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本国际公布:
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WO 2018/064964 A1

(54) **Title:** CYSTEINE MODIFIED ANTIBODY-DRUG CONJUGATE AND PREPARATION METHOD THEREOF

(54) **发明名称**: 半胱氨酸改造的抗体-毒素偶联物及其制备方法

(57) **Abstract:** By inserting cysteine (C) into a heavy chain and light chain of a target antibody, and performing site-specific conjugation through a free thiol group (-SH) of the site-specific inserted cysteine and a linker conjugated with a highly active small molecule cytotoxin, a cysteine modified antibody-drug conjugate with good homogeneity is formed. The insertion sites of cysteine are position 205 and/or position 206 (Kabat numbering) of the light chain of the antibody, and/or position 439 (Kabat numbering) of the heavy chain.

(57) **摘要**: 将目标抗体的重链和轻链定点插入半胱氨酸(C)，通过定点插入的半胱氨酸的游离巯基(-SH)与偶联了小分子高活性细胞毒素的连接子进行定点偶联，形成均一性优良的半胱氨酸改造的抗体-毒素偶联物。所述半胱氨酸插入位点为抗体轻链第205位和/或第206位(Kabat编号)，和/或重链第439位(Kabat编号)。

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CYSTEINE MODIFIED ANTIBODY-DRUG CONJUGATE AND PREPARATION METHOD THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application is a national stage application of International application number PCT/CN2017/104706, filed September 30, 2017, which claims the priority benefit of Chinese Patent Application No. 201610876568.9, filed on October 8, 2016, the entire disclosures of which are expressly incorporated by reference herein.

TECHNICAL FIELD

The present disclosure relates to therapeutic compounds and methods for the making thereof, and in particular to cysteine modified antibody-cytotoxin conjugates and methods of making thereof.

BACKGROUND

Reference to any prior art in the specification is not an acknowledgement or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be combined with any other piece of prior art by a skilled person in the art.

Antibody-drug conjugate (ADC) is a hotspot for targeted therapy. Two drugs, Adcetris and Kadcyla, have been approved for marketing in the United States, and have shown good clinical efficacy. There are more than 50 ADC drugs in Clinical trial stage.

SUMMARY

The present disclosure provides cysteine modified antibody-cytotoxin conjugates (TDC), the methods of making the antibody-cytotoxin conjugates, and methods of using thereof.

In one aspect, the application provides cysteine modified antibody-cytotoxin conjugates. In one embodiment, the cysteine insertion site includes one or more of the following three insertion sites or insertion positions in the target antibodies: light chain position 205 (Kabat numbering scheme, wherein the surrounding amino acid sequence comprises GLSSPCVTKSF, with C being the inserted cysteine), light chain 206 (Kabat numbering scheme, wherein the surrounding amino acid sequence comprises GLSSPVCTKSF, with C being the inserted cysteine), and heavy chain position 439 (474 Kabat numbering scheme, wherein the surrounding amino acid sequence comprises YTQKSLSCLSPGK, with C being the inserted cysteine).

An antibody comprising one or more of the above cysteine insertion mutations retains the ability to bind the antigen as the parental antibody does (affinity). In one embodiment, the present disclosure provides the site-directed coupling of antibody-cytotoxin conjugate (TDC) by a cysteine thiol group with a linker-drug (i.e., linker-cytotoxin), wherein the thiol group is from the cysteine inserted into position 205 or/and position 206 of the light chain or/and position 439 of the heavy chain.

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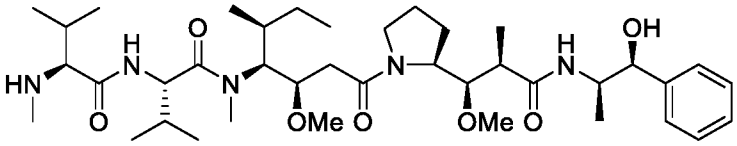
In one embodiment, the application provides cysteine modified antibody-cytotoxin conjugate, comprising an antibody that includes a site-specific inserted cysteine, where cysteine insertion site comprises one or more sites selected from the following three insertion sites: kappa/lambda light chain constant region position 205 (Kabat numbering scheme), kappa/lambda light chain constant region position 206 (Kabat numbering scheme), or the IgG antibody heavy chain constant region position 439 (474 Kabat numbering scheme).

The amino acid sequence surrounding the cysteine insertion site includes one or more of the following three sequences: LC-205ins:GLSSPCVTKS; LC-206ins:GLSSPVCTKSF or HC-439ins:TQKSLSCLSPGK.

