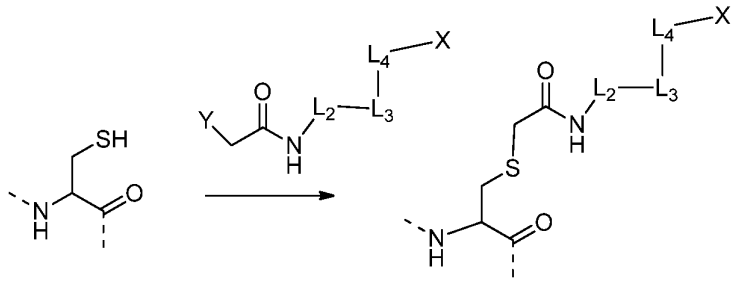
embodiments the self-immolative spacer is  , where n is 1 or 2.

Schemes (2a-2c) illustrate the post-translational modification of the modified antibodies or antibody fragment thereof provided herein wherein the Linker Unit (LU) is $-L_1-L_2-L_3-L_4-$, and L1 in each case is the group that reacts with the new Cys.

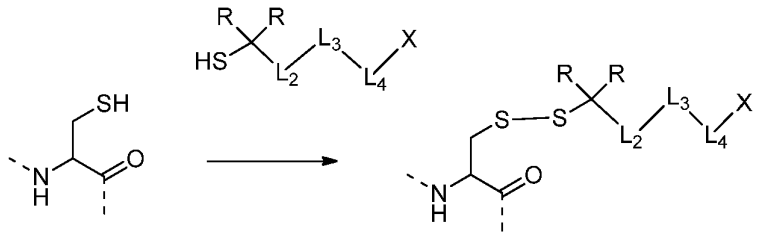
Scheme 2a.



Scheme 2b.



Scheme 3c.



In each of Schemes 2a-2c, the starting material is the replacement Cys residue in an antibody or antibody fragment modified as described herein, where the dashed bonds indicate connection to adjoining residues of the antibody or antibody fragment; each R is H or C_{1-4} alkyl, typically H or methyl; L_2, L_3 and L_4 are components of the linking unit LU, such as those described above; X is the payload; and the group connecting L_2 to the sulfur of the substitute Cys of the invention is L_1.

In some embodiments of the invention, X is a reactive functional group that can be used to connect the conjugated antibody to another chemical moiety, by interacting with a suitable complementary functional group. Table 4 depicts some examples of reactive functional groups that X can represent, along with a complementary functional group that can be used to connect a conjugate comprising X to another compound. Methods for using X to connect to the corresponding complementary functional group are well known in the art. Connections using azide are typically done using 'Click' or copper-free click chemistry; reactions involving hydrazines, alkoxyamines or acyl hydrazines typically proceed through the formation of a Schiff base with one of the carbonyl functional groups.

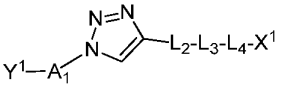
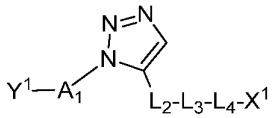
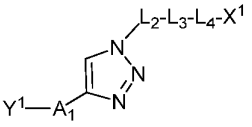
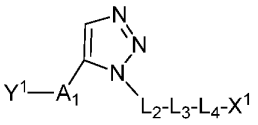
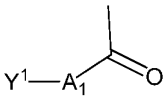
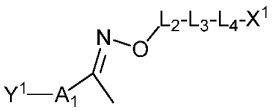
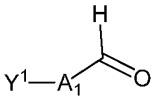
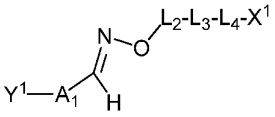
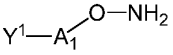
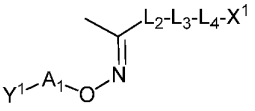
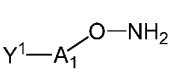
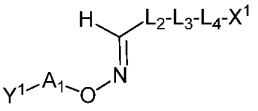
Table 4

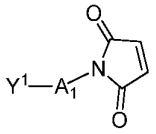
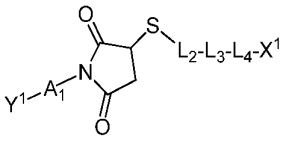
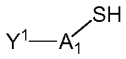
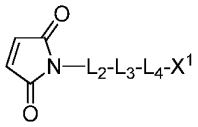
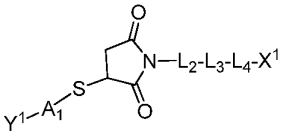
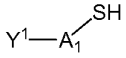
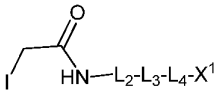
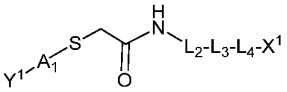
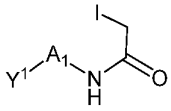
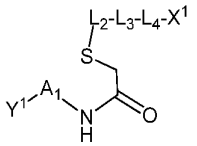
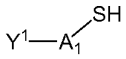
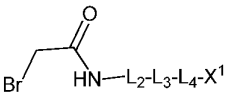
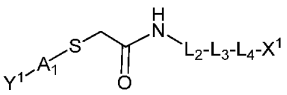
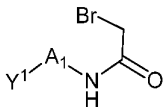
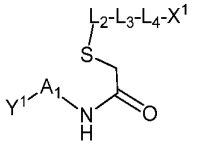
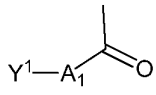
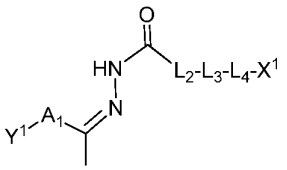
X	Complementary Reactive Functional Group for X

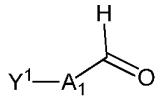
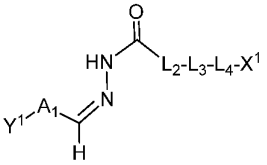
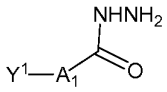
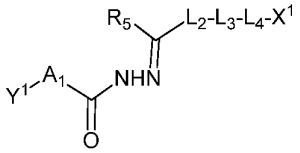
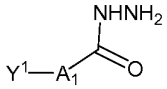
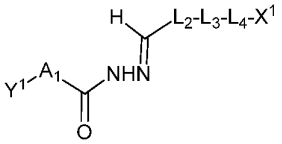
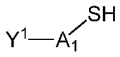
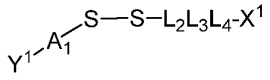
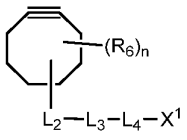
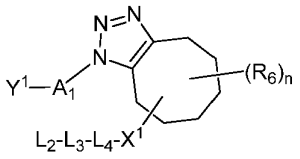
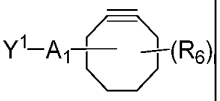
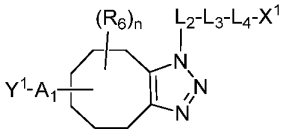
X	Complementary Reactive Functional Group for X
a thiol	a thiol, a maleimide, a haloacetamide, a vinyl sulfone, or a vinylpyridine
an azide	an alkene, alkyne, a phosphine-(thio)ester, a cyclooctyne, a cyclooctene or an oxanobornadiene
a phosphine-(thio)ester)	an azide
an oxanobornadiene	an azide or a tetrazine
an alkyne	an azide or a tetrazine
an alkene	a tetrazine
a cyclooctyne	an azide or a tetrazine
a cyclooctene	a tetrazine
a norbornene	a tetrazine
a tetrazine	a norbornene, an alkene, alkyne, a cyclooctyne or an oxanobornadiene
an aldehyde	a hydroxylamine, a hydrazine or NH_2-NH-C(=O)-
a ketone	a hydroxylamine, a hydrazine or NH_2-NH-C(=O)-
a hydroxylamine	an aldehyde or a ketone
a hydrazine	an aldehyde or a ketone
NH_2-NH-C(=O)-	an aldehyde or a ketone
a haloacetamide	a thiol
a thiol	a thiol
a maleimide	a thiol
a vinyl sulfone	a thiol
a vinylpyridine	a thiol

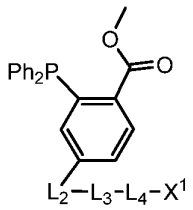
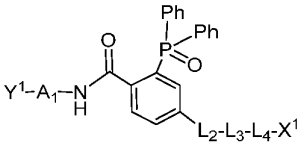
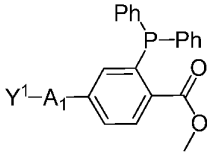
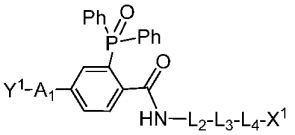
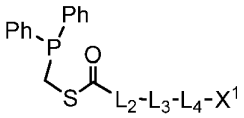
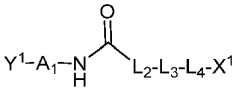
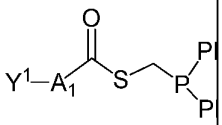
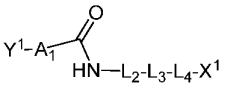
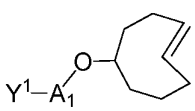
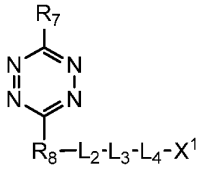
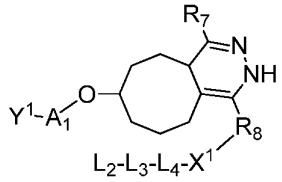
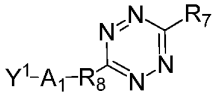
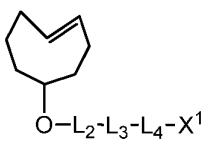
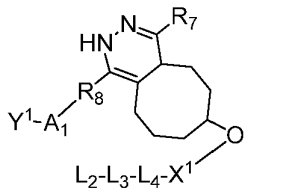
Exemplary products of the connections made using these components are depicted in Table 5, where Y^1 represents an antibody of the invention, A_1 represents a linking unit (LU) connecting the antibody to payload X^a, $-L_2-L_3-L_4-$ in Formula II-a represents a linker unit that can be present in a molecule to be connected to the conjugated antibody via X^a, and X^1 represents a payload. Payload X^a is a reactive functional group, and X^b on Formula II-a is the corresponding complementary functional group, and Formula II-a itself represents a molecule to be connected to the conjugated antibody. The third column in Table 5 depicts a product from reaction of X^a with X^b.

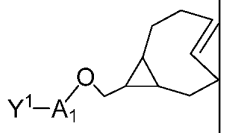
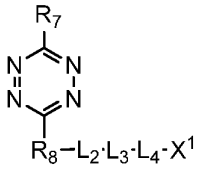
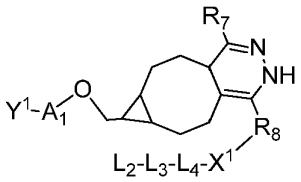
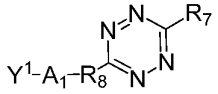
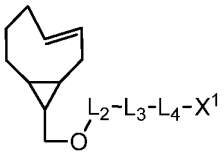
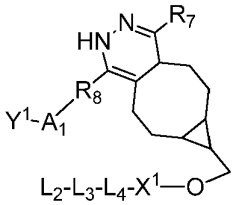
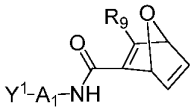
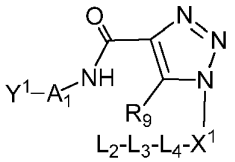
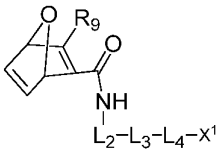
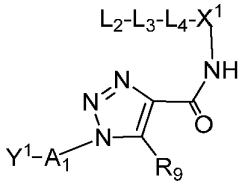
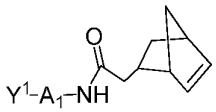
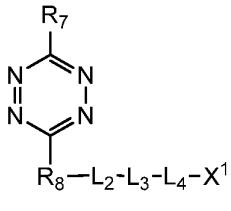
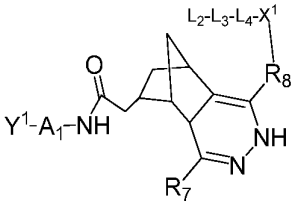
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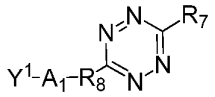
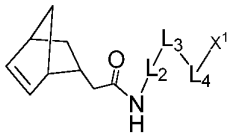
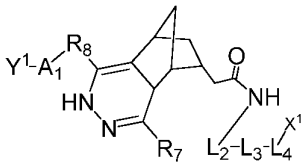
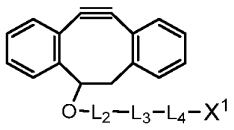
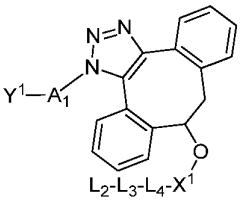
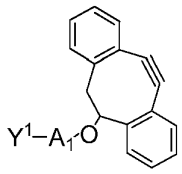
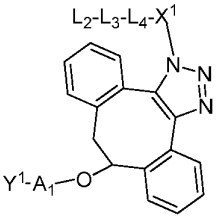
$Y^1-A_1-X^a$	X^b-L_2-L_3-L_4-X^1 Formula (II-a)	$Y^1-A_1-X^2-L_2-L_3-L_4-X^1$
$Y^1-A_1-N_3$	$HC\equiv C-L_2-L_3-L_4-X^1$	
$Y^1-A_1-N_3$	$HC\equiv C-L_2-L_3-L_4-X^1$	
$Y^1-A_1-C\equiv CH$	N_3-L_2-L_3-L_4-X^1	
$Y^1-A_1-C\equiv CH$	N_3-L_2-L_3-L_4-X^1	
	NH_2-O-L_2-L_3-L_4-X^1	
	NH_2-O-L_2-L_3-L_4-X^1	
	$CH_3C(=O)$-L_2-L_3-L_4-X^1	
	$HC(=O)$-L_2-L_3-L_4-X^1	

$Y^1–A_1—X^a$	X^b-L_2-L_3-L_4-X^1 Formula (II-a)	Y^1–A_1–X^2–L_2–L_3–L_4–X^1
	HS-L_2-L_3-L_4-X^1	
		
		
	HS—L_2-L_3-L_4-X^1	
		
	HS—L_2-L_3-L_4-X^1	
	NH_2-NH-C(=O)- L_2-L_3-L_4-X^1	

$Y^1-A_1-X^a$	X^b-L_2-L_3-L_4-X^1 Formula (II-a)	$Y^1-A_1-X^2-L_2-L_3-L_4-X^1$
	NH_2-NH-C(=O)-L_2-L_3-L_4-X^1	
	R_5C(=O)-L_2-L_3-L_4-X^1	
	HC(=O)-L_2-L_3-L_4-X^1	
	HS-L_2-L_3-L_4-X^1	
		
	N_3-L_2-L_3-L_4-X^1	

$Y^1-A_1-X^a$	$X^b-L_2-L_3-L_4-X^1$ Formula (II-a)	$Y^1-A_1-X^2-L_2-L_3-L_4-X^1$
$Y^1-A_1-N_3$		
	$N_3-L_2-L_3-L_4-X^1$	
$Y^1-A_1-N_3$		
	$N_3-L_2-L_3-L_4-X^1$	
		
		

$Y^1-A_1-X^a$	$X^b-L_2-L_3-L_4-X^1$ Formula (II-a)	$Y^1-A_1-X^2-L_2-L_3-L_4-X^1$
		
		
	$N_3-L_2-L_3-L_4-X^1$	
$Y^1-A_1-N_3$		
		

$Y^1-A_1-X^a$	$X^b-L_2-L_3-L_4-X^1$ Formula (II-a)	$Y^1-A_1-X^2-L_2-L_3-L_4-X^1$
		
$Y^1-A_1-N_3$		
	$N_3-L_2-L_3-L_4-X^1$	

In certain embodiments, the modified antibody or antibody fragment thereof provided herein is conjugated with an "X group-to-antibody" (payload to antibody) ratio between about 1 and 16, such as 1-12, or 1, 2, 3, 4, 5, 6, 7, or 8, wherein the modified antibody or antibody fragment thereof contains 1, 2, 3, 4, 5, 6, 7, or 8 cysteine residues incorporated at the specific sites disclosed herein. For example, an "X group-to-antibody" ratio of 4 can be achieved by incorporating two Cys residues into the heavy chain of an antibody, which will contain 4 conjugation sites, two from each heavy chain. Immunoconjugates of such antibodies will contain up to 4 payload groups, which may be alike or different and are preferably all alike. In another example, an "X group-to-antibody" ratio of 4 can be achieved by incorporating one Cys residue into the heavy chain and a second Cys residue into the light chain of an antibody resulting in 4 conjugation sites, two in the two heavy chains and two in the two light chains. A ratio 6, 8 or higher can be achieved by combinations of 3, 4 or more cysteine substitutions of the invention in heavy and light chain of the antibody. Substituting multiple cysteine groups into an antibody can lead to inappropriate disulfide formation and other problems. Thus for

loading more than 4 payload groups onto one antibody molecule, the methods of the invention can alternatively be combined with methods that do not rely upon reactions at cysteine sulfur, such as acylations at lysine, or conjugation via S6 tags or Pcl methodology.

While the payload to antibody ratio has an exact value for a specific conjugate molecule, it is understood that the value will often be an average value when used to describe a sample containing many molecules, due to some degree of inhomogeneity, typically in the conjugation step. The average loading for a sample of an immunoconjugate is referred to herein as the drug to antibody ratio, or DAR. In some embodiments, the DAR is between about 1 and about 16, and typically is about 1, 2, 3, 4, 5, 6, 7, or 8. In some embodiments, at least 50% of a sample by weight is compound having the average ratio plus or minus 2, and preferably at least 50% of the sample is a conjugate that contains the average ratio plus or minus 1. Preferred embodiments include immunoconjugates wherein the DAR is about 2 or about 8, e.g., about 2, about 4, about 6 or about 8. In some embodiments, a DAR of 'about n' means the measured value for DAR is within 10% of n (in Formula (I)).

3. Further Alteration of the Framework of Fc Region

The present invention provides site-specific labeled immunoconjugates. The immunoconjugates of the invention may comprise modified antibodies or antibody fragments thereof that further comprise modifications to framework residues within V_H and/or V_L, *e.g.* to improve the properties of the antibody. Typically such framework modifications are made to decrease the immunogenicity of the antibody. For example, one approach is to "back-mutate" one or more framework residues to the corresponding germline sequence. More specifically, an antibody that has undergone somatic mutation may contain framework residues that differ from the germline sequence from which the antibody is derived. Such residues can be identified by comparing the antibody framework sequences to the germline sequences from which the antibody is derived. To return the framework region sequences to their germline configuration, the somatic mutations can be "back-mutated" to the germline sequence by, for example, site-directed mutagenesis. Such "back-mutated" antibodies are also intended to be encompassed by the invention.

Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T-cell epitopes to thereby reduce the potential immunogenicity of the antibody. This approach is also referred to as "deimmunization" and is described in further detail in U.S. Patent Publication No. 20030153043 by Carr *et al.*

In addition or alternative to modifications made within the framework or CDR regions, antibodies of the invention may be engineered to include modifications within the Fc region,

typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody of the invention may be chemically modified (*e.g.*, one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody. Each of these embodiments is described in further detail below.

In one embodiment, the hinge region of CH1 is modified such that the number of cysteine residues in the hinge region is altered, *e.g.*, increased or decreased. This approach is described further in U.S. Patent No. 5,677,425 by Bodmer *et al.* The number of cysteine residues in the hinge region of CH1 is altered to, for example, facilitate assembly of the light and heavy chains or to increase or decrease the stability of the antibody.

In another embodiment, the Fc hinge region of an antibody is mutated to decrease the biological half-life of the antibody. More specifically, one or more amino acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody has impaired Staphylococcyl protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Patent No. 6,165,745 by Ward *et al.*

In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector functions of the antibody. For example, one or more amino acids can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand but retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in, *e.g.*, U.S. Patent Nos. 5,624,821 and 5,648,260, both by Winter *et al.*

In another embodiment, one or more amino acids selected from amino acid residues can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in, *e.g.*, U.S. Patent Nos. 6,194,551 by Idusogie *et al.*

In another embodiment, one or more amino acid residues are altered to thereby alter the ability of the antibody to fix complement. This approach is described in, *e.g.*, the PCT Publication WO 94/29351 by Bodmer *et al.* In a specific embodiment, one or more amino acids of an antibody or antibody fragment thereof of the present invention are replaced by one or more allotypic amino acid residues, such as those shown in FIG. 4 for the IgG1 subclass and the kappa isotype. Allotypic amino acid residues also include, but are not limited to, the constant

region of the heavy chain of the IgG1, IgG2, and IgG3 subclasses as well as the constant region of the light chain of the kappa isotype as described by Jefferis *et al.*, MAbs. 1:332-338 (2009).

