

efficacious as conventional ADC in xenograft models and are tolerated at higher doses in relevant preclinical models. THIOMAB™ antibodies have been engineered for drug attachment at different locations of the antibody (*e.g.*, specific amino acid positions (*i.e.*, sites) within the light chain-Fab, heavy chain-Fab and heavy chain-Fc). The *in vitro* & *in vivo* stability, efficacy and PK properties of THIOMAB™ antibodies provide a unique advantage over conventional ADCs due to their homogeneity and site-specific conjugation to cytotoxic drugs.

[0010] SUMMARY

[0011] This application includes disclosure of novel, isolated cysteine engineered antibodies comprising a free cysteine amino acid in the heavy chain or light chain (THIOMAB™ antibodies).

[0012] Disclosed is a process to prepare the isolated cysteine engineered antibodies (THIOMAB™ antibodies) by mutagenizing a nucleic acid sequence of a parent antibody by replacing one or more amino acid residues by cysteine to encode the cysteine engineered antibody; expressing the cysteine engineered antibody; and isolating the cysteine engineered antibody.

[0013] Also disclosed are conjugates of isolated cysteine engineered antibodies (THIOMAB™ antibodies) wherein the antibody is covalently attached to a capture label, a detection label, a drug moiety, or a solid support.

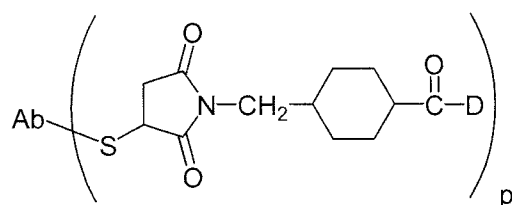
[0013a] In one aspect, there is provided a cysteine engineered antibody comprising a cysteine amino acid in the heavy chain selected from the group consisting of T114C, A140C, L174C, L179C, T187C, T209C, V262C, G371C, Y373C, E382C, S424C, N434C and Q438C according to EU numbering, or a cysteine amino acid in the light chain selected from the group consisting of I106C, R108C, R142C, and K149C according to Kabat numbering.

[0014] In certain embodiments, the disclosure is a cysteine engineered antibody comprising a cysteine mutation selected from the cysteine mutations identified in any of **Tables 1-4** and preferably in either **Table 1** or **2**. In specific embodiments, the cysteine mutation is a free cysteine amino acid. In certain embodiments, the cysteine mutation in the heavy chain selected from the cysteine mutations identified in **Tables 2** or **3**. In certain embodiments, the cysteine mutation in the in the light chain selected from the cysteine mutations identified in **Tables 1** or **4**. In certain embodiments, the cysteine mutation is selected from the group consisting of HC-I195C, HC-S420C, HC-Y432C, and LC-G64C (according to Kabat numbering). In certain embodiments, the cysteine mutation is selected from the group consisting of HC-Y432C and LC-G64C (according to Kabat numbering). In certain embodiments, the cysteine mutation is a heavy chain mutation and is selected from the group consisting of Y33C, G162C, V184C, I195C, S420C, Y432C, and Q434C (according to Kabat numbering). In certain embodiments, the cysteine mutation is a heavy chain mutation and is selected from the group consisting of R19C, E46C, T57C, Y59C,