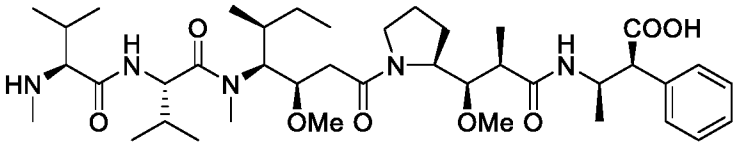
In one embodiment, a highly active cytotoxin is conjugated through a linker to a free thiol group from the modified cysteine inserted into specific cysteine insertion sites of the antibody, wherein the antibody light chain comprises amino acid sequence of GLSSPCVTKSF or GLSSPVCTKSF, and the antibody heavy chain comprises amino acid sequence of TQKSLSCLSPGK, and wherein the C is the cysteine inserted into the light chain position 205, the light chain position 206, or heavy chain position 439 of the antibody.

In one embodiment, the antibody light chain comprises a kappa (k) or a lambda (λ) isotype. In one embodiment, the antibody heavy chain comprises IgG1, IgG2, IgG3 or IgG4. In one embodiment, the inserted cysteine comprises a thiol group (-SH). In one embodiment, the thiol group (-SH) is capable of chemical conjugation.

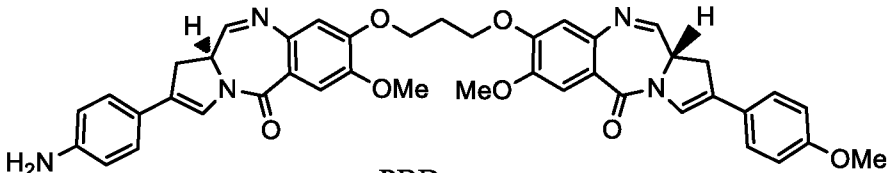
In one embodiment, a low molecular weight, high activity cytotoxin is site-specifically linked to the free thiol group of the inserted cysteine via a linker; the low molecular weight, high activity cytotoxin may include, without limitation, MMAE, MMAF, PBD, SN-38, Dox, and their derivatives thereof. The formula of example cytotoxins, MMAE, MMAF, PBD, SN-38, Dox, are shown below:



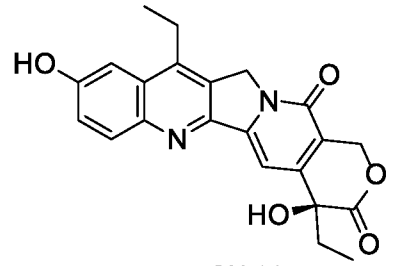
MMAE



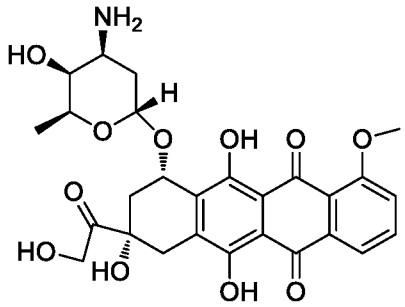
MMAF



PBD



SN-38



DOX

In a further aspect, the application provides methods producing cysteine modified antibody-cytotoxin conjugates. In one embodiment, the method includes the steps of: reducing the antibody with a reducing reagent (such as DTT, TCEP and the like) to provide a reduced antibody, removing the shielding group from the inserted cysteine of the antibody to provide free thiol group; removing the reducing reagent and the removed shielding group by cation exchange chromatography or ultrafiltration; oxidizing the reduced antibody with an oxidant (such as DHAA, $CuSO_4$) to re-connect interchain disulfide bonds of the antibody; adding a linker-drug (i.e., linker-cytotoxin) to conjugate with the free thiol group from the modified cysteine ; and removing unconjugated linker-drug by cation exchange chromatography or ultrafiltration.

Amino Acid List:

Name	Symbol and Abbreviation
Alanine	A and Ala
Arginine	R and Arg
Asparagine	N and Asn
Aspartic acid	D and Asp
Cysteine	C and Cys
Glutamine	Q and Gln
Glutamic acid	E and Glu
Glycine	G and Gly
Histidine	H and His
Isoleucine	I and Ile

Leucine	L and Leu
Lysine	K and Lys
Methionine	M and Met
Phenylalanine	F and Phe
Proline	P and Pro
Serine	S and Ser
Threonine	T and Thr
Tryptophan	W and Trp
Tyrosine	Y and Tyr
Valine	V and Val