In yet another embodiment, the Fc region is modified to increase the ability of the antibody to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to increase the affinity of the antibody for an Fcγ receptor by modifying one or more amino acids. This approach is described in, *e.g.*, the PCT Publication WO 00/42072 by Presta. Moreover, the binding sites on human IgG1 for FcγRl, FcγRII, FcγRIII and FcRn have been mapped and variants with improved binding have been described (see Shields *et al.*, J. Biol. Chem. 276:6591-6604, 2001).

In still another embodiment, the glycosylation of an antibody is modified. For example, an aglycosylated antibody can be made (*i.e.*, the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for "antigen." Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in, *e.g.*, U.S. Patent Nos. 5,714,350 and 6,350,861 by Co *et al.*

Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. For example, EP 1,176,195 by Hang *et al.* describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation. PCT Publication WO 03/035835 by Presta describes a variant CHO cell line, Lecl3 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields *et al.*, (2002) J. Biol. Chem. 277:26733-26740). PCT Publication WO 99/54342 by Umana *et al.* describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (*e.g.*, beta(1,4)-N acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies (see also Umana *et al.*, Nat. Biotech. 17:176-180, 1999).

In another embodiment, the antibody is modified to increase its biological half-life. Various approaches are possible. For example, one or more of the following mutations can be introduced: T252L, T254S, or T256F, as described in U.S. Patent No. 6,277,375 to Ward. Alternatively, to increase the biological half-life, the antibody can be altered within the CH1 or C_L region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patents 5,869,046 and 6,121,022 by Presta *et al.*

4. Antibody Conjugates

The present invention provides site-specific labeling methods, modified antibodies and antibody fragments thereof, and immunoconjugates prepared accordingly. Using the methods of the invention, a modified antibody or antibody fragments thereof can be conjugated to a label, such as a drug moiety, *e.g.*, an anti-cancer agent, an autoimmune treatment agent, an anti-inflammatory agent, an antifungal agent, an antibacterial agent, an anti-parasitic agent, an anti-viral agent, or an anesthetic agent, or an imaging reagent, such as a chelator for PET imaging, or a fluorescent label, or a MRI contrast reagent. An antibody or antibody fragments can also be conjugated using several identical or different labeling moieties combining the methods of the invention with other conjugation methods.

In certain embodiments, the immunoconjugates of the present invention comprise a drug moiety selected from a V-ATPase inhibitor, a HSP90 inhibitor, an IAP inhibitor, an mTor inhibitor, a microtubule stabilizer, a microtubule destabilizer, an auristatin, a dolastatin, a maytansinoid, a MetAP (methionine aminopeptidase), an inhibitor of nuclear export of proteins CRM1, a DPPIV inhibitor, proteasome inhibitors, an inhibitor of phosphoryl transfer reactions in mitochondria, a protein synthesis inhibitor, a kinase inhibitor, a CDK2 inhibitor, a CDK9 inhibitor, an HDAC inhibitor, a DNA damaging agent, a DNA alkylating agent, a DNA intercalator, a DNA minor groove binder, topoisomerase inhibitors, RNA synthesis inhibitors, kinesin inhibitors, inhibitors of protein-protein interactions, and a DHFR inhibitor.

Further, the modified antibodies or antibody fragments of the present invention may be conjugated to a drug moiety that modifies a given biological response. Drug moieties are not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be an immune modulator, such as an immune potentiator, a small molecule immune potentiator, a TLR agonist, a CpG oligomer, a TLR2 agonist, a TLR4 agonist, a TLR7 agonist, a TLR9 agonist, a TLR8 agonist, a T-cell epitope peptide or a like. The drug moiety may also be an oligonucleotide, a siRNA, a shRNA, a cDNA or a like. Alternatively, the drug moiety may be a protein, peptide, or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, cholera toxin, or diphtheria toxin, a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve

growth factor, platelet derived growth factor, tissue plasminogen activator, a cytokine, an apoptotic agent, an anti-angiogenic agent, or, a biological response modifier such as, for example, a lymphokine.

In one embodiment, the modified antibodies or antibody fragments of the present invention are conjugated to a drug moiety, such as a cytotoxin, a drug (*e.g.*, an immunosuppressant) or a radiotoxin. Examples of cytotoxin include but not limited to, taxanes (see, *e.g.*, International (PCT) Patent Application Nos. WO 01/38318 and PCT/US03/02675), DNA-alkylating agents (*e.g.*, CC-1065 analogs), anthracyclines, tubulysin analogs, duocarmycin analogs, auristatin E, auristatin F, maytansinoids, and cytotoxic agents comprising a reactive polyethylene glycol moiety (see, *e.g.*, Sasse *et al.*, J. Antibiot. (Tokyo), 53, 879-85 (2000), Suzawa *et al.*, Bioorg. Med. Chem., 8, 2175-84 (2000), Ichimura *et al.*, J. Antibiot. (Tokyo), 44, 1045-53 (1991), Francisco *et al.*, Blood (2003) (electronic publication prior to print publication), U.S. Pat. Nos. 5,475,092, 6,340,701, 6,372,738, and 6,436,931, U.S. Patent Application Publication No. 2001/0036923 A1, Pending U.S. patent application Ser. Nos. 10/024,290 and 10/116,053, and International (PCT) Patent Application No. WO 01/49698), taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, colchicine, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, and puromycin and analogs or homologs thereof. Therapeutic agents also include, for example, anti-metabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), ablating agents (*e.g.*, mechlorethamine, thiotepa chlorambucil, meiphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin, anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine). (See *e.g.*, Seattle Genetics US20090304721).

Other examples of therapeutic cytotoxins that can be conjugated to the modified antibodies or antibody fragments of the invention include duocarmycins, calicheamicins, maytansines and auristatins, and derivatives thereof. An example of a calicheamicin antibody conjugate is commercially available (Mylotarg^{Tm}; Wyeth-Ayerst).

For further discussion of types of cytotoxins, linkers and methods for conjugating therapeutic agents to antibodies, see also Saito *et al.,* (2003) Adv. Drug Deliv. Rev. 55:199-215; Trail *et al.,* (2003) Cancer Immunol. Immunother. 52:328-337; Payne, (2003) Cancer Cell 3:207-212; Allen, (2002) Nat. Rev. Cancer 2:750-763; Pastan and Kreitman, (2002) Curr. Opin. Investig. Drugs 3:1089-1091; Senter and Springer, (2001) Adv. Drug Deliv. Rev. 53:247-264.

According to the present invention, modified antibodies or antibody fragments thereof can also be conjugated to a radioactive isotope to generate cytotoxic radiopharmaceuticals, referred to as radioimmunoconjugates. Examples of radioactive isotopes that can be conjugated to antibodies for use diagnostically or therapeutically include, but are not limited to, iodine^{131}, indium^{111}, yttrium^{90}, and lutetium^{177}. Methods for preparing radioimmunoconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including Zevalin™ (DEC Pharmaceuticals) and Bexxar™ (Corixa Pharmaceuticals), and similar methods can be used to prepare radioimmunoconjugates using the antibodies of the invention. In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules are commonly known in the art and described in Denardo *et al.*, (1998) Clin. Cancer Res. 4(10):2483-90; Peterson *et al.*, (1999) Bioconjug. Chem. 10(4):553-7; and Zimmerman *et al.*, (1999) Nucl. Med. Biol. 26(8):943-50, each incorporated by reference in their entireties.

The present invention further provides modified antibodies or fragments thereof that specifically bind to an antigen. The modified antibodies or fragments may be conjugated or fused to a heterologous protein or polypeptide (or fragment thereof, preferably to a polypeptide of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids) to generate fusion proteins. In particular, the invention provides fusion proteins comprising an antibody fragment described herein (*e.g.*, a Fab fragment, Fd fragment, Fv fragment, F(ab)2 fragment, a V_H domain, a V_H CDR, a V_L domain or a V_L CDR) and a heterologous protein, polypeptide, or peptide.

In some embodiments, modified antibody fragments without antigen binding specificity, such as but not limited to, modified Fc domains with engineered cysteine residue(s) according to the present invention, are used to generate fusion proteins comprising such an antibody fragment (e.g., engineered Fc) and a heterologous protein, polypeptide, or peptide.

Additional fusion proteins may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to alter the activities of antibodies of the invention or fragments thereof (*e.g.*, antibodies or fragments thereof with higher affinities and lower dissociation rates). See, generally, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458; Patten *et al.*, (1997) Curr. Opinion Biotechnol. 8:724-33; Harayama, (1998) Trends Biotechnol. 16(2):76-82; Hansson *et al.*, (1999) J. Mol. Biol. 287:265-76; and Lorenzo and Blasco, (1998) Biotechniques 24(2):308- 313 (each of these patents and publications are hereby incorporated by reference in its entirety). Antibodies or fragments thereof, or the encoded antibodies or fragments thereof, may be altered by being subjected to

random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. A polynucleotide encoding an antibody or fragment thereof that specifically binds to an antigen may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

Moreover, the modified antibodies or antibody fragments thereof of the present invention can be conjugated to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, (1989) Proc. Natl. Acad. Sci. USA 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin ("HA") tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.*, (1984) Cell 37:767), and the "FLAG" tag (A. Einhauer *et al.*, J. Biochem. Biophys. Methods 49: 455–465, 2001). According to the present invention, antibodies or antibody fragments can also be conjugated to tumor-penetrating peptides in order to enhance their efficacy.

In other embodiments, modified antibodies or antibody fragments of the present invention are conjugated to a diagnostic or detectable agent. Such immunoconjugates can be useful for monitoring or prognosing the onset, development, progression and/or severity of a disease or disorder as part of a clinical testing procedure, such as determining the efficacy of a particular therapy. Such diagnosis and detection can accomplished by coupling the antibody to detectable substances including, but not limited to, various enzymes, such as, but not limited to, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as, but not limited to, streptavidin/biotin and avidin/biotin; fluorescent materials, such as, but not limited to, Alexa Fluor 350, Alexa Fluor 405, Alexa Fluor 430, Alexa Fluor 488, Alexa Fluor 500, Alexa Fluor 514, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 610, Alexa Fluor 633, Alexa Fluor 647, Alexa Fluor 660, Alexa Fluor 680, Alexa Fluor 700, Alexa Fluor 750, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as, but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as, but not limited to, iodine (^{131}I, ^{125}I, ^{123}I, and ^{121}I,), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{115}In, ^{113}In, ^{112}In, and ^{111}In,), technetium (^{99}Tc), thallium (^{201}Ti), gallium (^{68}Ga, ^{67}Ga), palladium (^{103}Pd), molybdenum (^{99}Mo), xenon (^{133}Xe), fluorine (^{18}F), ^{153}Sm, ^{177}Lu, ^{159}Gd, ^{149}Pm, ^{140}La, ^{175}Yb, ^{166}Ho, ^{90}Y, 47Sc, ^{186}Re, ^{188}Re, ^{142} Pr, ^{105}Rh, ^{97}Ru, ^{68}Ge, ^{57}Co, ^{65}Zn, ^{85}Sr, ^{32}P, ^{153}Gd, ^{169}Yb, ^{51}Cr, ^{54}Mn, ^{75}Se, ^{64}Cu, ^{113}Sn, and ^{117}Sn; and positron

emitting metals using various positron emission tomographies, and non-radioactive paramagnetic metal ions.

Modified antibodies or antibody fragments of the invention may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

5. Pharmaceutical Composition

To prepare pharmaceutical or sterile compositions including immunoconjugates, the immunoconjugates of the invention are mixed with a pharmaceutically acceptable carrier or excipient. The compositions can additionally contain one or more other therapeutic agents that are suitable for treating or preventing cancer (breast cancer, colorectal cancer, lung cancer, multiple myeloma, ovarian cancer, liver cancer, gastric cancer, pancreatic cancer, acute myeloid leukemia, chronic myeloid leukemia, osteosarcoma, squamous cell carcinoma, peripheral nerve sheath tumors (*e.g.*, schwannoma), head and neck cancer, bladder cancer, esophageal cancer, Barretts esophageal cancer, glioblastoma, clear cell sarcoma of soft tissue, malignant mesothelioma, neurofibromatosis, renal cancer, melanoma, prostate cancer, benign prostatic hyperplasia (BPH), gynacomastica, and endometriosis).

Formulations of therapeutic and diagnostic agents can be prepared by mixing with physiologically acceptable carriers, excipients, or stabilizers in the form of, *e.g.*, lyophilized powders, slurries, aqueous solutions, lotions, or suspensions (see, *e.g.*, Hardman *et al.*, Goodman and Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, N.Y., 2001; Gennaro, Remington: The Science and Practice of Pharmacy, Lippincott, Williams, and Wilkins, New York, N.Y., 2000; Avis, *et al.* (eds.), Pharmaceutical Dosage Forms: Parenteral Medications, Marcel Dekker, NY, 1993; Lieberman, *et al.* (eds.), Pharmaceutical Dosage Forms: Tablets, Marcel Dekker, NY, 1990; Lieberman, *et al.* (eds.) Pharmaceutical Dosage Forms: Disperse Systems, Marcel Dekker, NY, 1990; Weiner and Kotkoskie, Excipient Toxicity and Safety, Marcel Dekker, Inc., New York, N.Y., 2000).

Selecting an administration regimen for a therapeutic depends on several factors, including the serum or tissue turnover rate of the entity, the level of symptoms, the immunogenicity of the entity, and the accessibility of the target cells in the biological matrix. In certain embodiments, an administration regimen maximizes the amount of therapeutic delivered to the patient consistent with an acceptable level of side effects. Accordingly, the amount of biologic delivered depends in part on the particular entity and the severity of the condition being treated. Guidance in selecting appropriate doses of antibodies, cytokines, and small molecules is available (see, *e.g.*, Wawrzynczak, Antibody Therapy, Bios Scientific Pub.

Ltd, Oxfordshire, UK, 1996; Kresina (ed.), Monoclonal Antibodies, Cytokines and Arthritis, Marcel Dekker, New York, N.Y., 1991; Bach (ed.), Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases, Marcel Dekker, New York, N.Y., 1993; Baert *et al.*, New Engl. J. Med. 348:601-608, 2003; Milgrom *et al.*, New Engl. J. Med. 341:1966-1973, 1999; Slamon *et al.*, New Engl. J. Med. 344:783-792, 2001; Beniaminovitz *et al.,* New Engl. J. Med. 342:613-619, 2000; Ghosh *et al.*, New Engl. J. Med. 348:24-32, 2003; Lipsky *et al.*, New Engl. J. Med. 343:1594-1602, 2000).

Determination of the appropriate dose is made by the clinician, *e.g.*, using parameters or factors known or suspected in the art to affect treatment or predicted to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, *e.g.*, the inflammation or level of inflammatory cytokines produced.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors known in the medical arts.

Compositions comprising antibodies or fragments thereof of the invention can be provided by continuous infusion, or by doses at intervals of, *e.g.*, one day, one week, or 1-7 times per week. Doses may be provided intravenously, subcutaneously, topically, orally, nasally, rectally, intramuscular, intracerebrally, or by inhalation. A specific dose protocol is one involving the maximal dose or dose frequency that avoids significant undesirable side effects.

For the immunoconjugates of the invention, the dosage administered to a patient may be 0.0001 mg/kg to 100 mg/kg of the patient's body weight. The dosage may be between 0.0001 mg/kg and 20 mg/kg, 0.0001 mg/kg and 10 mg/kg, 0.0001 mg/kg and 5 mg/kg, 0.0001 and 2 mg/kg, 0.0001 and 1 mg/kg, 0.0001 mg/kg and 0.75 mg/kg, 0.0001 mg/kg and 0.5 mg/kg, 0.0001 mg/kg to 0.25 mg/kg, 0.0001 to 0.15 mg/kg, 0.0001 to 0.10 mg/kg, 0.001 to 0.5 mg/kg,

0.01 to 0.25 mg/kg or 0.01 to 0.10 mg/kg of the patient's body weight. The dosage of the antibodies or fragments thereof of the invention may be calculated using the patient's weight in kilograms (kg) multiplied by the dose to be administered in mg/kg.

Doses of the immunoconjugates the invention may be repeated and the administrations may be separated by at least 1 day, 2 days, 3 days, 5 days, 10 days, 15 days, 30 days, 45 days, 2 months, 75 days, 3 months, or at least 6 months. In a specific embodiment, does of the immunoconjugates of the invention are repeated every 3 weeks.

An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the method route and dose of administration and the severity of side effects (see, *e.g.*, Maynard *et al.*, A Handbook of SOPs for Good Clinical Practice, Interpharm Press, Boca Raton, Fla., 1996; Dent, Good Laboratory and Good Clinical Practice, Urch Publ., London, UK, 2001).

The route of administration may be by, *e.g.*, topical or cutaneous application, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, intracerebrospinal, intralesional, or by sustained release systems or an implant (see, *e.g.*, Sidman *et al.*, Biopolymers 22:547-556, 1983; Langer *et al.*, J. Biomed. Mater. Res. 15:167-277, 1981; Langer, Chem. Tech. 12:98-105, 1982; Epstein *et al.*, Proc. Natl. Acad. Sci. USA 82:3688-3692, 1985; Hwang *et al.*, Proc. Natl. Acad. Sci. USA 77:4030-4034, 1980; U.S. Pat. Nos. 6,350,466 and 6,316,024). Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. In addition, pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. See, *e.g.*, U.S. Pat. Nos. 6,019,968, 5,985,320, 5,985,309, 5,934,272, 5,874,064, 5,855,913, 5,290,540, and 4,880,078; and PCT Publication Nos. WO 92/19244, WO 97/32572, WO 97/44013, WO 98/31346, and WO 99/66903, each of which is incorporated herein by reference their entirety.

A composition of the present invention may also be administered via one or more routes of administration using one or more of a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. Selected routes of administration for the immunoconjugates of the invention include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal or other parenteral routes of administration, for example by injection or infusion. Parenteral administration may represent modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular,

subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Alternatively, a composition of the invention can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically. In one embodiment, the immunoconjugates of the invention is administered by infusion. In another embodiment, the immunoconjugates of the invention is administered subcutaneously.

If the immunoconjugates of the invention are administered in a controlled release or sustained release system, a pump may be used to achieve controlled or sustained release (see Langer, supra; Sefton, CRC Crit. Ref Biomed. Eng. 14:20, 1987; Buchwald *et al.*, Surgery 88:507, 1980; Saudek *et al.*, N. Engl. J. Med. 321:574, 1989). Polymeric materials can be used to achieve controlled or sustained release of the therapies of the invention (see *e.g.*, Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla., 1974; Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York, 1984; Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61, 1983; see also Levy *et al.*, Science 228:190, 1985; During *et al.*, Ann. Neurol. 25:351, 1989; Howard *et al.*, J. Neurosurg. 7 1:105, 1989; U.S. Pat. No. 5,679,377; U.S. Pat. No. 5,916,597; U.S. Pat. No. 5,912,015; U.S. Pat. No. 5,989,463; U.S. Pat. No. 5,128,326; PCT Publication No. WO 99/15154; and PCT Publication No. WO 99/20253. Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. In one embodiment, the polymer used in a sustained release formulation is inert, free of leachable impurities, stable on storage, sterile, and biodegradable. A controlled or sustained release system can be placed in proximity of the prophylactic or therapeutic target, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138, 1984).

Controlled release systems are discussed in the review by Langer, Science 249:1527-1533, 1990). Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more immunoconjugates of the invention. See, *e.g.*, U.S. Pat. No. 4,526,938, PCT publication WO 91/05548, PCT publication WO 96/20698, Ning *et al.*, Radiotherapy & Oncology 39:179-189, 1996; Song *et al.*, PDA Journal of Pharmaceutical Science & Technology 50:372-397, 1995; Cleek *et al.*, Pro. Int'l. Symp. Control. Rel. Bioact. Mater. 24:853-854, 1997; and Lam *et al.*, Proc. Int'l. Symp. Control Rel. Bioact. Mater. 24:759-760, 1997, each of which is incorporated herein by reference in their entirety.