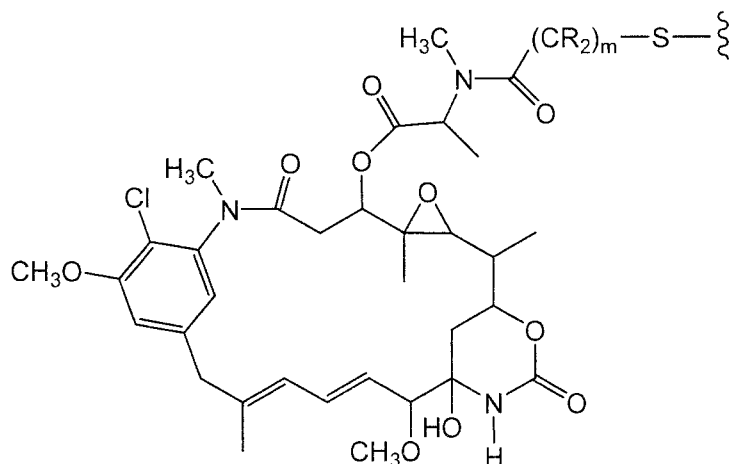
selected from ^3H , ^{11}C , ^{14}C , ^{18}F , ^{32}P , ^{35}S , ^{64}Cu , ^{68}Ga , ^{86}Y , ^{89}Zr , ^{99}Tc , ^{111}In , ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{133}Xe , ^{177}Lu , ^{211}At , and ^{213}Bi . In specific embodiments the antibody is covalently attached to a detection label by a chelating ligand. In specific embodiments the chelating ligand is selected from DOTA, DOTP, DOTMA, DTPA and TETA.

[0022a] In one aspect, there is provided an antibody drug conjugate, wherein the antibody drug conjugate is selected from one of the antibody drug conjugates recited in Table 18 or Table 19.

[0023] In certain embodiments, a cysteine engineered antibody as described herein is covalently attached to a drug moiety selected from a maytansinoid, an auristatin, a dolastatin, a trichothecene, CC1065, a calicheamicin, enediyne antibiotics, a taxane, and an anthracycline to form an antibody-drug conjugate having Formula I (*i.e.*, $\text{Ab}-(\text{L}-\text{D})_p$) where Ab is the antibody, L is a linker, D is the drug moiety, and p is 1, 2, 3, or 4. In specific embodiments the antibody-drug conjugate has the structure:



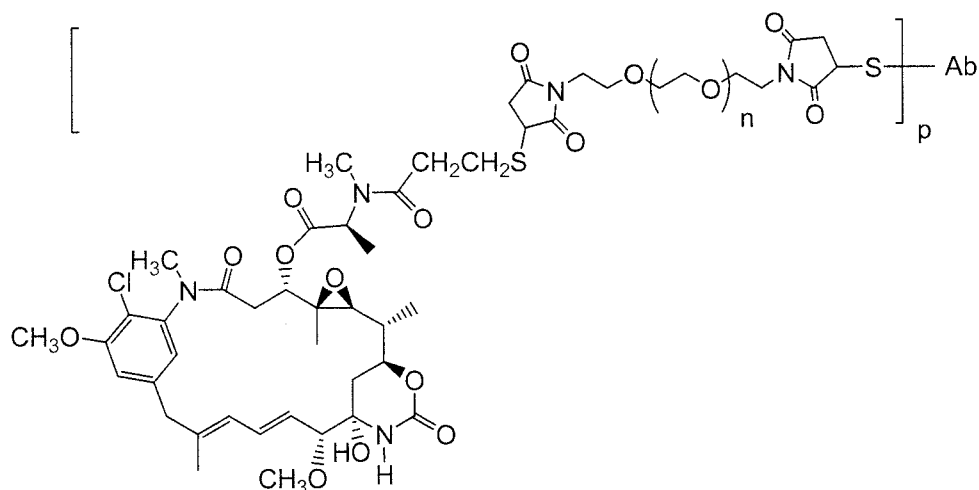
[0024] In specific embodiments, the drug (D) of Formula I of that is conjugated to a cysteine engineered antibody as described herein is a maytansinoid having the structure:



[0025] wherein the wavy line indicates the covalent attachment of the sulfur atom of D to the linker; R is independently selected from H, methyl, ethyl, 1-propyl, 2-propyl, 1-butyl, 2-methyl-1-propyl, 2-butyl, 2-methyl-2-propyl, 1-pentyl, 2-pentyl, 3-pentyl, 2-methyl-2-butyl, 3-methyl-2-butyl, 3-methyl-1-butyl, 2-methyl-1-butyl, 1-hexyl, 2-hexyl, 3-hexyl, 2-methyl-2-pentyl, 3-methyl-2-pentyl, 4-methyl-2-pentyl, 3-methyl-3-pentyl, 2-methyl-3-pentyl, 2,3-dimethyl-2-butyl, and 3,3-dimethyl-2-butyl; and m is 1, 2, or 3.