SEQ ID NO:6 LC-Cys205insc light chain constant region (Kappa) amino acid sequence
>LC-Cys205ins-Kappa
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSP**C**VTKSFNRGEC
wherein, the C in the GLSSPVCTKSFN is the site-specific conjugation position. In one embodiment, the cysteine is conjugated with mc-vc-PAB-payload site-specifically.
SEQ ID NO:8 LC-Cys206insc light chain constant region (Kappa) amino acid sequence
>LC-Cys206ins-Kappa
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSPV**C**TKSFNRGEC
wherein, the C in the GLSSPVCTKSFN is the site-specific conjugation position. In one embodiment, the cysteine is conjugated with mc-vc-PAB-payload site-specifically.
SEQ ID NO:10 IgG1-Fc-Cys439insheavy chain constant region (Fc) amino acid sequence
>IgG1-Fc-Cys439ins
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEALHNHYTQKSLS**C**LSPGK
wherein, the C in the TQKSLSCLSPGK is the site-specific conjugation position. In one embodiment, the cysteine is conjugated with mc-vc-PAB-payload site-specifically.
SEQ ID NO:12 LC-V205C light chain constant region (Kappa) amino acid sequence
>LC-V205C-Kappa
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSPCTKSFNRGEC
wherein, the C in the GLSSPCTKSFN is the site-specific conjugation position. In one embodiment, the cysteine is conjugated with mc-vc-PAB-payload site-.

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The present disclosure disclosed a novel cysteine modified antibody-cytotoxin conjugate (TDC) that, when compared to non-site-specific conjugated ADC, provides the significant advantages including, without limitation, good homogeneity and low side effect. Preclinical research confirmed that these novel antibody conjugates are significantly superior to non-site-specific conjugated ADC.

The aspects and advantages of the present application will become apparent from the following detailed description of preferred embodiments thereof in connection with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other features of this disclosure will become more fully apparent from the following description and appended claims, taken in conjunction with the accompanying drawings. Understanding that these drawings depict only several embodiments arranged in accordance with the disclosure and are, therefore, not to be considered limiting of its scope, the disclosure will be described with additional specificity and detail through use of the accompanying drawings, in which:

FIGURE 1 is an illustration showing the test result of detecting and measuring 2A1-LC-Cys205ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 2 is an illustration showing the test result of detecting and measuring 2A1-LC-Cys206ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 3 is an illustration showing the result of detecting and measuring 2A1-HC-Cys439ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 4 is an illustration showing the test result of detecting and measuring 4E1-LC-Cys205ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 5 is an illustration showing the test result of detecting and measuring 4E1-LC-Cys206ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 6 is an illustration showing the test result of detecting and measuring 4E1-HC-Cys439ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 7 is an illustration showing the test result of detecting and measuring 4D3-LC-Cys205ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 8 is an illustration showing the result of detecting and measuring 4D3-LC-Cys206ins-mc-vc-PAB-MMAE by HIC-HPLC method, as performed in Example 25;

FIGURE 9 is an illustration showing the test result of detecting and measuring toxin/antibody ratio of 4D3-HC-Cys439ins-mc-vc-PAB-MMAE by RP-HPLC method, as performed in Example 26.

FIGURE 10 is an illustration showing the test result of detecting and measuring TDC antibody skeleton 4D3 aggregation by SEC-HPLC method, as performed in Example 27;

FIGURE 11 is an illustration showing the result of detecting and measuring TDC antibody skeleton 4D3-LC-Cys205ins aggregation by SEC-HPLC method, as performed in Example 27;

FIGURE 12 is an illustration showing the test result of detecting and measuring TDC antibody skeleton 4D3-LC-Cys206ins aggregation by SEC-HPLC method, as performed in Example 27;

FIGURE 13 is an illustration showing the test result of detecting and measuring TDC antibody skeleton 4D3-HC-Cys439ins aggregation by SEC-HPLC method, as performed in Example 27;

FIGURE 14 is an illustration showing the test result of Example 28;

FIGURE 15 is an illustration showing the test result of Example 29, showing the affinity measurement between the antigen c-met and the antibody 4E1 and TDC 4E1-LC-Cys205ins-MVPM, 4E1-LC-Cys206ins-MVPM, and 4E1-HC-Cys439ins-MVPM;

FIGURE 16 is an illustration showing the test result of Example 29, showing the affinity measurement between the antigen Trop2 and the antibody 4D3 and TDC 4D3-LC-Cys205ins-MVPM, 4D3-LC-Cys206ins-MVPM, and 4D3-HC-Cys439ins-MVPM;

FIGURE 17 shows the IC_{50} of cytotoxicity of the ADCs against cancer cells, wherein the ADCs are 2A1-LC-V205C-mc-vc-PAB-MMAE, 2A1-LC-Cys205ins-mc-vc-PAB-MMAE, 2A1-LC-Cys206ins-mc-vc-PAB-MMAE, and 2A1-HC-Cys439ins-mc-vc-PAB-MMA, and the cancer cells are EGFRwt-overexpressing Human squamous cell carcinoma A431;