If the immunoconjugates of the invention are administered topically, they can be formulated in the form of an ointment, cream, transdermal patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form well-known to one of skill in the art. See, *e.g.*, Remington's Pharmaceutical Sciences and Introduction to Pharmaceutical Dosage Forms, 19th ed., Mack Pub. Co., Easton, Pa. (1995). For non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity, in some instances, greater than water are typically employed. Suitable formulations include, without limitation, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like, which are, if desired, sterilized or mixed with auxiliary agents (*e.g.*, preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, such as, for example, osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient, in some instances, in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (*e.g.*, a gaseous propellant, such as Freon^{TM}) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms if desired. Examples of such additional ingredients are well-known in the art.

If the compositions comprising the immunoconjugates are administered intranasally, it can be formulated in an aerosol form, spray, mist or in the form of drops. In particular, prophylactic or therapeutic agents for use according to the present invention can be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with a suitable propellant (*e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas). In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges (composed of, *e.g.*, gelatin) for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

Methods for co-administration or treatment with a second therapeutic agent, *e.g.*, a cytokine, steroid, chemotherapeutic agent, antibiotic, or radiation, are known in the art (see, *e.g.*, Hardman *et al.,* (eds.) (2001) Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10.sup.th ed., McGraw-Hill, New York, N.Y.; Poole and Peterson (eds.) (2001) Pharmacotherapeutics for Advanced Practice: A Practical Approach, Lippincott, Williams & Wilkins, Phila., Pa.; Chabner and Longo (eds.) (2001) Cancer Chemotherapy and Biotherapy, Lippincott, Williams & Wilkins, Phila., Pa.). An effective amount of therapeutic may decrease the symptoms by at least 10%; by at least 20%; at least about 30%; at least 40%, or at least 50%.

Additional therapies (*e.g.*, prophylactic or therapeutic agents), which can be administered in combination with the immunoconjugates of the invention may be administered less than 5 minutes apart, less than 30 minutes apart, 1 hour apart, at about 1 hour apart, at about 1 to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, at about 12 hours to 18 hours apart, 18 hours to 24 hours apart, 24 hours to 36 hours apart, 36 hours to 48 hours apart, 48 hours to 52 hours apart, 52 hours to 60 hours apart, 60 hours to 72 hours apart, 72 hours to 84 hours apart, 84 hours to 96 hours apart, or 96 hours to 120 hours apart from the immunoconjugates of the invention. The two or more therapies may be administered within one same patient visit.

In certain embodiments, the immunoconjugates of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Pat. Nos. 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, *e.g.*, Ranade, (1989) J. Clin. Pharmacol. 29:685). Exemplary targeting moieties include folate or biotin (see, *e.g.*, U.S. Pat. No. 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.,* (1988) Biochem. Biophys. Res. Commun. 153:1038); antibodies (Bloeman *et al.*, (1995) FEBS Lett. 357:140; Owais *et al.*, (1995) Antimicrob. Agents Chemother. 39:180); surfactant protein A receptor (Briscoe *et al.*, (1995) Am. J. Physiol. 1233:134); p 120 (Schreier *et al,* (1994) J. Biol. Chem. 269:9090); see also K. Keinanen; M. L. Laukkanen (1994) FEBS Lett. 346:123; J. J. Killion; I. J. Fidler (1994) Immunomethods 4:273.

The invention provides protocols for the administration of pharmaceutical composition comprising immunoconjugates of the invention alone or in combination with other therapies to a subject in need thereof. The therapies (*e.g.*, prophylactic or therapeutic agents) of the combination therapies of the present invention can be administered concomitantly or sequentially to a subject. The therapy (*e.g.*, prophylactic or therapeutic agents) of the combination therapies of the present invention can also be cyclically administered. Cycling therapy involves the administration of a first therapy (*e.g.*, a first prophylactic or therapeutic agent) for a period of time, followed by the administration of a second therapy (*e.g.*, a second prophylactic or therapeutic agent) for a period of time and repeating this sequential administration, *i.e.*, the cycle, in order to reduce the development of resistance to one of the

therapies (*e.g.*, agents) to avoid or reduce the side effects of one of the therapies (*e.g.*, agents), and/or to improve, the efficacy of the therapies.

The therapies (*e.g.*, prophylactic or therapeutic agents) of the combination therapies of the invention can be administered to a subject concurrently.

The term "concurrently" is not limited to the administration of therapies (*e.g.*, prophylactic or therapeutic agents) at exactly the same time, but rather it is meant that a pharmaceutical composition comprising antibodies or fragments thereof the invention are administered to a subject in a sequence and within a time interval such that the antibodies of the invention can act together with the other therapy or therapies to provide an increased benefit than if they were administered otherwise. For example, each therapy may be administered to a subject at the same time or sequentially in any order at different points in time; however, if not administered at the same time, they should be administered sufficiently close in time so as to provide the desired therapeutic or prophylactic effect. Each therapy can be administered to a subject separately, in any appropriate form and by any suitable route. In various embodiments, the therapies (*e.g.*, prophylactic or therapeutic agents) are administered to a subject less than 15 minutes, less than 30 minutes, less than 1 hour apart, at about 1 hour apart, at about 1 hour to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, 24 hours apart, 48 hours apart, 72 hours apart, or 1 week apart. In other embodiments, two or more therapies (*e.g.*, prophylactic or therapeutic agents) are administered to a within the same patient visit.

The prophylactic or therapeutic agents of the combination therapies can be administered to a subject in the same pharmaceutical composition. Alternatively, the prophylactic or therapeutic agents of the combination therapies can be administered concurrently to a subject in separate pharmaceutical compositions. The prophylactic or therapeutic agents may be administered to a subject by the same or different routes of administration.

The invention having been fully described, it is further illustrated by the following examples and claims, which are illustrative and are not meant to be further limiting.

EXAMPLES

Example 1. Selection of surface accessible sites for Cys mutation in human IgG1 heavy chain and kappa light chain.

Surface exposed residues in the constant region of human IgG1 heavy and human kappa light chains were identified in a crystal structure of an hIgG1/kappa antibody (Protein Databank structure entry 1HZH.pdb, Table 6, Table 7, FIG. 1) using the computer program Surface Racer 5.0, as described by Tsodikov et al, "A novel computer program for fast exact calculation of accessible and molecular surface areas and average surface curvature," *J. Comput. Chem.,* 23, 600-609 (2002). 88 residues were selected for Cys substitution, 59 sites in hIgG heavy chain and 29 in human kappa light chain, based on the following criteria: 1) select residues in CH1, CH2 and CH3 domains of the constant regions of heavy chain and the constant regions of light chain; 2) select surface exposed residues but circumvent globally exposed residues and the C-terminal region to avoid inter-antibody dimer formation; 3) focus on polar or charged residues such as Ser, Thr, Lys, Arg, Glu, and Asp; and 4) exclude residues in FcRn binding domain, Protein A binding domain, and heavy chain hinge region.

Criterion 1) namely the selection of Cys substitution sites in the constant region of the antibody, assures transferability of the conjugation sites to many different antibodies. Criterion 2) is based on observation of inter-antibody dimer formation for Cys substitutions of prominently exposed residues (residues excluded based on this criteria are listed in Table 6). Based on the IgG crystal structure, the putative orientation of the Cys side chain was taking into consideration: residues for which the Cys side chain may be partially shielded from interactions with another antibody but may still be reactive with a small molecular payload, were favored over residues with larger surface accessibility but with an orientation that may enable interactions with a large macromolecule such as dimer formation. Criterion 3) was implemented to favor conservative mutations in order to minimize destabilizing effects of the mutations on the antibody. Similarly, criterion 4) was used to avoid functional changes to the antibody such as effects on FcRn and Protein A binding which may affect the antibody's pharmacokinetic properties or may result in the loss of a purification handle, respectively. Residues excluded based on criterion 4 are listed in Table 6. The location of the 88 selected mutation sites in the structure model of hIgG1/kappa indicates that the selected sites are surface accessible (FIG. 2).

Table 6. Surface accessibility of amino acid residues in human IgG1 heavy chain. Surface accessibility was calculated using Surface Racer 5.0 and is expressed as Angstrom square [Å^2]. "Excluded sites" indicate the sites that are excluded from selection due to the reasons mentioned in example 1. "Selected sites" are the sites that are selected for substitution to Cys in the invention.

Eu number	Residue	Surface accessibility [Å²]	Reason for exclusion (if applicable)	Selected sites
117	SER	128		HC-S117C
118	ALA	2		
119	SER	79		HC-S119C
120	THR	71		
121	LYS	136		HC-K121C
122	GLY	21		
123	PRO	2		
124	SER	40		HC-S124C
125	VAL	0		
126	PHE	1		
127	PRO	0		
128	LEU	0		
129	ALA	0		
130	PRO	0		
131	SER	0		
132	SER	34		HC-S132C
133	LYS	87		
134	SER	123		HC-S134C
135	THR	1		
136	SER	183		HC-S136C
137	GLY	84		
138	GLY	40		
139	THR	33		HC-T139C
140	ALA	0		
141	ALA	0		
142	LEU	0		
143	GLY	0		
144	CYS	0		
145	LEU	0		
146	VAL	0		
147	LYS	0		
148	ASP	1		
149	TYR	0		
150	PHE	0		
151	PRO	0		
152	GLU	52		HC-E152C
153	PRO	89		HC-P153C
154	VAL	10		
155	THR	69		HC-T155C
156	VAL	0		
157	SER	39		HC-S157C
158	TRP	0		

Eu number	Residue	Surface accessibility [Å²]	Reason for exclusion (if applicable)	Selected sites
159	ASN	4		
160	SER	164	Dimer	
161	GLY	35	Dimer	
162	ALA	115	Dimer	
163	LEU	17		
164	THR	125		HC-T164C
165	SER	183		HC-S165C
166	GLY	20		
167	VAL	12		
168	HIS	5		
169	THR	60		HC-T169C
170	PHE	0		
171	PRO	33		HC-P171C
172	ALA	9		
173	VAL	0		
174	LEU	68		HC-L174C
175	GLN	0		
176	SER	162		HC-S176C
177	SER	68		HC-S177C
178	GLY	8		
179	LEU	0		
180	TYR	6		
181	SER	0		
182	LEU	2		
183	SER	0		
184	SER	0		
185	VAL	0		
186	VAL	0		
187	THR	30		
188	VAL	0		
189	PRO	86		HC-P189C
190	SER	21		
191	SER	127		HC-S191C
192	SER	17		
193	LEU	0		
194	GLY	18		
195	THR	111		HC-T195C
196	GLN	79		
197	THR	90		HC-T197C
198	TYR	0		
199	ILE	25		
200	CYS	0		

Eu number	Residue	Surface accessibility [Å²]	Reason for exclusion (if applicable)	Selected sites
201	ASN	8		
202	VAL	0		
203	ASN	22		
204	HIS	0		
205	LYS	217		HC-K205C
206	PRO	66		
207	SER	50		HC-S207C
208	ASN	91		
209	THR	24		
210	LYS	234	Dimer	
211	VAL	30		
212	ASP	97		HC-D212C
213	LYS	70		
214	LYS	146		
215	ALA	0		
216	GLU	79		
217	PRO	0		
218	LYS	4		
219	SER	149		
220	CYS	7		
221	ASP	0	Hinge	
222	LYS	208	Hinge	
223	THR	112	Hinge	
224	HIS	1	Hinge	
225	THR	22	Hinge	
226	CYS	12	Hinge	
227	PRO	22	Hinge	
228	PRO	133	Hinge	
229	CYS	7	Hinge	
230	PRO	84	Hinge	
231	ALA	114	Hinge	
232	PRO	49	Hinge	
233	GLU	114	Hinge	
234	LEU	90		
235	LEU	88		
236	GLY	9		
237	GLY	46		
238	PRO	14		
239	SER	9		
240	VAL	0		
241	PHE	0		
242	LEU	0		

Eu number	Residue	Surface accessibility [Å²]	Reason for exclusion (if applicable)	Selected sites
243	PHE	1		
244	PRO	34		
245	PRO	0		
246	LYS	55		HC-K246C
247	PRO	18		
248	LYS	47		
249	ASP	1		
250	THR	0	FcRn binding	
251	LEU	0		
252	MET	53	Protein A, FcRn binding	
253	ILE	155	Protein A binding	
254	SER	157	Protein A, FcRn binding	
255	ARG	103		
256	THR	86	FcRn binding	
257	PRO	0	FcRn binding	
258	GLU	42		HC-E258C
259	VAL	0	FcRn binding	
260	THR	0		
261	CYS	0		
262	VAL	0		
263	VAL	0		
264	VAL	0		
265	ASP	11	FcRn binding	
266	VAL	0		
267	SER	10		
268	HIS	79		
269	GLU	189		HC-E269C
270	ASP	23		
271	PRO	20		
272	GLU	152		
273	VAL	19		
274	LYS	138		HC-K274C
275	PHE	2		
276	ASN	1		
277	TRP	0		
278	TYR	14		
279	VAL	0		
280	ASP	66		
281	GLY	72		
282	VAL	141		

Eu number	Residue	Surface accessibility [Å²]	Reason for exclusion (if applicable)	Selected sites
283	GLU	80		
284	VAL	25		
285	HIS	133		
286	ASN	119		HC-N286C
287	ALA	67		
288	LYS	182		HC-K288C
289	THR	5		
290	LYS	177		HC-K290C
291	PRO	51		
292	ARG	252		HC-R292C
293	GLU	83		HC-E293C
294	GLU	73		HC-E294C
295	GLN	170		
296	TYR	29		
297	ASN	61	Glycosylation	
298	SER	125	Glycosylation	
299	THR	2	Glycosylation	
300	TYR	28		
301	ARG	18		
302	VAL	0		
303	VAL	10		
304	SER	0		
305	VAL	17		
306	LEU	0		
307	THR	27	FcRn binding	
308	VAL	0	FcRn binding	
309	LEU	122		
310	HIS	4	Protein A binding	
311	GLN	145	Protein A, FcRn binding	
312	ASP	14		
313	TRP	0		
314	LEU	6	Protein A binding	
315	ASN	151	Protein A binding	
316	GLY	12		
317	LYS	81		
318	GLU	49		
319	TYR	0		
320	LYS	55		HC-K320C
321	CYS	0		
322	LYS	78		HC-K322C

Eu number	Residue	Surface accessibility [Å²]	Reason for exclusion (if applicable)	Selected sites
323	VAL	0		
324	SER	0		
325	ASN	0		
326	LYS	213		HC-K326C
327	ALA	10		
328	LEU	9		
329	PRO	158		
330	ALA	96		HC-A330C
331	PRO	44		
332	ILE	32		
333	GLU	85		HC-E333C
334	LYS	50		HC-K334C
335	THR	70		HC-T335C
336	ILE	13		
337	SER	15		HC-S337C
338	LYS	0		
339	ALA	37		
340	LYS	217	Protein A binding	
341	GLY	37		
342	GLN	235		
343	PRO	42		
344	ARG	98		HC-R344C
345	GLU	105		
346	PRO	0		
347	GLN	24		
348	VAL	3		
349	TYR	3		
350	THR	0		
351	LEU	0		
352	PRO	38		
353	PRO	0		
354	SER	0		
355	ARG	249		HC-R355C
356	ASP	53		
357	GLU	0		
358	LEU	36		
359	THR	144	Dimer	
360	LYS	114		HC-K360C
361	ASN	155		
362	GLN	41		HC-Q362C
363	VAL	0		
364	SER	0		

Eu number	Residue	Surface accessibility [Å²]	Reason for exclusion (if applicable)	Selected sites
365	LEU	0		
366	THR	0		
367	CYS	0		
368	LEU	0		
369	VAL	0		
370	LYS	1		
371	GLY	0		
372	PHE	0		
373	TYR	23		
374	PRO	0		
375	SER	29		HC-S375C
376	ASP	9		
377	ILE	11		
378	ALA	11		
379	VAL	4		
380	GLU	18	FcRn binding	
381	TRP	0		
382	GLU	22		HC-E382C
383	SER	1		
384	ASN	147		
385	GLY	102	Dimer	
386	GLN	161		
387	PRO	99		
388	GLU	4		
389	ASN	189		HC-N389C
390	ASN	36		HC-N390C
391	TYR	44		
392	LYS	82		HC-K392C
393	THR	36		HC-T393C
394	THR	0		
395	PRO	72		
396	PRO	47		
397	VAL	9		
398	LEU	111		HC-L398C
399	ASP	0		
400	SER	81		HC-S400C
401	ASP	68		
402	GLY	29		
403	SER	0		
404	PHE	22		
405	PHE	0		
406	LEU	0		

Eu number	Residue	Surface accessibility [Å^2]	Reason for exclusion (if applicable)	Selected sites
407	TYR	0		
408	SER	0		
409	LYS	0		
410	LEU	0		
411	THR	0		
412	VAL	0		
413	ASP	80		HC-D413C
414	LYS	83		
415	SER	69		HC-S415C
416	ARG	53		
417	TRP	0		
418	GLN	108		
419	GLN	177		
420	GLY	39		
421	ASN	35		
422	VAL	81		HC-V422C
423	PHE	0		
424	SER	2		
425	CYS	0		
426	SER	0		
427	VAL	0		
428	MET	0	FcRn binding	
429	HIS	0		
430	GLU	14		
431	ALA	22		
432	LEU	1		
433	HIS	227	Protein A binding	
434	ASN	126	Protein A, FcRn binding	
435	HIS	28		
436	TYR	54		
437	THR	36		
438	GLN	82		
439	LYS	12		
440	SER	62		
441	LEU	2		
442	SER	34		
443	LEU	101		
444	SER	70	Dimer	

Table 7. Surface accessibility of amino acid residues in human kappa light chain. Surface

accessibility was calculated using Surface Racer 5.0 and is expressed as Angstrom squared [Å²].

"Selected sites" indicate the sites selected for substitution to Cys in the invention.

EU number	Residue	Surface accessibility [Å²]	Selected sites	EU number	Residue	Surface accessibility [Å²]	Selected sites
107	LYS	90	LC-K107C	161	GLU	66	LC-E161C
108	ARG	49	LC-R108C	162	SER	8	
109	THR	148	LC-T109C	163	VAL	14	
110	VAL	77		164	THR	5	
111	ALA	16		165	GLU	74	LC-E165C
112	ALA	50	LC-A112C	166	GLN	8	
113	PRO	2		167	ASP	13	
114	SER	39	LC-S114C	168	SER	170	LC-S168C
115	VAL	0		169	LYS	241	LC-K169C
116	PHE	0		170	ASP	48	LC-D170C
117	ILE	0		171	SER	1	
118	PHE	0		172	THR	0	
119	PRO	0		173	TYR	0	
120	PRO	0		174	SER	0	
121	SER	0		175	LEU	0	
122	ASP	90	LC-D122C	176	SER	0	
123	GLU	51	LC-E123C	177	SER	0	
124	GLN	0		178	THR	0	
125	LEU	21		179	LEU	0	
126	LYS	230		180	THR	13	
127	SER	101		181	LEU	21	
128	GLY	12		182	SER	59	LC-S182C
129	THR	41	LC-T129C	183	LYS	131	LC-K183C
130	ALA	0		184	ALA	32	
131	SER	0		185	ASP	52	
132	VAL	0		186	TYR	0	
133	VAL	0		187	GLU	77	
134	CYS	0		188	LYS	201	LC-K188C
135	LEU	0		189	HIS	42	
136	LEU	0		190	LYS	167	LC-K190C
137	ASN	5		191	VAL	58	LC-V191C
138	ASN	18		192	TYR	0	
139	PHE	0		193	ALA	0	
140	TYR	0		194	CYS	0	
141	PRO	3		195	GLU	12	
142	ARG	55	LC-R142C	196	VAL	0	

EU number	Residue	Surface accessibility [Å^2]	Selected sites	EU number	Residue	Surface accessibility [Å^2]	Selected sites
143	GLU	117	LC-E143C	197	THR	38	LC-T197C
144	ALA	7		198	HIS	4	
145	LYS	160	LC-K145C	199	GLN	127	LC-Q199C
146	VAL	11		200	GLY	11	
147	GLN	22		201	LEU	17	
148	TRP	0		202	ARG	343	
149	LYS	48		203	SER	110	LC-S203C
150	VAL	0		204	PRO	69	
151	ASP	59		205	VAL	30	
152	ASN	157	LC-N152C	206	THR	70	LC-T206C
153	ALA	51		207	LYS	44	
154	LEU	117	LC-L154C	208	SER	47	
155	GLN	26		209	PHE	5	
156	SER	122	LC-S156C	210	ASN	44	
157	GLY	114		211	ARG	89	
158	ASN	19		212	GLY	15	
159	SER	22	LC-S159C	213	GLU	107	
160	GLN	36		214	CYS	58	

Example 2. Preparation of trastuzumab Cys mutant antibodies.