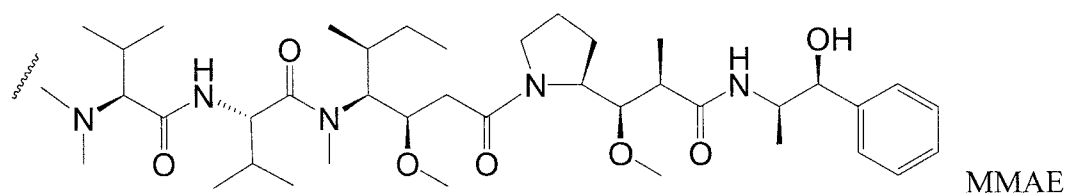
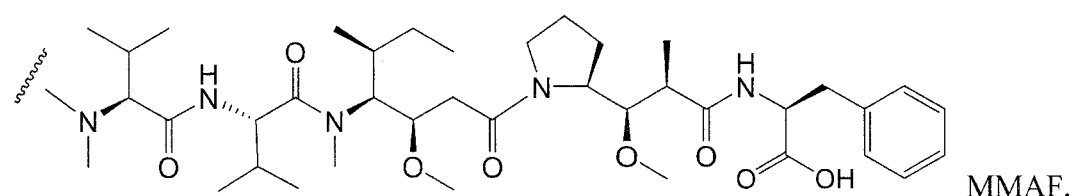
[0026] In specific embodiments, the drug (D) of Formula I of that is conjugated to a cysteine engineered antibody as described herein is selected from the structures:

cysteine engineered antibody as described herein has the following structure:

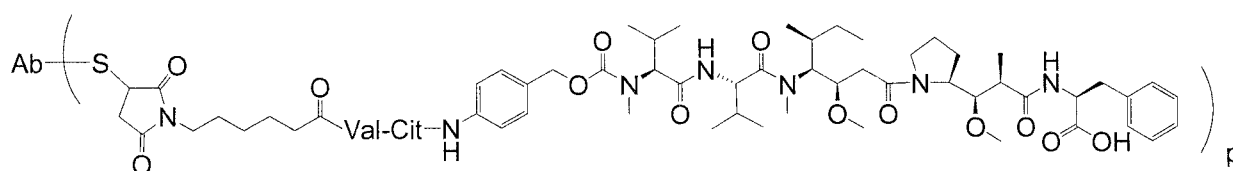


wherein n is 0, 1, or 2.

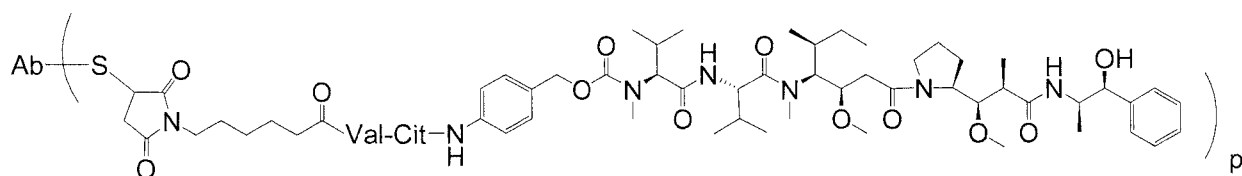
[0028] In specific embodiments, the drug (D) of Formula I of that is conjugated to a cysteine engineered antibody as described herein is a monomethylauristatin drug moiety MMAE or MMAF having the structures:



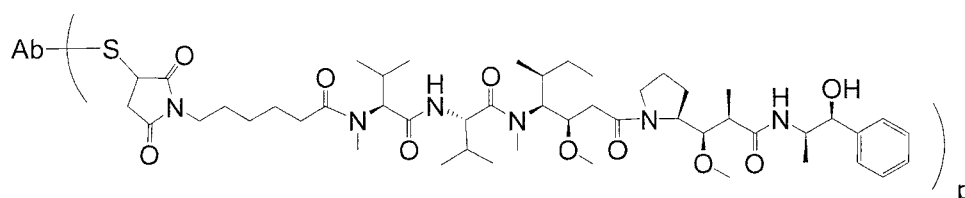
[0029] In certain embodiments, a cysteine engineered antibody as described herein has one of the following structures:



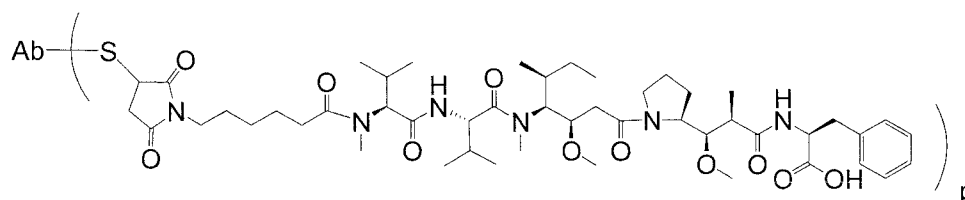
Ab-MC-vc-PAB-MMAF



Ab-MC-vc-PAB-MMAE

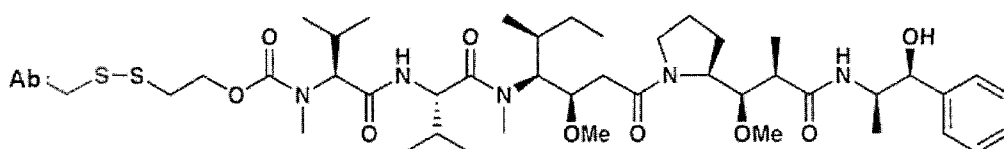


Ab-MC-MMAE



Ab-MC-MMAF

where Val is valine; Cit is citrulline; and p is 1, 2, 3, or 4. In certain embodiments, a cysteine engineered antibody as described herein has the following structures:



[0030] In certain embodiments, a cysteine engineered antibody as described herein is conjugated to a drug that falls into one of the following classes, that is described in further detail in a later section of this specification: auristatins, dolastatins, trichothecenes, CC1065, calicheamicins, enediyne antibiotics, taxanes, pyrrolobenzodiazepines (PBDs), 1-(Chloromethyl)-2,3-dihydro-1H-benzo[e]indole (CBI) dimers, CBI-PBD heterodimers, and anthracyclines.

[0031] In certain embodiments, the linker (L) of a cysteine engineered antibody as described herein comprises a thiol-reactive agent. In specific embodiments, the linker (L) is

selected from the group consisting of a maleimide, an iodoacetamide, and a pyridyl disulfide. In specific embodiments, the linker (L) is a pyridyl disulfide. In specific embodiments, the linker (L) is a pyridyl disulfide and the drug (D) is MMAE.

[0031a] In one aspect, there is provided a method of preparing an antibody-drug conjugate comprising reacting at least one cysteine of a cysteine engineered antibody (Ab) with a linker-drug intermediate to form an antibody-drug conjugate having Formula I:



wherein Ab is any cysteine engineered antibody as described herein, L is a linker, D is a drug moiety, and p is 1, 2, 3, or 4; and wherein the cysteine engineered antibody comprises one or more cysteine amino acids.