FIGURE 18 shows the I_{50} of cytotoxicity of the ADCs against cancer cells, wherein the ADCs are 2A1-LC-V205C-mc-vc-PAB-MMAE, 2A1-LC-Cys205ins-mc-vc-PAB-MMAE, 2A1-LC-Cys206ins-mc-vc-PAB-MMAE, and 2A1-HC-Cys439ins-mc-vc-PAB-MMAE, and the cancer cells are EGFRvIII-overexpressing Human glioma cell line U87-EGFRvIII;

FIGURE 19 shows the IC_{50} of cytotoxicity of the ADCs against cancer cells, wherein the ADCs are 4E1-LC-Cys205ins-mc-vc-PAB-MMAE, 4E1-LC-Cys206ins-mc-vc-PAB-MMAE, 4E1-HC-Cys439ins-mc-vc-PAB-MMAE, and 4E1, and the cancer cells are C-met high-expressing malignant glioma cell line U87-MG;

FIGURE 20 shows the IC_{50} of cytotoxicity of the ADCs against cancer cells, wherein the ADCs are D3-LC-Cys205ins-mc-vc-PAB-MMAE, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE, and 4D3, and the cancer cells are trop2 high-expressing pancreatic cancer cell line BXPC-3;

FIGURE 21 is an illustration showing the test result of stability measurement in human plasma for 4D3-LC-Cys205ins-mc-vc-PAB-MMAE;

FIGURE 22 is an illustration showing the test result of stability measurement in human plasma for 4D3-LC-Cys206ins-mc-vc-PAB-MMAE;

FIGURE 23 is an illustration showing the test result of stability measurement in human plasma for 4D3-HC-Cys439ins-mc-vc-PAB-MMAE;

FIGURE 24 is an illustration showing the test result of stability measurement in human plasma for 4E1-LC-Cys205ins-mc-vc-PAB-MMAE;

FIGURE 25 is an illustration showing the test result of stability measurement in human plasma for 4E1-LC-Cys206ins-mc-vc-PAB-MMAE;

FIGURE 26 is an illustration showing the test result of stability measurement in human plasma for 4E1-HC-Cys439ins-mc-vc-PAB-MMAE;

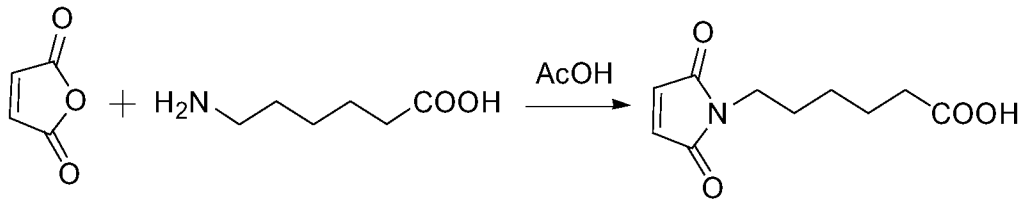
FIGURE 27 is an illustration showing the test result of pharmacodynamic effect measurement for 4D3-LC-Cys205ins-mc-vc-PAB-MMAE, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE, and 4D3 parental antibody in tumor-bearing mice; and

FIGURE 28 is an illustration showing the test result of pharmacodynamic effect measurement for 4D3-LC-Cys205ins-mc-vc-PAB-MMAE, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE, and 4D3 parental antibody in tumor-bearing mice.

DETAILED DESCRIPTION OF THE EMBODIMENTS

In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented herein. It will be readily understood that the aspects of the present disclosure, as generally described herein, and illustrated in the FIGUREs, can be arranged, substituted, combined, separated, and designed in a wide variety of different configurations, all of which are explicitly contemplated herein.