DNA encoding variable regions of heavy and light chains of trastuzumab were chemically synthesized and cloned into two mammalian expression vectors, pOG-HC and pOG-LC that contain constant regions of human IgG1 and human kappa light chain, resulting in two wild-type constructs, pOG-trastuzumab HC and pOG-trastuzumab LC, respectively. In the vectors the expression of antibody heavy and light chain constructs in mammalian cells is driven by a CMV promoter. The vectors contain a synthetic 24 amino acid signal sequence: MKTFILLLWVLLLWVIFLLPGATA (SEQ ID NO: 99), in the N-terminal of heavy chain or light chain to guide their secretion from mammalian cells. The signal sequence has been validated to be efficient in directing protein secretion in hundreds of mammalian proteins in 293 Freestyle™ cells. Oligonucleotide directed mutagenesis was employed to prepare Cys mutant constructs in trastuzumab. 88 pairs of mutation primers (Table 8) were chemically synthesized that correspond to the 88 Cys mutation sites selected in the constant regions of human IgG1 heavy chain and kappa light chain as described in Example 1. The sense and anti-sense mutation primer pairs were mixed prior to PCR amplification. PCR reactions were performed by using PfuUltra II Fusion HS DNA Polymerase (Stratagene) with pOG-trastuzumab HC and

pOG-trastuzumab LC as the templates. After PCR reactions, the PCR products were confirmed on agarose gels, and treated with DPN I followed by transformation in DH5a cells (Klock *et al.*, (2009) *Methods Mol Biol.* 498:91-103).

Sequences of 88 Cys mutant constructs were confirmed by DNA sequencing. The full length amino acid sequence of wild-type trastuzumab heavy chain is shown as SEQ ID NO:1 and that of the light chain as SEQ ID NO:90. The encoded protein sequence of the constant region of 59 trastuzumab HC Cys mutant constructs (SEQ ID NO:2 through SEQ ID NO:60) and 29 trastuzumab LC Cys mutant constructs (SEQ ID NO:61 to SEQ ID NO:89) are shown in Table 9 and Table 10, respectively. Amino acid residues in human IgG1 heavy chain and human kappa light chain are numbered by Eu numbering system (Edelman *et al*, (1969) *Proc Natl Acad Sci U S A*, 63:78-85).

Table 8. DNA sequences of mutation primers used to prepare 88 Cys mutations heavy and light chains of human IgG1.

Mutation sites	Primer name	Sequence	SEQ ID NO.
LC-K107C	LC-CYS-S1	GTGGAGATCTGTCGAACGGTGGCCGCTC CCAGCGTGTTCA	100
	LC-CYS-A1	ACCGTTCGACAGATCTCCACCTTGGTACC CTGTCCGAAC	101
LC-R108C	LC-CYS-S2	GGAGATCAAATGCACGGTGGCCGCTCCC AGCGTGTTCATCT	102
	LC-CYS-A2	GCCACCGTGCATTTGATCTCCACCTTGGT ACCCTGTCCGA	103
LC-T109C	LC-CYS-S3	GATCAAACGATGTGTGGCCGCTCCCAGC GTGTTCATCTTCC	104
	LC-CYS-A3	GCGGCCACACATCGTTTGATCTCCACCTT GGTACCCTGTC	105
LC-A112C	LC-CYS-S4	ACGGTGGCCTGTCCCAGCGTGTTCATCTT CCCCCCCAGCGA	106
	LC-CYS-A4	CACGCTGGGACAGGCCACCGTTCGTTTG ATCTCCACCTTG	107
LC-S114C	LC-CYS-S5	GCCGCTCCCTGCGTGTTCATCTTCCCCCC CAGCGACGAGCA	108
	LC-CYS-A5	ATGAACACGCAGGGAGCGGCCACCGTTC GTTTGATCTCCA	109
LC-D122C	LC-CYS-S6	CCCCCAGCTGTGAGCAGCTGAAGAGCGG CACCGCCAGCGT	110
	LC-CYS-A6	CAGCTGCTCACAGCTGGGGGGGAAGATG AACACGCTGGGA	111
LC-E123C	LC-CYS-S7	CCCAGCGACTGTCAGCTGAAGAGCGGCA CCGCCAGCGTG	112
	LC-CYS-A7	TTCAGCTGACAGTCGCTGGGGGGGAAGA TGAACACGCTG	113
LC-T129C	LC-CYS-S10	AGAGCGGCTGTGCCAGCGTGGTGTGCCT GCTGAACAACTT	114
	LC-CYS-A10	CACGCTGGCACAGCCGCTCTTCAGCTGCT CGTCGCTGGGG	115
LC-R142C	LC-CYS-S11	TCTACCCCTGTGAGGCCAAGGTGCAGTG	116

Mutation sites	Primer name	Sequence	SEQ ID NO.
		GAAGGTGGACAA	
	LC-CYS-A11	TTGGCCTCACAGGGGTAGAAGTTGTTCA GCAGGCACACCA	117
LC-E143C	LC-CYS-S12	TACCCCCGGTGTGCCAAGGTGCAGTGGA AGGTGGACAAC	118
	LC-CYS-A12	ACCTTGGCACACCGGGGGTAGAAGTTGT TCAGCAGGCACA	119
LC-K145C	LC-CYS-S13	CGGGAGGCCTGCGTGCAGTGGAAGGTGG ACAACGCCCTGC	120
	LC-CYS-A13	CACTGCACGCAGGCCTCCCGGGGGTAGA AGTTGTTCAGCA	121
LC-N152C	LC-CYS-S14	AAGGTGGACTGTGCCCTGCAGAGCGGCA ACAGCCAGGAGA	122
	LC-CYS-A14	TGCAGGGCACAGTCCACCTTCCACTGCAC CTTGGCCTCCC	123
LC-L154C	LC-CYS-S15	GACAACGCCTGTCAGAGCGGCAACAGCC AGGAGAGCGTCA	124
	LC-CYS-A15	TGCCGCTCTGACAGGCGTTGTCCACCTTC CACTGCACCTTG	125
LC-S156C	LC-CYS-S16	GCCCTGCAGTGTGGCAACAGCCAGGAGA GCGTCACCGAGCA	126
	LC-CYS-A16	GCTGTTGCCACACTGCAGGGCGTTGTCCA CCTTCCACTGCA	127
LC-S159C	LC-CYS-S18	AGCGGCAACTGTCAGGAGAGCGTCACCG AGCAGGACAGCAA	128
	LC-CYS-A18	CTCTCCTGACAGTTGCCGCTCTGCAGGGC GTTGTCCACCT	129
LC-E161C	LC-CYS-S19	AACAGCCAGTGCAGCGTCACCGAGCAGG ACAGCAAGGACT	130
	LC-CYS-A19	GTGACGCTGCACTGGCTGTTGCCGCTCTG CAGGGCGTTGT	131
LC-E165C	LC-CYS-S20	GAGCGTCACCTGTCAGGACAGCAAGGAC TCCACCTACAGC	132
	LC-CYS-A20	CTGTCCTGACAGGTGACGCTCTCCTGGCT GTTGCCGCTCT	133
LC-S168C	LC-CYS-S21	GAGCAGGACTGCAAGGACTCCACCTACA GCCTGAGCAGCA	134
	LC-CYS-A21	GAGTCCTTGCAGTCCTGCTCGGTGACGCT CTCCTGGCTGT	135
LC-K169C	LC-CYS-S22	CAGGACAGCTGTGACTCCACCTACAGCC TGAGCAGCACC	136
	LC-CYS-A22	GTGGAGTCACAGCTGTCCTGCTCGGTGAC GCTCTCCTGG	137
LC-D170C	LC-CYS-S23	ACAGCAAGTAGTCCACCTACAGCCTGAG CAGCACCCTGAC	138
	LC-CYS-A23	TAGGTGGACTACTTGCTGTCCTGCTCGGT GACGCTCTCCT	139
LC-S182C	LC-CYS-S24	TGACCCTGTGCAAGGCCGACTACGAGAA GCATAAGGTGTA	140
	LC-CYS-A24	GTCGGCCTTGCACAGGGTCAGGGTGCTG CTCAGGCTGTAG	141
LC-K183C	LC-CYS-S25	GACCCTGAGCTGTGCCGACTACGAGAAG CATAAGGTGTAC	142
	LC-CYS-A25	TAGTCGGCACAGCTCAGGGTCAGGGTGC TGCTCAGGCTGT	143

Mutation sites	Primer name	Sequence	SEQ ID NO.
LC-K188C	LC-CYS-S26	GACTACGAGTGCCATAAGGTGTACGCCT GCGAGGTGAC	144
	LC-CYS-A26	ACCTTATGGCACTCGTAGTCGGCCTTGCT CAGGGTCAGG	145
LC-K190C	LC-CYS-S27	GAGAAGCATTGCGTGTACGCCTGCGAGG TGACCCACCAG	146
	LC-CYS-A27	GGCGTACACGCAATGCTTCTCGTAGTCGG CCTTGCTCAGG	147
LC-V191C	LC-CYS-S28	AGCATAAGTAGTACGCCTGCGAGGTGAC CCACCAGGGCCT	148
	LC-CYS-A28	CAGGCGTACTACTTATGCTTCTCGTAGTC GGCCTTGCTCA	149
LC-T197C	LC-CYS-S29	GCGAGGTGTGTCACCAGGGCCTGTCCAG CCCCGTGACCAA	150
	LC-CYS-A29	CCCTGGTGACACACCTCGCAGGCGTACA CCTTATGCTTCT	151
LC-Q199C	LC-CYS-S30	GTGACCCACTGTGGCCTGTCCAGCCCCGT GACCAAGAGCT	152
	LC-CYS-A30	GACAGGCCACAGTGGGTCACCTCGCAGG CGTACACCTTAT	153
LC-S203C	LC-CYS-S31	GGCCTGTCCTGTCCCGTGACCAAGAGCTT CAACAGGGGCGA	154
	LC-CYS-A31	GTCACGGGACAGGACAGGCCCTGGTGGG TCACCTCGCAGG	155
LC-T206C	LC-CYS-S32	CAGCCCCGTGTGCAAGAGCTTCAACAGG GGCGAGTGCTAA	156
	LC-CYS-A32	AAGCTCTTGCACACGGGGCTGGACAGGC CCTGGTGGGTC	157
HC-S117C	HC-CYS-S1	CCGTCTCCTGCGCTAGCACCAAGGGCCC CAGCGTGTTC	158
	HC-CYS-A1	GGTGCTAGCGCAGGAGACGGTGACCAGG GTTCCTTGAC	159
HC-S119C	HC-CYS-S2	TCCTCGGCTTGTACCAAGGGCCCCAGCGT GTTCCCCCTGG	160
	HC-CYS-A2	CCCTTGGTACAAGCCGAGGAGACGGTGA CCAGGGTTCCTT	161
HC-K121C	HC-CYS-S3	CTAGCACCTGTGGCCCCAGCGTGTTCCCC CTGGCCCCCA	162
	HC-CYS-A3	GCTGGGGCCACAGGTGCTAGCCGAGGAG ACGGTGACCAG	163
HC-S124C	HC-CYS-S4	AGGGCCCCTGTGTGTTCCCCCTGGCCCCC AGCAGCAAGA	164
	HC-CYS-A4	GGGGAACACACAGGGGCCCTTGGTGCTA GCCGAGGAGACG	165
HC-S132C	HC-CYS-S5	CCCCCAGCTGCAAGAGCACCAGCGGCGG CACAGCCGCCCT	166
	HC-CYS-A5	GGTGCTCTTGCAGCTGGGGGCCAGGGGG AACACGCTGGGG	167
HC-S134C	HC-CYS-S6	AGCAGCAAGTGTACCAGCGGCGGCACAG CCGCCCTGGGCT	168
	HC-CYS-A6	CCGCTGGTACACTTGCTGCTGGGGGCCA GGGGGAACACG	169
HC-S136C	HC-CYS-S7	AGAGCACCTGTGGCGGCACAGCCGCCCT GGGCTGCCTGGT	170
	HC-CYS-A7	GTGCCGCCACAGGTGCTCTTGCTGCTGGG GGCCAGGGGGA	171

Mutation sites	Primer name	Sequence	SEQ ID NO.
HC-T139C	HC-CYS-S8	AGCGGCGGCTGTGCCGCCCTGGGCTGCC TGGTGAAGGACT	172
	HC-CYS-A8	CAGGGCGGCACAGCCGCCGCTGGTGCTC TTGCTGCTGGGG	173
HC-E152C	HC-CYS-S9	TACTTCCCCTGTCCCGTGACCGTGTCCTG GAACAGCGGA	174
	HC-CYS-A9	GGTCACGGGACAGGGGAAGTAGTCCTTC ACCAGGCAGC	175
HC-P153C	HC-CYS-S10	TCCCCGAGTGCGTGACCGTGTCCTGGAAC AGCGGAGCCCT	176
	HC-CYS-A10	CACGGTCACGCACTCGGGGAAGTAGTCC TTCACCAGGCAG	177
HC-T155C	HC-CYS-S11	GAGCCCGTGTGCGTGTCCTGGAACAGCG GAGCCCTGACCT	178
	HC-CYS-A11	CAGGACACGCACACGGGCTCGGGGAAGT AGTCCTTCACCA	179
HC-S157C	HC-CYS-S12	TGACCGTGTGCTGGAACAGCGGAGCCCT GACCTCCGGCGT	180
	HC-CYS-A12	CTGTTCCAGCACACGGTCACGGGCTCGG GGAAGTAGTCCT	181
HC-T164C	HC-CYS-S13	GGAGCCCTGTGCTCCGGCGTGCACACCTT CCCCGCCGTGCT	182
	HC-CYS-A13	ACGCCGGAGCACAGGGCTCCGCTGTTCC AGGACACGGTCA	183
HC-S165C	HC-CYS-S14	CCCTGACCTGTGGCGTGCACACCTTCCCC GCCGTGCTGCA	184
	HC-CYS-A14	TGTGCACGCCACAGGTCAGGGCTCCGCT GTTCCAGGACAC	185
HC-T169C	HC-CYS-S15	GCGTGCACTGCTTCCCCGCCGTGCTGCAG AGCAGCGGCCT	186
	HC-CYS-A15	GGCGGGGAAGCAGTGCACGCCGGAGGTC AGGGCTCCGCTG	187
HC-P171C	HC-CYS-S16	CACACCTTCTGTGCCGTGCTGCAGAGCAG CGGCCTGTACA	188
	HC-CYS-A16	CAGCACGGCACAGAAGGTGTGCACGCCG GAGGTCAGGGCT	189
HC-L174C	HC-CYS-S17	CCGCCGTGTGTCAGAGCAGCGGCCTGTA CAGCCTGTCCA	190
	HC-CYS-A17	GCTGCTCTGACACACGGCGGGGAAGGTG TGCACGCCGGAG	191
HC-S176C	HC-CYS-S18	TGCTGCAGTGCAGCGGCCTGTACAGCCT GTCCAGCGTGGT	192
	HC-CYS-A18	ACAGGCCGCTGCACTGCAGCACGGCGGG GAAGGTGTGCACG	193
HC-S177C	HC-CYS-S19	CTGCAGAGCTGTGGCCTGTACAGCCTGTC CAGCGTGGTGA	194
	HC-CYS-A19	TACAGGCCACAGCTCTGCAGCACGGCGG GGAAGGTGTGCA	195
HC-P189C	HC-CYS-S21	TGACAGTGTGCAGCAGCAGCCTGGGCAC CCAGACCTACAT	196
	HC-CYS-A21	CTGCTGCTGCACACTGTCACCACGCTGGA CAGGCTGTACA	197
HC-S191C	HC-CYS-S22	TGCCCAGCTGCAGCCTGGGCACCCAGAC CTACATCTGCAA	198
	HC-CYS-A22	CCCAGGCTGCAGCTGGGCACTGTCACCA CGCTGGACAGGCT	199

Mutation sites	Primer name	Sequence	SEQ ID NO.
HC-T195C	HC-CYS-S23	GCCTGGGCTGTCAGACCTACATCTGCAAC GTGAACCACAA	200
	HC-CYS-A23	GTAGGTCTGACAGCCCAGGCTGCTGCTG GGCACTGTCACCA	201
HC-T197C	HC-CYS-S24	GCACCCAGTGCTACATCTGCAACGTGAA CCACAAGCCCA	202
	HC-CYS-A24	GCAGATGTAGCACTGGGTGCCCAGGCTG CTGCTGGGCACT	203
HC-K205C	HC-CYS-S25	TGAACCACTGTCCCAGCAACACCAAGGT GGACAAGAGAGT	204
	HC-CYS-A25	TGTTGCTGGGACAGTGGTTCACGTTGCAG ATGTAGGTCTGG	205
HC-S207C	HC-CYS-S26	ACAAGCCCTGCAACACCAAGGTGGACAA GAGAGTGGAGC	206
	HC-CYS-A26	CTTGGTGTTGCAGGGCTTGTGGTTCACGT TGCAGATGTAG	207
HC-D212C	HC-CYS-S27	ACCAAGGTGTGCAAGAGAGTGGAGCCCA AGAGCTGCGACA	208
	HC-CYS-A27	CACTCTCTTGCACACCTTGGTGTTGCTGG GCTTGTGGTTCA	209
HC-K246C	HC-CYS-S28	TCCCCCCCTGTCCCAAGGACACCCTGATG ATCAGCAGGA	210
	HC-CYS-A28	GTCCTTGGGACAGGGGGGGAACAGGAAC ACGGAGGGTCCG	211
HC-E258C	HC-CYS-S29	AGGACCCCCTGCGTGACCTGCGTGGTGG TGGACGTGAG	212
	HC-CYS-A29	CAGGTCACGCAGGGGGTCCTGCTGATCA TCAGGGTGTCCT	213
HC-E269C	HC-CYS-S30	TGAGCCACTGTGACCCAGAGGTGAAGTT CAACTGGTACG	214
	HC-CYS-A30	CTCTGGGTCACAGTGGCTCACGTCCACCA CCACGCAGGTC	215
HC-K274C	HC-CYS-S32	CCAGAGGTGTGCTTCAACTGGTACGTGG ACGGCGTGGAGG	216
	HC-CYS-A32	CCAGTTGAAGCACACCTCTGGGTCCTCGT GGCTCACGTCCA	217
HC-N286C	HC-CYS-S35	GAGGTGCACTGTGCCAAGACCAAGCCCA GAGAGGAGCAGT	218
	HC-CYS-A35	GGTCTTGGCACAGTGCACCTCCACGCCGT CCACGTACCAGT	219
HC-K288C	HC-CYS-S36	CACAACGCCTGTACCAAGCCCAGAGAGG AGCAGTACAACA	220
	HC-CYS-A36	GGCTTGGTACAGGCGTTGTGCACCTCCAC GCCGTCCACGT	221
HC-K290C	HC-CYS-S37	GCCAAGACCTGTCCCAGAGAGGAGCAGT ACAACAGCACCT	222
	HC-CYS-A37	CTCTCTGGGACAGGTCTTGGCGTTGTGCA CCTCCACGCCGT	223
HC-R292C	HC-CYS-S38	ACCAAGCCCTGTGAGGAGCAGTACAACA GCACCTACAGGGT	224
	HC-CYS-A38	CTGCTCCTCACAGGGCTTGGTCTTGGCGT TGTGCACCTCCA	225
HC-E293C	HC-CYS-S39	CAAGCCCAGATGCGAGCAGTACAACAGC ACCTACAGGGTG	226
	HC-CYS-A39	GTACTGCTCGCATCTGGGCTTGGTCTTGG CGTTGTGCACCT	227