[0032] In certain embodiments, this disclosure comprises a method of preparing an antibody-drug conjugate comprising reacting at least one free cysteine of a cysteine engineered antibody (Ab) with a linker-drug (L-D) reagent to form an antibody-drug conjugate having Formula (*i.e.*, $\text{Ab}-(\text{L}-\text{D})_p$) wherein Ab is the cysteine engineered antibody, L is a linker, D is a drug moiety, and p is 1, 2, 3, or 4; and wherein the cysteine engineered antibody comprises one or more free cysteine amino acids, wherein at least one free cysteine amino acid is selected from the cysteine mutations identified in any of **Tables 1 or 2** or alternatively is selected from an alternate stable cysteine mutation identified in **Tables 3 or 4**. In certain embodiments, this disclosure comprises a method of preparing an antibody-drug conjugate comprising reacting at least one free cysteine of a cysteine engineered antibody (Ab) with a linker-drug (L-D) reagent to form an antibody-drug conjugate having Formula (*i.e.*, $\text{Ab}-(\text{L}-\text{D})_p$) wherein Ab is the cysteine engineered antibody, L is a linker, D is a drug moiety, and p is 1, 2, 3, or 4; and wherein the cysteine engineered antibody comprises one or more free cysteine amino acids, wherein at least one free cysteine amino acid is preferably selected from the cysteine mutations identified in **Tables 1 and 2** or alternatively is selected from an alternate stable cysteine mutation identified in **Tables 3 or 4**.

[0033] In specific embodiments, the methods can be used to make a cysteine engineered antibody, wherein the in the cysteine mutation is in the heavy chain and is selected from the cysteine mutations identified in **Tables 2 or 3**. In specific embodiments, the methods can be used to make a cysteine engineered antibody, wherein the in the cysteine mutation is in the light chain and is selected from the cysteine mutations identified in **Tables 1 and 4**. In a preferred embodiment, the methods can be used to make a cysteine engineered antibody, wherein the in the cysteine mutation is selected from the cysteine mutations identified in **Figure 21**. In other embodiments, the methods can be used to make a cysteine engineered antibody, wherein the in the cysteine mutation is selected from the cysteine mutations identified in **Table 5**. In certain embodiments, the methods can be used to make a cysteine engineered antibody, wherein the in the cysteine mutation is selected from the group consisting of HC-I195C, HC-S420C, HC-Y432C, and LC-G64C (according to Kabat

and **21** and **Table 2**).

[0053] In a preferred embodiment, any cysteine engineered antibody as described herein has one of the following cysteine mutations: LC-K149C according to Kabat numbering and HC-A140C according to EU numbering (*see* **Tables 1** and **2** and **Figures 1a** and **1b**).

[0054] In certain embodiments, any cysteine engineered antibody as described herein has a thiol reactivity value of 0.8-1.0. In certain embodiments, any cysteine engineered antibody as described herein has a thiol reactivity value of 0.9-1.0. In certain embodiments, any cysteine engineered antibody as described herein has a thiol reactivity value of 0.6-0.9. In certain embodiments, any cysteine engineered antibody as described herein has a thiol reactivity value of 0.5-0.7. In certain embodiments, any cysteine engineered antibody as described herein has a thiol reactivity value of 0.4-0.6. In certain embodiments, any cysteine engineered antibody as described herein has a thiol reactivity value of 0.3-0.5. In certain embodiments, any cysteine engineered antibody as described herein has a thiol reactivity value of 0.2-0.4.

[0055] In one aspect, there is provided a pharmaceutical composition comprising any cysteine engineered antibody or antibody drug conjugate as described herein.

[0056] BRIEF DESCRIPTION OF THE DRAWINGS

[0057] **Figure 1a** shows the Kabat numbering scheme for the 4D5 light chain.

Figure 1b shows a sequential numbering scheme (left row) starting at the N-terminus in comparison with the Kabat numbering scheme (middle row) and EU numbering scheme (right row) for the 4D5 antibody.

[0058] **Figure 2** shows exemplary sites for cysteine mutagenesis and drug conjugation in an antibody. The bold and underlined residues are exemplary sites for cysteine mutagenesis. The underlined residues are additional exemplary sites for cysteine mutagenesis. Figure 2A shows the exemplary and additional exemplary residues for cysteine mutagenesis in the heavy chain. Figure 2B shows the exemplary and additional exemplary residues for cysteine mutagenesis in the light chain. The preferred sites for cysteine mutagenesis are shaded in grey and include HC-T114C, HC-A140C, HC-L174C, HC-L179C, HC-T187C, HC-T209C, HC-V262C, HC-G371C, HC-Y373C, HC-E382C, HC-S424C, HC-