Mutation sites	Primer name	Sequence	SEQ ID NO.
HC-E294C	HC-CYS-S40	GCCCAGAGAGTGTCAGTACAACAGCACC TACAGGGTGGT	228
	HC-CYS-A40	TTGTACTGACACTCTCTGGGCTTGGTCTT GGCGTTGTGCA	229
HC-K320C	HC-CYS-S41	CAAGGAATACTGCTGCAAGGTCTCCAAC AAGGCCCTGCCA	230
	HC-CYS-A41	GACCTTGCAGCAGTATTCCTTGCCGTTCA GCCAGTCCTGGT	231
HC-K322C	HC-CYS-S42	TACAAGTGCTGCGTCTCCAACAAGGCCCT GCCAGCCCCCA	232
	HC-CYS-A42	GTTGGAGACGCAGCACTTGTATTCCTTGC CGTTCAGCCAGT	233
HC-K326C	HC-CYS-S43	GGTCTCCAACTGTGCCCTGCCAGCCCCCA TCGAAAAGACC	234
	HC-CYS-A43	GGCAGGGCACAGTTGGAGACCTTGCACT TGTATTCCTTGC	235
HC-A330C	HC-CYS-S44	GCCCTGCCATGTCCCATCGAAAAGACCA TCAGCAAGGCCA	236
	HC-CYS-A44	TTCGATGGGACATGGCAGGGCCTTGTTG GAGACCTTGCACT	237
HC-E333C	HC-CYS-S45	GCCCCCATCTGCAAGACCATCAGCAAGG CCAAGGGCCAGC	238
	HC-CYS-A45	GATGGTCTTGCAGATGGGGGCTGGCAGG GCCTTGTTGGAGA	239
HC-K334C	HC-CYS-S46	CCCATCGAATGCACCATCAGCAAGGCCA AGGGCCAGCCA	240
	HC-CYS-A46	GCTGATGGTGCATTCGATGGGGGCTGGC AGGGCCTTGTTG	241
HC-T335C	HC-CYS-S47	TCGAAAAGTGCATCAGCAAGGCCAAGGG CCAGCCACGGGA	242
	HC-CYS-A47	CTTGCTGATGCACTTTTCGATGGGGGCTG GCAGGGCCTTGT	243
HC-S337C	HC-CYS-S48	AGACCATCTGCAAGGCCAAGGGCCAGCC ACGGGAGCCCCA	244
	HC-CYS-A48	CCTTGGCCTTGCAGATGGTCTTTTCGATG GGGGCTGGCAGG	245
HC-R344C	HC-CYS-S50	GGCCAGCCATGCGAGCCCCAGGTGTACA CCCTGCCTCCAT	246
	HC-CYS-A50	CTGGGGCTCGCATGGCTGGCCCTTGGCCT TGCTGATGGTCT	247
HC-R355C	HC-CYS-S51	CTCCATCCTGCGACGAGCTGACCAAGAA CCAGGTGTCCCT	248
	HC-CYS-A51	CAGCTCGTCGCAGGATGGAGGCAGGGTG TACACCTGGGGCT	249
HC-K360C	HC-CYS-S52	AGCTGACCTGCAACCAGGTGTCCCTGAC CTGTCTGGTGA	250
	HC-CYS-A52	CACCTGGTTGCAGGTCAGCTCGTCCCGGG ATGGAGGCAGG	251
HC-Q362C	HC-CYS-S53	CCAAGAACTGCGTGTCCCTGACCTGTCTG GTGAAGGGCTT	252
	HC-CYS-A53	TCAGGGACACGCAGTTCTTGGTCAGCTCG TCCCGGGATGGA	253
HC-S375C	HC-CYS-S54	TTCTACCCCTGCGACATCGCCGTGGAGTG GGAGAGCAACG	254
	HC-CYS-A54	GGCGATGTCGCAGGGGTAGAAGCCCTTC ACCAGACAGGTCA	255

Mutation sites	Primer name	Sequence	SEQ ID NO.
HC-E382C	HC-CYS-S55	TGGAGTGGTGCAGCAACGGCCAGCCCGAGAACAACTACA	256
	HC-CYS-A55	GGCCGTTGCTGCACCACTCCACGGCGATGTCGCTGGGGTAG	257
HC-N389C	HC-CYS-S56	AGCCCGAGTGCAACTACAAGACCACCCCCCCCAGTGCTGGA	258
	HC-CYS-A56	CTTGTAGTTGCACTCGGGCTGGCCGTTGCTCTCCCACTCCA	259
HC-N390C	HC-CYS-S57	CCCGAGAACTGCTACAAGACCACCCCCCCAGTGCTGGACA	260
	HC-CYS-A57	GGTCTTGTAGCAGTTCTCGGGCTGGCCGTTGCTCTCCCACT	261
HC-K392C	HC-CYS-S58	GAACAACTACTGCACCACCCCCCCAGTGCTGGACAGCGAC	262
	HC-CYS-A58	GGGGTGGTGCAGTAGTTGTTCTCGGGCTGGCCGTTGCTCT	263
HC-T393C	HC-CYS-S59	AACTACAAGTGTACCCCCCCAGTGCTGGACAGCGACGGCA	264
	HC-CYS-A59	TGGGGGGGTACACTTGTAGTTGTTCTCGGGCTGGCCGTTG	265
HC-L398C	HC-CYS-S60	CCCCAGTGTGTGACAGCGACGGCAGCTTCTTCCTGTACA	266
	HC-CYS-A60	GTCGCTGTCACACACTGGGGGGGTGGTCTTGTAGTTGTTCT	267
HC-S400C	HC-CYS-S61	TGCTGGACTGCGACGGCAGCTTCTTCCTGTACAGCAAGCT	268
	HC-CYS-A61	GCTGCCGTCGCAGTCCAGCACTGGGGGGGTGGTCTTGTAGT	269
HC-D413C	HC-CYS-S62	TGACCGTGTGCAAGTCCAGGTGGCAGCAGGGCAACGTGTT	270
	HC-CYS-A62	ACCTGGACTTGCACACGGTCAGCTTGCTGTACAGGAAGAAG	271
HC-S415C	HC-CYS-S63	TGGACAAGTGCAGGTGGCAGCAGGGCAACGTGTTCAGCT	272
	HC-CYS-A63	CTGCCACCTGCACTTGTCCACGGTCAGCTTGCTGTACAGG	273
HC-V422C	HC-CYS-S64	AGGGCAACTGCTTCAGCTGCAGCGTGATGCACGAGGCCCT	274
	HC-CYS-A64	GCAGCTGAAGCAGTTGCCCTGCTGCCACCTGGACTTGTCCA	275

Table 9. Amino acid sequences of the constant region of Cys mutant constructs in human IgG1 heavy chain. SEQ ID NO:1 is the sequence for full-length trastuzumab (human IgG1). SEQ ID NO:2 to SEQ ID NO:60 indicate the sequence ID numbers for 59 Cys mutant constructs in human IgG1 heavy chain, showing only the sequences of the constant region.

SEQ ID NO:1 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYP TNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGF YAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
SEQ ID NO:2 CASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:3 SACTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:4 SASTCGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:5 SASTKGPCVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:6 SASTKGPSVFPLAPSCKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:7 SASTKGPSVFPLAPSSKCTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK

SEQ ID NO:8 SASTKGPSVFPLAPSSKSTCGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:9 SASTKGPSVFPLAPSSKSTSGGCAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:10 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPCPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:11 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPECVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:12 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVCVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:13 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVCWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:14 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALCSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK

SEQ ID NO:15 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTCGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:16 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHCF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:17 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF CAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
SEQ ID NO:18 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVCQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
SEQ ID NO:19 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQCSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
SEQ ID NO:20 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSCGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
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SEQ ID NO:22 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSCSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
SEQ ID NO:23 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGCQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
SEQ ID NO:24 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQCYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
SEQ ID NO:25 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHCPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:26 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPCNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
SEQ ID NO:27 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVCKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:28 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPCPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK

SEQ ID NO:29 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPCVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:30 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHCDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:31 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVCFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:32 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHCAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:33 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNACTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:34 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTCPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:35 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPCEEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK

SEQ ID NO:36 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPRCEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:37 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPRECQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:38 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYCCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:39 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCCVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:40 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNCALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:41 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP CPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:42 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APICKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK

SEQ ID NO:43 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIECTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:44 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKCISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:45 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTICKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:46 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPCEPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:47 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSCEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:48 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTCNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:49 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNCVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK

SEQ ID NO:50 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPCDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:51 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWCS NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:52 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPECNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:53 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENCYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:54 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYCTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:55 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKCTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:56 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVCDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK

SEQ ID NO:57 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDCDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:58 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVCKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:59 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKCRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO:60 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNCFSCSVMHEALHNH YTQKSLSLSPGK
SEQ ID NO 290: SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE**C** TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP**C**DIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL SLSPGK
SEQ ID NO: 291 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE**C** TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NY**C**TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPGK
SEQ ID NO: 292 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE**C** TISKAKGQPREPQVYTLPPSREEMT**C**NQVSLTCLVKGFYP**C**DIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL SLSPGK

SEQ ID NO: 293 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE**C**TISKAKGQP REPQVYTLPPSREEMT**C**NQVSLTCLVKGFYPSDIAVEWESNGQPE NNY**C**TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSPGK
SEQ ID NO: 294 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE**C**TISKAKGQP REPQVYTLPPSREEMTKNQVSLTCLVKGFYP**C**DIAVEWESNGQPE NNY**C**TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSPGK
SEQ ID NO: 295 SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP**C**PVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSREEMTKNQVSLTCLVKGFYP**C**DIAVEWESNGQP ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGK

Table 10. Amino acid sequences of the constant region of 29 human kappa light chain Cys mutant constructs. SEQ ID NO:61 is the sequence of the constant region of wild-type human kappa light chain. SEQ ID NO: 62 to SEQ ID NO:90 indicate the sequence ID numbers for 29 Cys mutant constructs in the constant region of human kappa light chain.

SEQ ID NO:61 CRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:62 KCTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC

SEQ ID NO:63 KRCVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:64 KRTVACPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:65 KRTVAAPCVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:66 KRTVAAPSVFIFPPSCEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:67 KRTVAAPSVFIFPPSDCQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:68 KRTVAAPSVFIFPPSDEQLKSGCASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:69 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPCEAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:70 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRCAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:71 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREACVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:72 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDCALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:73 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNACQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:74 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQCG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:75 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NCQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:76 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQCSVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC

SEQ ID NO:77 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTCQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:78 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDCKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:79 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSCDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:80 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKCSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:81 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLCKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:82 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSCADYEKHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:83 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYECHKVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:84 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHCVYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:85 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKCYACEVTHQGLSSPVTKSF NRGEC
SEQ ID NO:86 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVCHQGLSSPVTKSF NRGEC
SEQ ID NO:87 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHCGLSSPVTKSF NRGEC
SEQ ID NO:88 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSCPVTKSF NRGEC
SEQ ID NO:89 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVCKSF NRGEC
SEQ ID NO:90 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSAS FLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEI KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC

Example 3. Transfer of the trastuzumab heavy chain and light chain Cys mutations to different antibodies.

For trastuzumab, all Cys mutations for the attachment of drug payloads were chosen to be in the constant region of its human IgG1 heavy and human kappa light chain. Because constant regions of antibodies are highly conserved in primary sequence and structure, Cys mutant residues that are identified as good payload attachment sites in the context of trastuzumab will also serve as preferred attachment residues in other antibodies. To demonstrate the transferability of these generic conjugation sites to other antibodies, we cloned a set of Cys mutations into antibody 14090. Antibody 14090 is an antibody with a human IgG1 heavy chain and a human lambda light chain that binds to a different target protein than trastuzumab. The DNA encoding variable region of antibody 14090 was cloned into seven selected pOG trastuzumab HC Cys mutant plasmid constructs (SEQ ID NO listed in Table 11) to replace the variable regions of trastuzumab constructs in the plasmids as described in Example 2. As result, the amino acid sequences of the heavy chain constant regions in corresponding seven Cys constructs of antibody 14090 and trastuzumab are identical (FIG. 3). Subsequent examples show that these sites can be conjugated readily. Conversely, due to a high degree of similarity in primary sequences and in tertiary structures for different human IgG isotypes (FIG. 4), Cys mutations on the kappa light chain of trastuzumab can readily be transferred to equivalent light chains on human antibodies containing different isotype heavy chains. In the same way, the sites identified in the constant region of IgG1 may be transferred to IgG2, IgG3 and IgG4.

Example 4. Cysteine mutations in human lambda light chains.

Human lambda and kappa light chains have little amino acid sequence similarity (FIG. 5A). Mutations in the lambda light chain of antibody 14090 were selected based on the approximate similarity of the locations of the residues in a protein crystal structure model (Protein Databank structure entry 3G6D.pdb) of a Fab containing the human lambda light chain in reference to the desirable residues in the kappa light chain of trastuzumab(FIG. 5 A and B). Seven additional Cys mutant constructs were generated in antibody 14090-lambda light chain plasmid using oligonucleotide directed mutagenesis (Higuchi *et al.* 1988) in combination with PIPE cloning strategy (Klock and Lesley, 2009). The mutation primers used to generate Cys point mutations in the lambda light chain are listed in Table 12. The secretion of antibody 14090 is also directed by the synthetic 24 amino acid signal sequence: MKTFILLLWVLLLWVIFLLPGATA (SEQ ID NO: 99). Sequences of antibody 14090 Cys constructs were confirmed by DNA sequencing. The sequence for the constant region of human wild-type lambda light chain is shown as SEQ ID NO:91. The encoded protein sequences of

seven Cys mutant constructs in the light chain (SEQ ID NO:92 to SEQ ID NO:98) are shown in Table 13. Subsequent examples will show that these Cys mutants are efficiently conjugated with an ADC payload. Because all of these mutants are in the constant region of the human lambda light chain, these conjugation sites can readily be transferred to other antibodies with lambda light chains.

Table 11. Sequence ID numbers of trastuzumab heavy chain Cys constructs used for cloning of the variable region of antibody 14090.

Sequence ID NO: of trastuzumab HC Cys construct
SEQ ID NO:5
SEQ ID NO:8
SEQ ID NO:9
SEQ ID NO:10
SEQ ID NO:18
SEQ ID NO:48
SEQ ID NO:50

Table 12. Nucleotide sequences of primers used in mutagenesis of seven Cys mutant constructs in lambda light chain of human IgG1.

Mutation sites	Primer name	Sequence	SEQ ID NO.
LC-A143C	Seq-0017	CCGGGATGCGTGACAGTGGCCTGG AAGGCAGATAGC	276
	Seq-0018	TGTCACGCATCCCGGGTAGAAGTCA CTTATGAGACA	277
LC-T145C	Seq-0019	GCCGTGTGTGTGGCCTGGAAGGCA GATAGCAGCCCC	278
	Seq-0020	GGCCACACACACGGCTCCCGGGTA GAAGTCACTTAT	279
LC-A147C	Seq-0021	ACAGTGTGTTGGAAGGCAGATAGC AGCCCCGTCAAG	280
	Seq-0022	CTTCCAACACACTGTCACGGCTCCC GGGTAGAAGTC	281
LC-K156C	Seq-0023	CCCGTCTGTGCGGGAGTGGAGACC ACCACACCCTCC	282
	Seq-0024	TCCCGCACAGACGGGGCTGCTATCT GCCTTCCAGGC	283
LC-V159C	Seq-0025	GCGGGATGTGAGACCACCACACCC TCCAAACAAAGC	284
	Seq-0026	GGTCTCACATCCCGCCTTGACGGGG CTGCTATCTGC	285
LC-T163C	Seq-0027	ACCACCTGTCCCTCCAAACAAAGCA ACAACAAGTAC	286
	Seq-0028	GGAGGGACAGGTGGTCTCCACTCC CGCCTTGACGGG	287
LC-S168C	Seq-0029	AAACAATGCAACAACAAGTACGCG GCCAGCAGCTAT	288

Mutation sites	Primer name	Sequence	SEQ ID NO.
	Seq-0030	GTTGTTGCATTGTTTGGAGGGTGTG GTGGTCTCCAC	289

Table 13. Amino acid sequence of the constant region of Cys mutant constructs in antibody 14090 lambda light chain. SEQ ID NO:91 is the sequence for the constant region of wild-type human lambda light chain. SEQ ID NO:91 to SEQ ID NO:98 indicate the sequences of the 7 Cys mutants in the constant region of human lambda light chain of antibody 14090.

SEQ ID NO:91 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAG VETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS
SEQ ID NO:92 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGCVTVAWKADSSPVKAG VETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS
SEQ ID NO:93 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVCVAWKADSSPVKAG VETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS
SEQ ID NO:94 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVCWKADSSPVKAG VETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS
SEQ ID NO:95 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVCAG VETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS
SEQ ID NO:96 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAG CETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS
SEQ ID NO:97 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAG VETTCPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS
SEQ ID NO:98 QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAG VETTTPSKQCNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPT ECS

Example 5. Expression and purification of Cys mutant antibodies in 293 Freestyle™ cells.

Cys mutants of the trastuzumab antibody were expressed in 293 Freestyle™ cells by co-transfecting heavy chain and light chain plasmids using transient transfection method as described previously (Meissner, *et al.*, *Biotechnol Bioeng.* 75:197-203 (2001)). The DNA plasmids used in co-transfection were prepared using Qiagen plasmid preparation kit according

to manufacturer's protocol. 293 Freestyle™ cells were cultured in suspension in Freestyle™ expression media (Invitrogen) at 37°C under 5% CO_2. On the day before transfection, cells were split to 0.7 x 10^6 cells /ml into fresh media. On the day of transfection, the cell density typically reached 1.5 x 10^6 cells/ml. The cells were transfected with a mixture of heavy chain and light chain plasmids at the ratio of 1:1 using PEI method (Meissner *et al.*, 2001). The transfected cells were further cultured for five days. The media from the culture was harvested by centrifugation of the culture at 2000x g for 20 min and filtered through 0.2 micrometer filters. The expressed antibodies were purified from the filtered media using Protein A-Sepharose™ (GE Healthcare Life Sciences). Antibody IgGs were eluted from the Protein A-Sepharose™ column by the elution buffer (pH 3.0) and immediately neutralized with 1 M Tris-HCl (pH 8.0) followed by a buffer exchange to PBS.

Expression levels of 88 Cys trastuzumab mutant antibodies in transiently transfected 293 Freestyle™ are similar to that of wild-type trastuzumab, with an average yield at 18.6 mg/L +/- 9.5 mg/L (Table 14), suggesting that single point mutations in the selected sites did not significantly alter retention of the expressed antibody by the cells' secretion machinery. Analysis of the purified trastuzumab Cys mutant antibodies using non-reducing SDS PAGE indicates that the Cys mutant antibodies did not form oligomers disulfide-linked by the engineered cysteines (FIG. 6). Size exclusion chromatography (FIG. 7) further supported the conclusion that all Cys mutant trastuzumab antibodies are monomeric. HPLC reverse phase analysis of the mutant antibodies also suggests that majority of the Cys mutant antibodies are indistinguishable from wild-type trastuzumab in terms of retention time and homogeneity (FIG. 8). Analysis of non-reduced deglycosylated full length trastuzumab LC-R108C by mass spectrometry (intact LC-MS) revealed that the majority of the antibody was modified by two cysteines (FIG. 9 and Table 15). These observations are consistent with a previous publication indicating that the thiol group of the engineered cysteine in the trastuzumab Cys mutant antibodies is modified by cysteine when expressed in 293 freestyle™ cells, and that the modification needs to be removed by reducing reagents before conjugation with any thiol reactive reagents (Chen, *et al.*, *mAbs* 1:6, 563-571, 2009).

The Cys mutants of antibody 14090 were also expressed in in 293 Freestyle™ cells by co-transfecting HC and LC plasmids using PEI method as described (Meissner *et al.*, 2001). The expression levels of the Cys mutants of antibody 14090 are similar to that of wild-type antibody 14090 (Table 16).

Table 14. Yield of trastuzumab Cys mutant antibodies transiently expressed in 293 Freestyle™ cells. Yields were measured by UV absorbance at 280 nm after Protein A purification.

trastuzumab Cys mutant	Purified Ab (mg/L)	trastuzumab Cys mutant	Purified Ab (mg/L)
HC-S117C	46.9	HC-R355C	30.1
HC-S119C	22.5	HC-K360C	32.0
HC-K121C	22.1	HC-Q362C	20.7
HC-S124C	17.8	HC-S375C	33.3
HC-S132C	30.9	HC-E382C	35.3
HC-S134C	18.6	HC-N389C	28.7
HC-S136C	21.2	HC-N390C	34.5
HC-T139C	25.9	HC-K392C	28.2
HC-E152C	13.0	HC-T393C	6.6
HC-P153C	10.8	HC-L398C	5.1
HC-T155C	18.4	HC-S400C	4.1
HC-S157C	16.9	HC-D413C	27.6
HC-T164C	20.2	HC-S415C	10.6
HC-S165C	20.6	HC-V422C	5.0
HC-T169C	8.2	LC-K107C	11.0
HC-P171C	24.6	LC-R108C	27.0
HC-L174C	15.2	LC-T109C	13.1
HC-S176C	13.4	LC-A112C	10.5
HC-S177C	30.0	LC-S114C	21.2
HC-P189C	11.7	LC-D122C	25.5
HC-K205C	13.3	LC-E123C	20.1
HC-S207C	2.5	LC-T129C	7.1
HC-D212C	26.5	LC-R142C	14.6
HC-K246C	12.0	LC-E143C	10.0
HC-E258C	18.7	LC-K145C	13.0
HC-E269C	6.3	LC-N152C	12.0
HC-K273C	20.7	LC-L154C	13.1
HC-N286C	15.0	LC-S156C	12.0
HC-K288C	20.9	LC-S159C	26.6
HC-K290C	20.0	LC-E161C	20.0
HC-R292C	21.0	LC-E165C	5.0
HC-E293C	31.2	LC-S168C	12.0
HC-E294C	37.5	LC-K169C	4.0
HC-K320C	23.6	LC-D170C	5.0
HC-K322C	35.1	LC-S182C	8.8
HC-K326C	28.0	LC-K183C	12.6
HC-A330C	27.1	LC-K188C	12.0
HC-E333C	10.3	LC-K190C	5.2
HC-K334C	14.0	LC-V191C	29.9
HC-T335C	7.0	LC-T197C	19.0
HC-S337C	6.9	LC-Q199C	16.8
HC-R344C	32.6	LC-S203C	26.2

trastuzumab Cys mutant	Purified Ab (mg/L)	trastuzumab Cys mutant	Purified Ab (mg/L)
		LC-T206C	27.8

Table 15. Theoretical and observed mass for trastuzumab LC-R108C antibody after purification from 293 Freestyle™ cells.

Antibody species		Theoretical mass (Da)	Observed mass (Da)
LC-R108C		145063	145071
LC-R108C-Cys adduct	+1 Cys	145181	145189
LC-R108C-Cys double adduct	+2 Cys	145299	145311

Table 16: Yield of antibody 14090 Cys mutants transiently expressed in 293 Freestyle™ cells.

Antibody 14090 Cys mutant	Ab yield (mg/L)
HC-S124C	4.72
HC-S136C	3.64
HC-T139C	4.59
HC-E152C	2.93
HC-L174C	5.26
HC-E258C	5.86
HC-K360C	4.86
LC-A143C	4.63
LC-T145C	6.98
LC-A147C	8.37
LC-K156C	5.74
LC-V159C	9.67
LC-T163C	9.98
LC-S168C	5.61

Example 6. Reduction, re-oxidation and conjugation of Cys mutant antibodies with MC-MMAF.

Because engineered Cys in antibodies expressed in mammalian cells are modified by adducts (disulfides) such as glutathione (GSH) and/or Cysteine during their biosynthesis (Chen *et al*. 2009), the modified Cys in the product as initially expressed is unreactive to thiol reactive reagents such as maleimido or bromo-or iodo-acetamide groups. To conjugate the engineered cysteine after expression, the glutathione or cysteine adducts need to be removed by reducing these disulfides, which generally entails reducing all of the disulfides in the expressed protein. This can be accomplished by first exposing the antibody to a reducing agent such as dithiothreitol (DTT) followed by a procedure that allows for the re-oxidation of all native disulfide bonds of the antibody to restore and/or stabilize the functional antibody structure. Accordingly, in order to reduce all native disulfide bonds and the disulfide bound between the cysteine or GSH adducts of the engineered cysteine residue, freshly prepared DTT was added to

previously purified Cys mutants of trastuzumab and antibody 14090, to a final concentration of 20 mM. After the antibody incubation with DTT at 37°C for 1 hour, the mixtures were dialyzed at 4°C against PBS for three days with daily buffer exchange to remove DTT and re-oxidize the native disulfide bonds. An alternative method is to remove the reducing reagents through a desalting column, Sephadex G-25. Once the protein is fully reduced, 1 mM oxidized ascorbate (dehydro-ascorbic acid) is added to the desalted samples and the re-oxidation incubations are carried out for 20 hours. Both methods have produced similar results. However, attempts to follow the re-oxidation protocols previously described in the literature using $CuSO_4$ resulted in protein precipitation. All examples herein use the dialysis protocol described above. Reoxidation restores intra-chain disulfides, while dialysis allows cysteines and glutathiones connected to the newly-introduced cysteine(s) to dialyze away.

After re-oxidation, the antibodies are ready for conjugation. Maleimide-MMAF (MC-MMAF, 10 equivalents relative to the antibody, FIG. 10) was added to re-oxidized antibodies in PBS buffer (pH7.2). The incubations were carried out from 1 hour to 24 hours. The conjugation process was monitored by reverse-phase HPLC, which is able to separate conjugated antibodies from non-conjugated ones. The conjugation reaction mixtures were analyzed on a PRLP-S 4000A column (50 mm x 2.1 mm, Agilent) heated to 80°C and elution of the column was carried out by a linear gradient of 30-60% acetonitrile in water containing 0.1% TFA at a flow rate of 1.5 ml/min. The elution of proteins from the column was monitored at 280 nm, 254 nm and 215 nm. The reverse-phase HPLC trace of a typical conjugation mixture is shown in FIG. 11.

When the conjugation mixtures were analyzed by reverse-phase HPLC, many Cys sites generated homogenous conjugation products, as suggested by uniform, single peak elution profiles (FIG. 11), while some Cys sites generated heterogeneous conjugation products (FIG. 12). The procedures described above involve reduction and re-oxidation of native disulfide bonds as well as the reduction of bonds between the cysteine and GSH adducts of the engineered cysteine residues. During the re-oxidation process, the engineered cysteine residue may interfere with reforming of the proper native disulfide bonds through a process of disulfide shuffling. This may lead to the formation of mismatched disulfide bonds, either between the engineered cysteine and a native cysteine residue or between incorrectly matched native disulfide bonds. Such mismatched disulfide bonds may affect the retention of the antibody on the reverse-phase HPLC column. The mismatch processes may also result in unpaired cysteine residues other than the desired engineered cysteine. Attachment of the maleimide-MMAF to different positions on the antibody affects the retention time differently (see discussion of homogenously conjugated ADCs below). In addition, incomplete re-oxidation will leave the antibody with native cysteine residues that will react with maleimide-MMAF in addition to the

desired conjugation with the engineered cysteine residue. Any process that hinders proper and complete formation of the native disulfide bonds will result in a complex HPLC profile (FIG. 11) upon conjugation to Maleimide-MMAF. The yield of the uniform ADC as measured by UV absorption of the unpurified reaction mixtures, varied depending on the Cys mutations (Table 17). Using the reduction/re-oxidation protocol and conjugation procedures described above 65 of the 88 Cys mutant trastuzumab antibodies resulted in homogeneous conjugation products and these sites are advantageous sites for Cys replacements to be made when making cysteine-engineered antibodies for conjugation.

These 65 Cys-MMAF ADCs were analyzed in details in various assays: Differential scanning fluorimetry (DSF) was used to measure thermal stability. Analytical size exclusion chromatograph (AnSEC) was used to measure aggregation. *In vitro* antigen dependent cell killing potency was measured by cell viability assays and pharmacokinetics behavior was measured in mice. These assays and the respective results are described in more detail below.

To evaluate the aggregation state of trastuzumab Cys-MMAF ADCs, the ADCs were analyzed in a size exclusion chromatography column (GE, Superdex200, 3.2/30) at a flow rate of 0.1 ml/min in PBS. All 65 Cys-MMAF ADCs were monomeric. The majority of the ADCs contain less than 10% oligomer (FIG. 13, Table 18), indicating that conjugation of MC-MMAF to trastuzumab Cys mutant constructs at the selected sites did not cause aggregation of the antibody.

Table 17. Yield of MMAF ADCs generated with trastuzumab Cys mutant constructs. "Hetero" indicates a heterogeneous mixture of species shown in reverse phase HPLC with different retention times.

trastuzumab Cys-MMAF ADC	Yield (mg/L)	Cys construct	Yield (mg/L)
HC-S117C	6.9	HC-R344C	33.4
HC-S119C	15.3	HC-R355C	24.3
HC-K121C	4.4	HC-K360C	26.5
HC-S124C	13.2	HC-Q362C	hetero
HC-S132C	Hetero	HC-S375C	34.3
HC-S134C	Hetero	HC-E382C	34.9
HC-S136C	Hetero	HC-N389C	hetero
HC-T139C	11.1	HC-N390C	33.1
HC-E152C	7.8	HC-K392C	20.8
HC-P153C	8.2	HC-T393C	hetero
HC-T155C	12.9	HC-L398C	3.4
HC-S157C	13.5	HC-S400C	1.7
HC-T164C	13.7	HC-D413C	hetero
HC-S165C	Hetero	HC-S415C	hetero

trastuzumab Cys-MMAF ADC	Yield (mg/L)	Cys construct	Yield (mg/L)
HC-T169C	4.7	HC-V422C	3.6
HC-P171C	14.7	LC-K107C	1.6
HC-L174C	9.1	LC-R108C	12.2
HC-S176C	Hetero	LC-T109C	8.4
HC-S177C	Hetero	LC-A112C	hetero
HC-P189C	7.7	LC-S114C	16.9
HC-S191C	Hetero	LC-D122C	Hetero
HC-T195C	Hetero	LC-E123C	Hetero
HC-T197C	Hetero	LC-T129C	4.0
HC-K205C	11.3	LC-R142C	11.3
HC-S207C	1.0	LC-E143C	4.0
HC-D212C	Hetero	LC-K145C	8.7
HC-K246C	9.0	LC-N152C	7.2
HC-E258C	10.1	LC-L154C	1.3
HC-E269C	5.6	LC-S156C	7.2
HC-K274C	15.3	LC-S159C	12.3
HC-N286C	12.9	LC-E161C	12.0
HC-K288C	14.4	LC-E165C	2.0
HC-K290C	8.0	LC-S168C	3.1
HC-R292C	10.3	LC-K169C	2.5
HC-E293C	15.0	LC-D170C	2.2
HC-E294C	Hetero	LC-S182C	7.9
HC-K320C	18.9	LC-K183C	3.8
HC-K322C	29.1	LC-K188C	7.2
HC-K326C	22.8	LC-K190C	Hetero
HC-A330C	Hetero	LC-V191C	Hetero
HC-E333C	7.4	LC-T197C	16.4
HC-K334C	11.2	LC-Q199C	10.3
HC-T335C	5.2	LC-S203C	13.5
HC-S337C	1.4	LC-T206C	Hetero

Table 18. Percentage of oligomer in trastuzumab Cys-MMAF ADC preparations as determined by analytical size-exclusion chromatography.

trastuzumab Cys-MMAF ADC	Oligomer (%)	Conjugation site	Oligomer (%)
HC-S117C	b.d.	HC-R344C	9.5
HC-S119C	3.2	HC-R355C	b.d.
HC-K121C	b.d.	HC-K360C	b.d.
HC-S124C	b.d.	HC-S375C	b.d.
HC-T139C	4.8	HC-E382C	b.d.

trastuzumab Cys-MMAF ADC	Oligomer (%)	Conjugation site	Oligomer (%)
HC-E152C	b.d.	HC-N390C	b.d.
HC-P153C	b.d.	HC-K392C	b.d.
HC-T155C	b.d.	HC-L398C	b.d.
HC-S157C	b.d.	HC-S400C	9.2
HC-T164C	b.d.	HC-V422C	b.d.
HC-T169C	b.d.	LC-K107C	b.d.
HC-P171C	b.d.	LC-R108C	b.d.
HC-L174C	b.d.	LC-T109C	b.d.
HC-P189C	b.d.	LC-S114C	b.d.
HC-K205C	b.d.	LC-T129C	b.d.
HC-S207C	b.d.	LC-R142C	b.d.
HC-K246C	b.d.	LC-E143C	13.1
HC-E258C	b.d.	LC-K145C	b.d.
HC-E269C	b.d.	LC-N152C	b.d.
HC-K274C	11.7	LC-L154C	7.3
HC-N286C	9.2	LC-S156C	6.1
HC-K288C	b.d.	LC-S159C	2.8
HC-K290C	b.d.	LC-E161C	b.d.
HC-R292C	b.d.	LC-E165C	b.d.
HC-E293C	b.d.	LC-S168C	b.d.
HC-K320C	b.d.	LC-K169C	b.d.
HC-K322C	b.d.	LC-D170C	b.d.
HC-K326C	b.d.	LC-S182C	6.9
HC-E333C	b.d.	LC-K183C	b.d.
HC-K334C	b.d.	LC-K188C	b.d.
HC-T335C	b.d.	LC-T197C	b.d.
HC-S337C	b.d.	LC-Q199C	6.3
		LC-S203C	b.d.

b.d.: Below detection limit.

Example 7. *In vitro* thermal stability assay of trastuzumab Cys-MMAF ADCs.

Conjugation of MMAF payload to trastuzumab may stabilize or destabilize the antibody, leading to changes in melting temperature of the antibody, which can be determined by differential scanning fluorimetry (DSF) that is based on temperature induced denaturation monitored by an environmentally sensitive dye, such as sypro orange. ADC samples were aliquoted in triplicate to 384-well plates into PBS (6.7 mM sodium phosphate pH7.2; 150 mM NaCl). In each well, 8 µl of 0.25 mg/ml antibody was mixed with 2 µl 25x sypro orange dye (Invitrogen). Plates were sealed and analyzed in a Roche LightCycler 480 system with a

temperature ramp from 30 to 85°C with 20 fluorescence scans recorded per degree C. Melting temperatures were determined from the first derivative of the fluorescence intensity vs. time curves.

A typical thermal shift assay for wild-type trastuzumab revealed two melting transitions (Tm), Tm1 at 69.7°C and Tm2 at 81.2°C, respectively (Table 19). When trastuzumab Cys-MMAF ADCs were subjected to protein thermal stability assays, it was evident that conjugation of MC-MMAF to the antibodies induced different Tm changes depending on the sites of conjugation (Table 19). When MC-MMAF was conjugated to majority of the Cys sites in either CH1 or CH3 domains, the resulting ADCs, for example HC-K356C-MMAF, showed a similar pattern as that of wild-type anti-Her with little changes in Tm1 and Tm2. However, when MC-MMAF was conjugated to Cys sites located in the CH2 domain, a decrease in Tm1 was observed for most sites while the Tm2 remained largely unchanged. The Tm1 decrease observed for most CH2 domain Cys-MMAF conjugates ranged from 5°C to 26°C. The two ADCs with the largest decrease in Tm1 are HC-T335C-MMAF and HC-S337C-MMAF, with Tm1 at 42°C and 45°C, respectively (Figure 14). The results indicate that the location of MC-MMAF conjugation can have a significant impact on the stability of the ADCs.

Table 19. Melting temperatures Tm1 and Tm2 of trastuzumab Cys-MMAF ADCs observed by differential scanning fluorimetry (DSF).

trastuzumab Cys-MMAF ADC	HC domain	Tm1 [°C]	Tm2 [°C]
wild-type antibody	n.a.	69.71	81.18
HC-S117C	CH1	69.09	79.85
HC-S119C	CH1	69.28	78.58
HC-K121C	CH1	69.63	78.52
HC-S124C	CH1	69.27	80.56
HC-T139C	CH1	69.09	80.74
HC-E152C	CH1	69.63	80.83
HC-P153C	CH1	69.71	78.52
HC-T155C	CH1	69.27	80.83
HC-S157C	CH1	69.72	80.81
HC-T164C	CH1	69.17	80.7
HC-T169C	CH1	68.74	80.47
HC-P171C	CH1	69.27	77.18
HC-L174C	CH1	69.89	80.03
HC-P189C	CH1	69.09	81.27
HC-K205C	CH1	69.54	80.65
HC-S207C	CH1	69.00	80.65
HC-K246C	CH2	64.65	80.74

trastuzumab Cys-MMAF ADC	HC domain	Tm1 [°C]	Tm2 [°C]
HC-E258C	CH2	65.32	81.03
HC-E269C	CH2	65.36	81.01
HC-K274C	CH2	67.14	81.09
HC-N286C	CH2	67.22	81.09
HC-K288C	CH2	65.54	80.83
HC-K290C	CH2	69.00	80.65
HC-R292C	CH2	67.49	80.56
HC-E293C	CH2	64.34	81.03
HC-K320C	CH2	60.60	80.59
HC-K322C	CH2	62.41	80.70
HC-K326C	CH2	63.05	80.74
HC-E333C	CH2	63.67	80.92
HC-K334C	CH2	64.65	80.47
HC-T335C	CH2	42.93	80.04
HC-S337C	CH2	45.56	80.48
HC-R344C	CH3	69.50	80.92
HC-R355C	CH3	68.18	81.25
HC-K360C	CH3	69.28	80.92
HC-S375C	CH3	68.20	81.36
HC-E382C	CH3	69.36	80.74
HC-N390C	CH3	68.73	80.92
HC-K392C	CH3	67.05	80.92
HC-L398C	CH3	68.47	81.36
HC-S400C	CH3	68.65	81.27
HC-V422C	CH3	69.98	81.45
LC-K107C	n.a.	69.45	80.29
LC-R108C	n.a.	70.10	n.d.[1]
LC-T109C	n.a.	68.47	80.21
LC-T129C	n.a.	68.47	80.12
LC-R142C	n.a.	69.00	78.61
LC-E143C	n.a.	69.83	80.59
LC-K145C	n.a.	69.00	80.65
LC-N152C	n.a.	67.49	81.09
LC-L154C	n.a.	68.47	80.65
LC-S156C	n.a.	68.83	80.47
LC-S159C	n.a.	69.50	79.93
LC-E161C	n.a.	68.65	80.12
LC-E165C	n.a.	69.27	79.76
LC-S168C	n.a.	69.54	79.67
LC-K169C	n.a.	69.09	80.29

trastuzumab Cys-MMAF ADC	HC domain	Tm1 [°C]	Tm2 [°C]
LC-D170C	n.a.	68.83	80.12
LC-S182C	n.a	69.18	80.29
LC-K183C	n.a	69.09	80.47
LC-K188C	n.a	68.74	80.65
LC-T197C	n.a	69.63	80.74
LC-Q199C	n.a	69.54	80.21
LC-S203C	n.a	68.84	80.92

n.d. Not determined because a broad transition in Tm2 prevented accurate Tm determination.,
n.a. Not applicable

Example 8. Cell proliferation assays to measure *In vitro* cell killing potency of Cys ADCs

Cells that naturally express target antigens or cell lines engineered to express target antigens are frequently used to assay the activity and potency of ADCs. For evaluation of the cell killing potency of trastuzumab ADCs *in vitro*, two engineered cell lines, MDA-MB231 clone 16 and clone 40, and HCC1954 cells were employed (Clinchy B, Gazdar A, Rabinovsky R, Yefenof E, Gordon B, Vitetta ES. Breast Cancer Res Treat. (2000) 61:217-228). MDA-MB231 clone 16 cells stably express high copy numbers (~$5x10^5$ copies /cell) of recombinant human Her2 while clone 40 expresses low copy numbers (~$5x10^3$ copies /cell) of human Her2. HCC1954 cells endogenously express high level (~$5x10^5$ copies /cell) of human Her2 in the surface. For determination of the cell killing potency of antibody 14090 ADCs, CMK11-5 and Jurkat cells were used. While CMK11-5 cells express a high level of the antigen for antibody 14090 in the cell surface there is no detectable antigen expression in Jurkat cells. An antigen dependent cytotoxic effect should only kill cells that express sufficient antigen in the cell surface and not cells lacking the antigen. The cell proliferation assays were conducted with Cell-Titer-Glo™ (Promega) five days after cells were incubated with various concentrations of ADCs (Riss *et al.*, (2004) Assay Drug Dev Technol. 2:51-62). In some studies, the cell based assays are high throughput and conducted in an automated system (Melnick *et al.*, (2006) Proc Natl Acad Sci U S A. 103:3153-3158).

Trastuzumab Cys-MMAF ADCs specifically killed MDA-MB231 clone 16 and HCC1954 but not MDA-MB231 clone 40 cells (FIG. 15). IC_{50} of the trastuzumab Cys-MMAF ADCs in MDA-MB231 clone 16 cell assays ranges from 30 pM to 200 pM (Table 20, FIG. 16). Similarly, antibody 14090 Cys-MMAF ADC displayed antigen dependent cell killing in cell proliferation assays. The antibody 14090 Cys-MMAF ADCs killed antigen expressing

CMK11-5 cells but not antigen negative Jurkat cells (FIG. 17). The IC_{50} of the antibody 14090-MMAF ADC in CMK11-5 proliferation assay is in the range of 400 pM to 1 nM (Table 21).

Table 20. IC_{50} of trastuzumab Cys-MMAF ADCs in MDA-MB231 clone 16 $Her2^{+}$ cell proliferation assay.

trastuzumab Cys-MMAF ADC	IC_{50} (μM)	trastuzumab Cys-MMAF ADC	IC_{50} (μM)
HC-S117C	1.55E-04	HC-R344C	2.75E-04
HC-S119C	1.18E-04	HC-R355C	1.21E-04
HC-K121C	1.06E-04	HC-K360C	1.92E-04
HC-S124C	9.78E-05	HC-S375C	1.42E-04
HC-T139C	1.48E-04	HC-E382C	2.53E-04
HC-E152C	7.62E-05	HC-N390C	1.58E-04
HC-P153C	9.27E-05	HC-K392C	1.43E-04
HC-T155C	1.33E-04	HC-L398C	1.08E-04
HC-S157C	1.47E-04	HC-S400C	1.43E-04
HC-T164C	1.34E-04	HC-V422C	1.72E-04
HC-T169C	1.98E-04	LC-K107C	2.59E-05
HC-P171C	1.33E-04	LC-R108C	2.96E-05
HC-L174C	1.19E-04	LC-T109C	8.12E-05
HC-P189C	1.82E-04	LC-S114C	3.37E-05
HC-K205C	1.02E-04	LC-T129C	2.73E-05
HC-S207C	2.16E-04	LC-R142C	2.64E-05
HC-K246C	9.54E-05	LC-E143C	3.76E-05
HC-E258C	9.40E-05	LC-K145C	3.87E-05
HC-E269C	8.98E-05	LC-N152C	2.14E-05
HC-K274C	9.99E-05	LC-L154C	3.52E-05
HC-N286C	9.94E-05	LC-S156C	4.28E-05
HC-K288C	7.47E-05	LC-S159C	4.34E-05
HC-K290C	3.55E-04	LC-E161C	3.62E-05
HC-R292C	9.69E-05	LC-E165C	4.68E-05
HC-E293C	1.10E-04	LC-S168C	2.50E-04
HC-K320C	9.79E-05	LC-K169C	7.74E-05
HC-K322C	1.16E-04	LC-D170C	1.64E-04
HC-K326C	1.73E-04	LC-S182C	9.07E-05
HC-E333C	1.28E-04	LC-K183C	8.39E-05
HC-K334C	1.43E-04	LC-K188C	9.71E-05
HC-T335C	8.69E-05	LC-T197C	1.07E-04
HC-S337C	7.79E-05	LC-Q199C	1.31E-04
		LC-S203C	1.18E-04

Table 21. IC_{50} of antibody 14090 Cys-MMAF ADCs in CMK11-5 cell proliferation assay

Antibody 14090 Cys-MMAF ADC	IC_{50} (μM)
HC-S124C	9.26E-04
HC-T139C	1.22E-03
HC-E152C	4.60E-04
HC-L174C	6.02E-04
HC-K360C	8.56E-04
HC-S375C	4.38E-04
LC-A143C	7.09E-04
LC-A147C	1.14E-03
LC-V159C	5.41E-04
LC-T163C	6.38E-04
LC-S168C	1.06E-03

Example 9. Pharmacokinetic study of trastuzumab Cys-MMAF ADCs

It has been demonstrated that a long serum half-life is critical for high *in vivo* efficacy of ADCs (Hamblett, *et al.*, "Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate," *Clin Cancer Res.*, 10:7063-7070 (2004); Alley *et al.*, *Bioconjug Chem.* 19:759-765 (2008)). Attaching an usually hydrophobic drug payload to an antibody could significantly affect the properties of an antibody, and this may lead to a fast clearance of the ADCs *in vivo* (Hamblett *et al.*, 2004) and poor *in vivo* efficacy. To evaluate the effects of different conjugation site on clearance of MMAF ADCs *in vivo*, pharmacokinetic studies in non-tumor bearing mice were carried out with 65 trastuzumab Cys-MMAF ADCs. To detect MMAF containing ADCs in murine plasma, an anti-MMAF antibody was generated. ELISA assays for the detection of ADCs were developed using the extracellular domain of human HER2 to capture trastuzumab IgG molecules from the plasma and an anti-human IgG (anti-hIgG) antibody and the anti-MMAF antibody for signal generation in two separate assays. The two ELISA assays measure the serum concentration of the trastuzumab antibody and the "intact" ADC respectively as discussed in more detail below.

Three mice per group were administered with a single dose of a trastuzumab Cys-MMAF ADC at 1 mg/kg. Ten plasma samples were collected over the course of two weeks and assayed by ELISA using the extracellular domain of human HER2 to capture all trastuzumab IgG molecules including trastuzumab Cys-MMAF ADCs and trastuzumab lacking MMAF. An anti-MMAF and an anti-hIgG antibody were then used for detection in two separate assays. The anti-MMAF antibody ELISA measures the concentration of trastuzumab MMAF conjugates only and the anti-hIgG ELISA quantitates both trastuzumab Cys-MMAF conjugates

and trastuzumab antibodies that lack MMAF. Standard curves were generated for each ADC separately using the same material as injected into the mice. The assays with anti-MMAF and anti-hIgG should therefore yield identical concentration readouts if no changes to the drug loading of the trastuzumab Cys-MMAF ADC occur after injection into mice. For trastuzumab Cys-MMAF ADCs that lost some of the MMAF payload, the ELISA assay with the anti-MMAF antibody will measure a lower concentration than the anti-hIgG ELISA. A comparison of the two concentration readouts therefore allows to measure drug-release from trastuzumab Cys-MMAF ADCs during *in vivo* incubation in the mouse.

As measured by anti-hIgG ELISA, 63 out of 65 ADCs displayed a pharmacokinetic profile similar to unconjugated wild-type trastuzumab antibody (FIGs. 18, 19, 20), indicating that MC-MMAF payload conjugation to these sites did not significantly affect the antibody's clearance. The two exceptions are HC-T335C and HC-S337C. Conjugation of MC-MMAF to these two sites results in rapid clearance of the ADCs as measured by the anti-MMAF and the anti-hIgG ELISA (FIG. 21). The protein thermal shift assay revealed that the Tm1 for trastuzumab HC-T335C-MMAF and trastuzumab HC-S337C-MMAF decreased from 69°C in wild-type trastuzumab antibody to 42°C and 45°C, respectively (FIG. 14). Conjugation of MC-MMAF to the two sites dramatically reduces the thermal stability of the ADC (by 27 °C and 24°C, respectively). For the 63 ADCs that show a pharmacokinetic profile similar to unconjugated antibody, Tm1 changes were smaller than 8°C suggesting that fast clearance may possibly correlate with low thermal stability of the ADC.

To determine the chemical stability of linkage between the MMAF payload and the antibody at the various Cys sites, the concentrations of trastuzumab Cys-MMAF ADC as measured by the anti-MMAF ELISA and of all trastuzumab molecules as measured by the anti-hIgG ELISA were compared to each other for each sample. Many trastuzumab Cys-MMAF ADCs, within the error of the measurements, displayed a good overlap between the two concentrations over the course of two weeks, suggesting that the bond between MC-MMAF and the cysteine introduced at these sites was stable during circulation in mice over this period (FIGs. 18, 19). In contrast, some trastuzumab Cys-MMAF ADCs displayed a significant drug loss as indicated by the higher anti-hIgG readout than the anti-MMAF readout (FIG. 20). For some trastuzumab Cys-MMAF ADCs, the concentration of ADC was about 50% of that of hIgG. These results suggest that there are significant differences in stability of a thiol-maleimide bond of drug payloads conjugated to different sites as has been suggested previously (Shen et al. Nat. Biotechnol. 2012, 30 (2):184-9). Sites having good stability are preferred sites for use of preparing ADCs as described herein.

In pharmacokinetic studies, the area-under-the-plasma-concentration-versus-time-curve (AUC) is an important parameter in estimating total clearance and bioavailability of an

administered drug. In our pharmacokinetic studies, for each trastuzumab Cys-MMAF ADC two AUC values, AUC-MMAF and AUC-hIgG, were calculated separately from measurements with the anti-MMAF and the anti-hIgG ELISA. The ratios of AUC-MMAF to AUC-hIgG for all trastuzumab Cys-MMAF ADCs varied from 0.4 to 1.2 (Table 20). FIGs 18, 19 and 20 include PK curves for ADCs over the full range of observed AUC-MMAF/AUC-hIgG ratios and illustrate the variability and uncertainty of the measurements. Ratios of AUC-MMAF to AUC-hIgG >1 (Table 20) suggest uncertainties of >25% since the ratio should remain near 1 if no drug loss occurs. As shown in Table 20, out of 63 trastuzumab Cys-MMAF ADCs with measurable AUCs from both ELISAs, 40 ADCs show a ratio of AUC-MMAF/AUC-hIgG >0.7, indicating that within the accuracy of the measurement, little MMAF drug loss was observed at these after administration in mice. However, 23 ADCs displayed a ratio of AUC-MMAF/AUC-hIgG <0.7, suggesting that the amount of MMAF payload conjugate at these 23 sites decreased significant during the *in vivo* incubation in the mouse.

Differences in stability of the maleimide linkage at different conjugation sites has previously been reported for Cys engineered ADCs (see Shen *et al.*, (2012) Nat Biotechnol. 22;30(2):184-9 for discussion and references). For the preferred sites that exhibit enhanced serum stability, the antibody environment likely catalyzes the hydrolysis of the succinimide ring formed by the reaction of maleimide with cysteine. The hydrolyzed form cannot revert back and cannot release the maleimide drug. As such, the ability of the antibody environment to catalyze the ring hydrolysis cannot be predicted and is an unexpected property of certain engineered Cys sites. Sites in Table 22 having an AUC(MMAF)/AUC(hIgG) ratio greater than 0.7 are therefore particularly suitable sites for cysteine substitution based on this criterion, and sites having a ratio of about 0.9 or above are especially preferred cysteine substitution sites for purposes of the invention when applying. These include heavy chain sites 322, 334, 121, 288, 171, 139, 360, 117, 392, 375, 292, 333, 174, 258, 337, 422, 320, 390, and 335; and light chain sites 107, 203, 108 and 114.

Table 22. AUC-MMAF and AUC-hIgG of trastuzumab Cys-MMAF ADCs in mice

trastuzumab Cys-MMAF ADC	AUC-MMAF (hrs*μg/ml)	AUC-hIgG (hrs*μg/ml)	AUC(MMAF)/AUC(hIgG)
HC-K246C-MMAF	1515	3587	0.4
HC-K205C-MMAF	2109	4893	0.4
LC-S168C-MMAF	1688	3619	0.5
LC-E143C-MMAF	1589	3254	0.5
HC-E382C-MMAF	1364	2541	0.5
HC-T155C-MMAF	2930	5308	0.6
HC-S119C-MMAF	2230	4045	0.6

trastuzumab Cys-MMAF ADC	AUC-MMAF (hrs*μg/ml)	AUC-hIgG (hrs*μg/ml)	AUC(MMAF)/AUC(hIgG)
LC-T129C-MMAF	2375	4332	0.6
LC-T109C-MMAF	1588	2716	0.6
LC-K169C-MMAF	2858	4855	0.6
HC-S400C-MMAF	2363	3922	0.6
HC-R355C-MMAF	2344	3777	0.6
HC-R344C-MMAF	1994	3215	0.6
LC-Q199C-MMAF	2042	3261	0.6
LC-S182C-MMAF	2434	3722	0.7
HC-P153C-MMAF	2201	3402	0.7
HC-N286C-MMAF	2286	3535	0.7
HC-T169C-MMAF	2113	3190	0.7
LC-K183C-MMAF	2014	3053	0.7
LC-T197C-MMAF	2126	3177	0.7
LC-K145C-MMAF	2339	3454	0.7
HC-L398C-MMAF	2063	2979	0.7
HC-P189C-MMAF	2042	2968	0.7
HC-S157C-MMAF	2625	3640	0.7
HC-E269C-MMAF	2373	3293	0.7
LC-S159C-MMAF	2063	2809	0.7
LC-E161C-MMAF	1974	2632	0.8
LC-E165C-MMAF	2481	3244	0.8
HC-T164C-MMAF	2514	3290	0.8
LC-R142C-MMAF	2903	3786	0.8
LC-S156C-MMAF	2217	2847	0.8
HC-S207C-MMAF	2378	3001	0.8
LC-N152C-MMAF	2303	2862	0.8
HC-E152C-MMAF	3403	4202	0.8
LC-L154C-MMAF	1959	2387	0.8
LC-K188C-MMAF	2230	2680	0.8
HC-K326C-MMAF	2621	3157	0.8
LC-D170C-MMAF	2048	2420	0.9
HC-K290C-MMAF	2668	3090	0.9
HC-E293C-MMAF	2167	2523	0.9
HC-S124C-MMAF	2107	2463	0.9
HC-K274C-MMAF	3080	3554	0.9
HC-K322C-MMAF	3108	3437	0.9
HC-K334C-MMAF	4527	5048	0.9
HC-K121C-MMAF	2647	2952	0.9
HC-K288C-MMAF	2681	2902	0.9
HC-P171C-MMAF	2312	2481	0.9

trastuzumab Cys-MMAF ADC	AUC-MMAF (hrs*μg/ml)	AUC-hIgG (hrs*μg/ml)	AUC(MMAF)/AUC(hIgG)
LC-K107C-MMAF	2621	2817	0.9
HC-T139C-MMAF	2951	3186	0.9
HC-K360C-MMAF	3791	4014	0.9
HC-S117C-MMAF	2661	2828	0.9
LC-S203C-MMAF	2730	2919	0.9
HC-K392C-MMAF	3148	3302	1.0
HC-S375C-MMAF	2593	2644	1.0
HC-R292C-MMAF	2816	2806	1.0
HC-E333C-MMAF	3850	3796	1.0
HC-L174C-MMAF	2604	2541	1.0
HC-E258C-MMAF	3941	3732	1.1
HC-S337C-MMAF	34.38	32.14	1.1
HC-V422C-MMAF	2662	2424	1.1
HC-K320C-MMAF	3181	2776	1.2
HC-N390C-MMAF	3627	3105	1.2
LC-R108C-MMAF	3711	2992	1.2
LC-S114C-MMAF	n.a.	2567	n.a.
HC-T335C-MMAF	6.71	n.a.	n.a.

n.a: not applicable.

Example 10: Combination of Cys sites to produce antibody drug conjugates with drug-to-antibody-ratios greater than 2.

Antibody conjugates produced through conjugation to lysine residues or partially reduced native disulfide bonds often feature drug-to-antibody-ratios (DAR) of between 3 and 4. Cys engineered antibodies more typically feature a DAR of 2. For certain indications, it may be desirable to produce ADCs with higher DAR which can in principle be achieved by introducing multiple Cys mutations in the antibody. As the number of Cys mutation increases, the likelihood that such mutations interfere with the required re-oxidation process during ADC preparation and hence result in heterogeneous products also increases. In this study, a large number of single site heavy and light chain Cys mutants with good re-oxidation behavior were identified.

To demonstrate that several conjugation sites can be combined for the production of ADCs with DAR greater than two, several preferred single site Cys constructs of light and heavy chain of trastuzumab and antibody 14090 (Table 23) were coexpressed in 293 Freestyle™ cells as described in Example 5. Purified antibodies which all contain one Cys mutation on the heavy chain and one Cys mutation on the light chain were reduced, re-oxidized

and conjugated with MC-MMAF as described in Example 6. Reverse phase high-pressure liquid chromatography demonstrated a single defined elution peak suggesting efficient re-oxidation of the native disulfide bonds. Reverse phase high-pressure liquid chromatography after MC-MMAF conjugation also showed predominantly a single elution peak for the DAR 4 ADC species. The DAR of all ADCs in Table 23 was confirmed to be 4 by mass spectrometry. Production yields varied from 16 to 24 mg/L transient cell culture. The ADCs were predominantly monomeric as determined by analytical size exclusion chromatography; only for 2 of the 8 antibodies could small amounts of aggregates be detected (Table 23). Trastuzumab and 14090 ADCs exhibited antigen-dependent cell killing in MDA-MB231 clone 16 and CMK1105 cell proliferation assays, respectively (Table 23).

Table 23. Properties of Cys engineered MMAF ADCs with DAR of 4.

Cys-MMAF ADC (DAR=4)	LC SEQ ID NO	HC SEQ ID NO	Yield (mg/L)	AnSEC % Monomer	AnSEC % Multimer	IC_{50} MDA-MB231-16 cells (μM)	IC_{50} CMK11-5 cells (μM)
trastuzumab LC-S159C-HC-E258C	75	29	17.3	100	Not detected	4.91e-4	No potency
trastuzumab LC-S159C-HC-S375C	75	50	17.8	100	Not detected	2.44e-4	No potency
trastuzumab LC-E165C-HC-E258C	77	29	16.5	100	Not detected	3.24e-4	No potency
trastuzumab LC-E165C-HC-S375C	77	50	16.9	100	Not detected	2.15e-4	No potency
Antibody 14090 LC-A143C-HC-K360C	92	48	16.1	94.8	5.2	No potency	4.92e-4
Antibody 14090 LC-A143C-HC-S375C	92	50	21.8	100	Not detected	No potency	4.76e-4
Antibody 14090 LC-V159C-HC-K360C	96	48	24.0	100	Not detected	No potency	4.55e-4
Antibody 14090 LC-V159C-HC-S375C	96	50	21.7	97.1	2.9	No potency	3.99e-4

n.d.: not detectable,

no potency: no sign of cell killing at highest concentration evaluated (66 nM)

SEQ ID NOs only specify constant regions of antibody sequences.

Example 11. Selection of Cys sites based on ADC hydrophobicity

To further optimize the selection Cys mutants and mutant combinations for the preparation of ADCs with DAR 2, 4, 6 and 8, the properties of MMAF ADCs prepared with single site trastuzumab Cys and Pcl mutants (Preparation of Pcl ADCs is described in patent application 55573) were analyzed, and accessibility and solvent exposure of conjugation sites was inspected in the crystal structures of IgG.

One of the most informative data was the observation that the hydrophobicity of trastuzumab Pcl-MMAF ADCs varied greatly when the payload was attached different sites (Figure 23). The hydrophobicity of these ADCs was measured by hydrophobic interaction chromatography (HIC) using a TSKgel Phenyl-5PW column (Tosoh Bioscience, TSKgel Phenyl-5PW, 13 m, 21x150 mm, stainless steel, Cat# 07656; running buffer A: 1.5 M ammonium sulfate in 20 mM NaPi (pH7.4); buffer B: 20% isopropanol in 20 mM NaPi (pH7.4); flow rate 5 ml/min; linear gradient from 20% to 80% buffer B over 90 mins; monitored by UV absorbance at 280 nm). Surprisingly, it was observed that retention times of the DAR 2 species varied greatly among ADCs although the only difference is the site of ABA-MMAF attachment (Figure 23). HIC separates molecules on the basis of the hydrophobicity. All DAR 2 ADCs have a HIC retention time larger than that of unconjugated antibody (WT = 45 min, Figure 23) which is to be expected when a hydrophobic drug molecule such as ABA-MMAF is attached to an antibody. However, attaching the payload at different sites increases the hydrophobicity of the ADC to various extends.

The surprisingly large differences in retention times can be rationalized from the inspection of location of the attachment sites on the structure of an antibody (Figure 24): The retention times are higher if the drug payload is attached at an exposed site on the outside of an antibody, for example at HC-K288Pcl, HC-N286Pcl, HC-V422Pcl, HC-L398Pcl and HC-S415Pcl where retention time between 87 and 94 min were measured for the respective ADCs (Figure 23). Conversely, if the payload is attached at an interior site such as the cavity formed between variable and CH1 domains (for examples, HC-P153Pcl, HC-E152Pcl, HC-L174Pcl, HC-P171Pcl, LC-R142Pcl, LC-E161Pcl, LC-E165Pcl, LC-S159Pcl) or the large opening between CH2 and CH3 domains of the antibody (for examples, HC-K246C, HC-S375Pcl, HC-T393Pcl, HC-K334Pcl), the HIC retention time increased to only 47 to 57 mins

because the payload is partially sequestered from interacting with solvent and the HIC column. For other sites, for example, the relatively exposed sites, LC-K107Pcl and HC-K360Pcl, intermediate retention time of 70 and 83 min were measured.

Reducing hydrophobicity of a protein drug is generally considered beneficial because it may reduce aggregation and clearance from circulation. We propose that the HIC data presented in Figure 23 enables selection of preferred payload attachment sites. Conjugating drug payloads at sites where they are sequestered from solvent interactions and attachment minimally increases the hydrophobicity of the antibody upon drug attachment should be beneficial independent of the conjugation chemistry and payload class. Carefully selecting attachment sites that result in minimal changes in hydrophobicity may be particularly beneficial when 4, 6 or 8 drugs are attached per antibody, or when particularly hydrophobic payloads are used.

Cys sites selected for ADCs with low hydrophobicity:

To minimize hydrophobicity of ADCs, sites were chosen that would point toward the interior of the various protein domains of the antibody. Selection was based on analysis of the antibody structure and behavior of the existing ADCs with DAR=2 where applicable (behavior = retention time on HIC and/or delayed retention time on AnSEC with conjugates that interact with SEC resins). Of the Cys sites identified in Table 1 and Table 2, sites listed in Table 24 fulfill the above criteria.

All ADCs were analyzed by hydrophobic interaction chromatography (HIC). Trastuzumab MMAF ADCs conjugated at the exposed sites HC-K360C, LC-K107C, HC-E258C and HC-R292C were used for comparison purposes. The results are shown in Table 25. The trastuzumab Cys-MMAF ADCs and unconjugated, wild-type antibody were analyzed on a TSKgel Butyl-NPR column as described below. For comparison, HIC data previously obtained for Pcl-MMAF ADCs on a TSKgel Phenyl-5PW (Figure 23) are also listed. Despite different instrumentation and protocols, and although some variability is expected due to the different geometry and structures of the two linker, the ratio of retention times for the ADC conjugated at the same position but through different conjugation methods remains nearly constant. The HIC data suggests that retention times are indeed a measure of how well a payload is sequestered in the interior of the antibody

independent of attachment chemistry and linker structure. As expected the relative ranking of the different attachment sites remains largely identical for Pcl-MMAF and Cys-MMAF ADCs.

Attachment to site selected in Table 24, HC-E333C, HC-K392C, and HC-K326C results in MMAF ADCs that have HIC retention times that are similar to the exposed site ADCs LC-K107C-MMAF, HC-E258C-MMAF, HC-R292C-MMAF and HC-K360C-MMAF (Table 28). Attachment to the HC-E152C, LC-E165C, HC-P171C, LC-R142C, LC-E161C, HC-L174C and HC-S124C sites increases the retention time of the resulting ADC by less than 15% compared to the unconjugated, wild-type antibody. These sites are all located on in the CH1 domain or on the light chain (LC) and HIC retention time data suggests them as preferred attachment sites. Of the CH3 domain sites, HC-K334C and HC-S375C exhibit to lowest increase in hydrophobicity upon conjugation making them preferred attachment sites.

Table 24. Cys mutant sites

Cys mutant site	Site (EU No.)
LC-R142C	142
LC-S159C	159
LC-E161C	161
LC-E165C	165
HC-S124C	124
HC-E152C	152
HC-P171C	171
HC-L174C	174
HC-K326C	326
HC-E333C	333
HC-K334C	334
HC-S375C	375
HC-K392C	392

Table 25. Hydrophobic interaction chromatography (HIC) retention time of the DAR 2 species of trastuzumab MMAF ADCs.

Comparing Cys and Pcl conjugation chemistry, the two sets agree well: Sites that hide the drug conjugated by one chemistry also tend to hide the drug when conjugated by the other chemistry. Some variability is expected due to the different geometry of the two linker systems.

Trastuzumab ADC	DAR2 HIC retention[a] (min)	Trastuzumab ADC	DAR2 HIC retention[b] (min)	Ratio
WT	19.5	WT	45	0.43
HC-E152C-MMAF	20.4	HC-E152Pcl-MMAF	50	0.41
LC-E165C-MMAF	20.8	LC-E165Pcl-MMAF	55	0.38
HC-P171C-MMAF	21.0	HC-P171Pcl-MMAF	51	0.41
HC-K334C-MMAF	21.5	HC-K334Pcl-MMAF	56	0.38
HC-S375C-MMAF	21.6	HC-S375Pcl-MMAF	52	0.42
LC-R142C-MMAF	21.7	LC-R142Pcl-MMAF	51	0.42
LC-E161C-MMAF	22.0	LC-E161Pcl-MMAF	55	0.40
HC-L174C-MMAF	22.0	HC-L174Pcl-MMAF	50	0.44
HC-S124C-MMAF	22.4	HC-S124Pcl-MMAF	59	0.38
HC-E333C-MMAF	23.1	HC-E333Pcl-MMAF	63	0.37
HC-K392C-MMAF	23.1	HC-K392Pcl-MMAF	60	0.38
HC-R292C-MMAF	23.8	HC-R292Pcl-MMAF	69	0.35
HC-K326C-MMAF	24.5	HC-K326Pcl-MMAF	72	0.34
LC-K107C-MMAF	24.8	LC-K107Pcl-MMAF	70	0.35
HC-E258C-MMAF	24.9	HC-E258Pcl-MMAF	69	0.36
HC-K360C-MMAF	26.8	HC-K360Pcl-MMAF	83	0.32

[a] Analytical HIC: Tosoh Bioscience (King of Prussia, PA, USA) TSKgel Butyl-NPR column (100 mm × 4.6 mm, 2.5 µm), running buffer A: 50 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0; buffer B: 50 mM sodium phosphate, pH 7.0; gradient consisted of 5 min holding at 100% A, followed by a linear gradient of 20 to 100% B over 40 min; monitored by UV absorption at 280 nm.

[b] Semi-prep HIC: Tosoh Bioscience (King of Prussia, PA, USA), TSKgel Phenyl-5PW, 13 µm, 21x150 mm; running buffer A: 1.5 M ammonium sulfate in 20 mM NaPi (pH7.4); buffer B: 20% isopropanol in 20 mM NaPi (pH7.4); flow rate 5 ml/min; linear gradient from 20% to 80% buffer B over 90 mins; monitored by UV absorption at 280 nm.

Analytical HIC protocol in detail:

Analytical HIC data for trastuzumab Cys-MMAF ADCs were collected using a Tosoh Bioscience (King of Prussia, PA, USA) TSKgel Butyl-NPR column (100 mm × 4.6 mm, 2.5 μm) installed on a Dionex UltiMate 3000 HPLC (Sunnyvale, CA, USA). The method consisted of a binary gradient of buffer A (50 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0) and buffer B (50 mM sodium phosphate, pH 7.0). Samples were prepared by diluting approximately 50 μg of antibody (PBS) with an equal volume of 3 M ammonium sulfate. The gradient consisted of 5 min holding at 100% A, followed a linear gradient of 20 to 100% B over 40 min and finally re-equilibrating at initial conditions for 10 min prior to the next injection. The separation was monitored by UV absorption at 280 nm.

Preparation and characterization of DAR 4, 6 and 8 Cys ADCs

Cys mutations can be combined for the preparation of DAR 4, 6 and 8 ADCs. In general, the preferred combination is a combination of two Cys mutations resulting in ADCs with DAR 4. Some examples that involve combining a heavy chain (HC) Cys mutant with a light chain (LC) Cys mutant for the preparation of DAR 4 ADCs are shown in Example 10 for trastuzumab and for antibody 14090. Additional data is provided in Table 26. Based on the HIC data and the inspection of attachment sites in the IgG crystal structures, additional Cys combinations were prepared using the protocols described in Examples 2, 5 and 6. Data for selected examples of MMAF ADCs are shown in Table 26. In addition, selected heavy chain sites were combined and double Cys mutations of the heavy chain were cloned following protocols listed in Example 2. Antibodies featuring two HC Cys mutations were prepared and conjugated following protocols described in Example 5 and 6.

For the preparation of DAR 4 ADCs, combinations include single site mutations listed in Table 24. Combinations of single sites resulted in ADCs with low hydrophobicity (Table 25). In thesome combination, one Cys mutation is located in the CH1 domain or on the light chain and the second site is located in the CH3 domain. Examples of such combinations are antibodies featuring Cys

mutant combinations of HC-E152C and HC-S375C, and LC-E165C and HC-S375C, and HC-E152C and HC-K334C, and LC-E165C and HC-K334C.

ADCs with DAR 6 and 8 can also be prepared when three or four Cys mutations are combined in one antibody. Selected heavy chain combinations were combined for the preparations of DAR 4, 6 and 8 ADCs. Double and triple Cys mutations of the heavy chain were cloned following protocols listed in Example 2. Antibodies featuring two, three and four Cys mutations were prepared and conjugated following protocols described in Example 5 and 6. The characteristics of some DAR 4, DAR 6 and DAR 8 ADC examples are summarized in Table 26. Some of these ADCs have surprisingly good PK properties as shown in Figure 25. Antibody 14090 is mouse cross-reactive and therefore, antibody 14090 ADCs, as expected, are cleared more rapidly than trastuzumab ADCs which do not bind to any mouse antigens.

Combinations include those with three and four of the single site mutations listed in Table 24. Combinations include those sites that resulted in ADCs with low hydrophobicity (Table 25). Combinations include one Cys mutation is located in the CH1 domain or on the light chain (LC), and optionally an additional one to three sites are in the CH3 domain. Examples of such combinations include antibodies featuring Cys mutant combinations of HC-E152C or LC-E165C, with HC-S375C, with HC-K334C, and/or HC-K392C. Preferred combinations for the preparation of DAR 6 and DAR 8 ADCs are shown in Table 27 and Table 28 respectively.

With a few of exceptions, attachment of MMAF at all Cys sites studied results in ADCs with high thermal stability (Example 7, Table 19), low propensity to aggregate (Example 6, Table 18) and good pharmacokinetic properties of DAR 2 ADCs (Example 9, Table 22, Figure 18). Differences in ADC hydrophobicity apparently do not translate into large differences in biophysical and pharmacokinetic properties when a relatively soluble payload such as MMAF is used. In fact, as is shown above, DAR 4, DAR 6 and DAR 8 MMAF ADCs with acceptable pharmacokinetic properties can be prepared even using exposed, "hydrophobic" sites such as HC-K360C in combination with more preferred attachment sites. However, when less well behaved, more hydrophobic payloads are used, carefully selecting attachment sites that result in minimal changes in hydrophobicity may be essential to allow the preparation of non-aggregating ADCs with good pharmacokinetic properties. For such hydrophobic payloads, using combination of sites that reduce hydrophobicity increases might be beneficial when 4, 6 or 8 drugs are attached per antibody.

Table 26. Characterization of selected DAR 4, 6 and 8 MMAF ADCs prepared with combinations of Cys mutations.

Cys-MMAF ADC name	DAR	% Multimer AnSEC	AUC MMAF/ AUC hIgG
trastuzumab-HC-E258C-LC-S159C-MMAF	4.0	n.d.	0.9
trastuzumab-HC-S375C-LC-S159C-MMAF	4.0	n.d.	0.8
trastuzumab-HC-E258C-LC-E165C-MMAF	4.0	n.d.	0.9
trastuzumab-HC-S375C-LC-E165C-MMAF	4.0	n.d.	0.8
trastuzumab-HC-E152C-LC-R142C-MMAF	3.8	n.d.	0.9
trastuzumab-HC-P171C-LC-R142C-MMAF	3.8	0.1	1.1
trastuzumab-HC-E152C-LC-S159C-MMAF	3.8	n/a	0.7
Antibody 14090-HC-S375C-LC-A143C-MMAF	4.0	n.d.	0.9
Antibody 14090-HC-K360C-LC-V159C-MMAF	4.0	n.d.	1.0
Antibody 14090-HC-S375C-LC-V159C-MMAF	4.0	2.9	1.0
trastuzumab-HC-K334C-S375C-LC-E165C-MMAF	6.0	n.d.	0.8
trastuzumab-HC-K334C-K392C-LC-E165C-MMAF	5.8	11	0.4
trastuzumab-HC-K334C-K360C-S375C-LC-E165C-MMAF	8.0	5	0.6
trastuzumab-HC-K334C-K360C-K392C-LC-E165C-MMAF	7.8	n.d.	0.8
trastuzumab-HC-K334C-S375C-K392C-LC-E165C-MMAF	8.0	n.d.	0.7

*AUC calculations based on mouse PK measurements with anti-MMAF and anti-IgG ELISA assays.

n.d.; not detected, below limited of quantitation.

Table 27. Preferred combinations of Cys sites for the preparation of DAR 6 ADCs.

ADC combination	Site 1	Site 2	Site 3
1	HC-E152C	HC-S375C	HC-K392C
2	HC-E152C	HC-S375C	HC-K334C
3	HC-E152C	HC-K334C	HC-K392C
4	LC-E165C	HC-S375C	HC-K392C
5	LC-E165C	HC-S375C	HC-K334C
6	LC-E165C	HC-K334C	HC-K392C

Table 28. Preferred combinations of Cys sites for the preparation of DAR 8 ADCs.

ADC combination	Site 1	Site 2	Site 3	Site 4
1	HC-E152C	HC-S375C	HC-K334C	HC-K392C
2	HC-E152C	HC-S375C	HC-E333C	HC-K392C
3	LC-E165C	HC-S375C	HC-K334C	HC-K392C
4	LC-E165C	HC-S375C	HC-E333C	HC-K392C

Example 12. *In vivo* efficacy studies of trastuzumab Cys-MMAF ADCs.

In vivo xenograft tumor models simulate biological activity observed iby grafting relevant and well characterized human primary tumors or tumor cell lines into immune-deficient nude mice. Studies on treatment of tumor xenograft mice with anti-cancer reagents have provided valuable information regarding *in vivo* efficacy of the tested reagents (Sausville and Burger, 2006). Since MDA-MB231 clone 16 cells were sensitive to trastuzumab Cys-MMAF ADCs in antigen dependent manner (Figure 15), the cell line was chosen as the *in vivo* model to evaluate the trastuzumab Cys-MMAF ADCs. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication; National Academy Press, 8^{th} edition, 2001). MDA-MB231 clone 16 cells were implanted in nu/nu mice subcutaneously (Morton and Houghton, 2007). After the tumor size reached ~200 mm^3, trastuzumab Cys-MMAF ADCs were administered into the mice by IV injection in a single dose at 3 mg/kg. The tumor growth was measured weekly after ADC injection. Each treatment group included 9 mice. An example of the in vivo efficacy study is indicated in Figure 22 with three trastuzumab Cys-MMAF ADCs. Treatment of mice with 3 mg/kg trastuzumab Cys-MMAF ADCs caused tumor regression for all three tested Cys-MMAF ADCs(Figure 22). No weight loss was observed associated with the ADC treatment. The results confirmed that with a single dose treatment at 3 mg/kg, trastuzumab Cys-MMAF ADCs effectively caused regression of MDA-MB231 clone 16 tumors.

CLAIMS

1. An immunoconjugate comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment comprises a substitution of one or more amino acids with cysteine on its constant region selected from positions 124, 152, 164, 174, and 189 of the heavy chain of said antibody or antibody fragment, and wherein said positions are numbered according to the EU system.

2. The immunoconjugate of claim 1. wherein said antibody or antibody fragment comprises a sequence selected from the group consisting of SEQ ID NOs: 5, 10, 14, 18, and 21.

3. The immunoconjugate of claim 1 or claim 2, wherein said modified antibody or antibody fragment comprises a combination of substitutions of two or more amino acids with cysteine:
   a. on a constant region of a heavy chain at positions 152 and 375, wherein said positions are numbered according to EU system;
   b. on a constant region of a heavy chain at positions 327 and 375, wherein said positions are numbered according to the EU system;
   c. on a constant region at position 107 of a light chain and position 360 of a heavy chain, wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system;
   d. on a constant region at position 375 of a heavy chain and position 165 of a light chain, wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system; or
   e. on a constant region at position 334 of a heavy chain and at position 165 of a light chain, wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

4. The immunoconjugate of any one of claims 1 to 3, comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment thereof comprises a combination of substitution of three or more amino acids with cysteine on its

constant regions wherein the combinations comprise substitutions selected from the group consisting of:

a. positions 375 and 392 of an antibody heavy chain and position 165 of an antibody light chain;
b. positions 334 and 375 of an antibody heavy chain and position 165 of an antibody light chain;
c. positions 334 and 392 of an antibody heavy chain and position 165 of an antibody light chain;
d. positions 152, 375 and 392 of the an antibody heavy chain,
e. positions 152, 334 and 375 of an antibody heavy chain, and
f. positions 152, 334 and 392 of an antibody heavy chain,

wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

5. The immunoconjugate of any one of claims 1 to 4. comprising a modified antibody or antibody fragment thereof, wherein said modified antibody or antibody fragment thereof comprises a combination of substitution of four or more amino acids with cysteine:
   a. on its constant region at positions 334, 375, and 392 of a heavy chain and position 165 of a light chain;
   b. on its constant region at positions 333, 375, and 392 of a heavy chain and at position 165 of a light chain;
   c. on its constant region at positions 152, 334, 375, and 392 of a heavy chain; or
   d. on its constant region at positions 152, 333, 375, and 392 of a heavy chain;

   wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

6. The immunoconjugates of any one of claims 1 to 5, wherein the drug antibody ratio is about 4, about 6 or about 8.

7. The immunoconjugate of any one of claims 1 to 6, further comprising a drug moiety.

8. The immunoconjugate of claim 7, wherein a drug moiety is attached to the modified antibody or antibody fragment through the sulfur of said cysteine and an optional linker.

9. The immunoconjugate of claim 8, wherein said drug moiety is connected to said sulfur of said cysteine through a cleavable or non-cleavable linker.

10. The immunoconjugate of claim 8 or claim 9, wherein said immunoconjugate comprises a thiol-maleimide linkage, a $-S-CH_2-C(=O)-$ linkage or a disulfide linkage.

11. The immunoconjugate of any one of claims 7 to 10, wherein said drug moiety is a cytotoxic agent.

12. The immunoconjugate of claim 11, wherein said drug moiety is selected from the group consisting of taxanes, DNA-alkylating agent, anthracyclines, tubulysin analogs, duocarmycin analogs, auristatins, and maytansinoids.

13. The immunoconjugate of any one of claims 1 to 12, wherein said antibody is a monoclonal antibody, a chimeric antibody, a humanized antibody, a fully humanized antibody, a bispecific antibody, or a multi-specific antibody.

14. The immunoconjugate of any one of claims 1 to 13, wherein said antibody or antibody fragment specifically binds to a cell surface marker characteristic of a tumor.

15. A pharmaceutical composition comprising the immunoconjugate of any one of claims 1 to 14.

16. A modified antibody or antibody fragment thereof comprising a substitution of one or more amino acids with cysteine on its constant region selected from positions 124, 152, 164, 174, and 189 of a heavy chain of said antibody or antibody fragment, and wherein said positions are numbered according to the EU system.

17. The modified antibody or antibody fragment of claim 16, wherein substitution is selected from the group consisting of:

a. positions 152 and 375 of a heavy chain;
b. positions 327 and 375 of a heavy chain;
c. position 107 of a light chain and position 360 of a heavy chain;
d. position 375 of a heavy chain and position 165 of a light chain;
e. at position 334 of a heavy chain and position 165 of a light chain;
f. positions 375 and 392 of a heavy chain and position 165 of a light chain;
g. positions 334 and 375 of a heavy chain and position 165 of a light chain;
h. positions 334 and 392 of a heavy chain and position 165 of a light chain;
i. positions 152, 375 and 392 of a heavy chain;
j. positions 152, 334 and 375 of a heavy chain;
k. positions 152, 334 and 392 of an antibody heavy chain;
l. positions 334, 375, and 392 of a heavy chain and position 165 of a light chain;
m. positions 333, 375, and 392 of a heavy chain and at position 165 of a light chain;
n. positions 152, 334, 375, and 392 of a heavy chain; and
o. positions 152, 333, 375, and 392 of a heavy chain;

wherein said light chain is a kappa chain, and wherein said positions are numbered according to the EU system.

18. A nucleic acid encoding the modified antibody or antibody fragment of any one of claims 1 to 17.

19. A host cell comprising the nucleic acid of claim 18.

20. A method of producing a modified antibody or antibody fragment comprising incubating the host cell of claim 19 under suitable conditions for expressing the antibody or antibody fragment, and isolating said antibody or antibody fragment.

21. Use of the immunoconjugate of any one of claims 1 to 14 in the manufacture of a medicament for treating or preventing cancer in a subject in need thereof.

22. A modified antibody or antibody fragment produced by the method of claim 20.