Example 1: Synthesis and preparation of mc

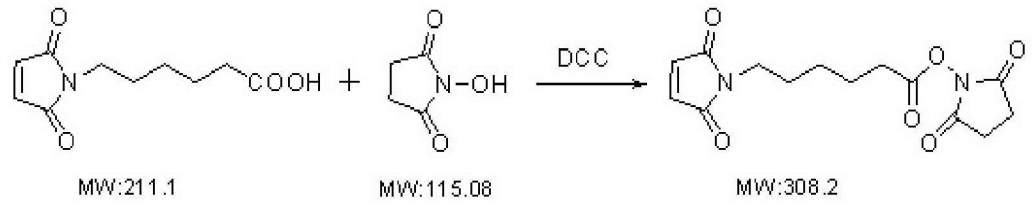


MW : 98.06 MW : 131.17 MW:211.1

6-aminocaproic acid (3.9 g, 0.03 mol) and maleic anhydride (3.5 g, 0.036 mol) were added to glacial acetic acid (30 ml). After stirring at 120°C for 4-6 h, the reaction was cooled to room temperature. Most of the acetic acid was removed by concentration in vacuum at 60 °C. The obtained brownish yellow viscous liquid was poured into water, and then extracted with ethyl acetate (20 ml × 3), and the organic layers were combined. The organic layers were washed with water and brine, dried over anhydrous sodium sulfate, filtered and evaporated in vacuo to yield a brown-yellow oil, which was stirred in 50 ml of water, and white solid materials precipitated out of the solution, the white solid materials is filtered, and the product was dried under reduced pressure at 50 ° C, 5.08 g, yield 80%. Mp: 89-92 °C. m/z: 212.2 [M+H]+. 1H NMR (400Mz, DMSO):

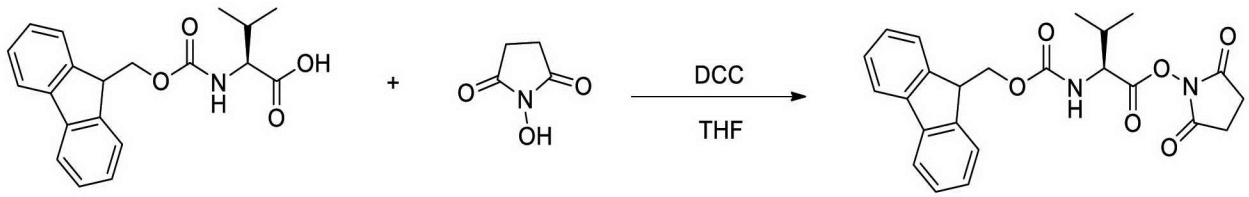
13.21 (br, 1H, COOH), 6.75 (s, 2H, COCH=CHCO), 3.63 (t, 2H, J = 7.2 Hz, NCH2CH2), 2.42 (t, 2H, J = 7.4 Hz, CH2COOH), 1.52 - 1.68 (m, 4H, NCH2CH2CH2CH2), 1.30 - 1.42 (m, 2H, NCH2CH2CH2CH2).

Example 2: Synthesis and preparation of Mc-OSu



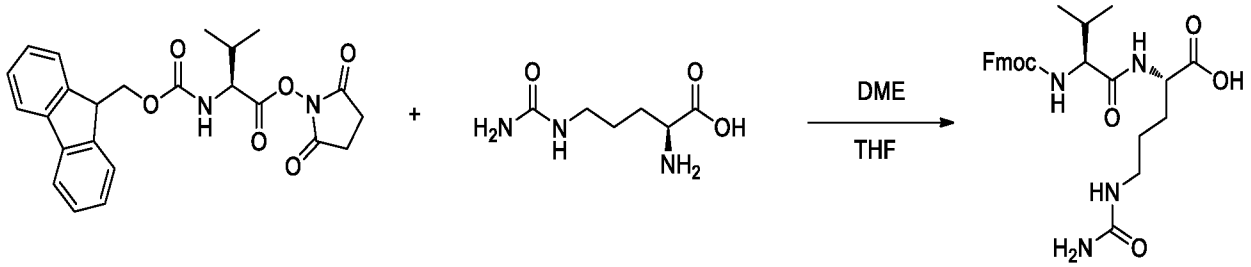
Under nitrogen atmosphere, to a solution of a mixture of MC (4.7 g, 22 mmol) and HOSu (25 g, 22 mmol) in acetonitrile (50 mL) at 0°C was slowly added DCC (4.5 g, 22 mmol) dissolved in 25 ml acetonitrile. The reaction solution was reacted at 0 ° C for 2 hours and then allowed to reacted at room temperature overnight. After filtering, the filter cake was washed with acetonitrile (10 ml × 3). The filtrate was concentrated to dry under reduced pressure. The obtained oil was dried under reduced pressure at room temperature for 6 h to afford 6.4 g of pale brown solid, and yield 95%. (To be used directly in the next step without purification) m/z：309.2 [M+H]+。1HNMR（400Mz ,CDCl3）：1～2（m,6H,CCH2CH2CH2C）, 2.68 (t,2H,CH2CO, 2.95（s,4H,COCH2CH2CO）, 3.68（t,2H,CH2N）, 6.81（s,2H,CH=CH）

Example 3: Synthesis and preparation of Fmoc-Val-OSu



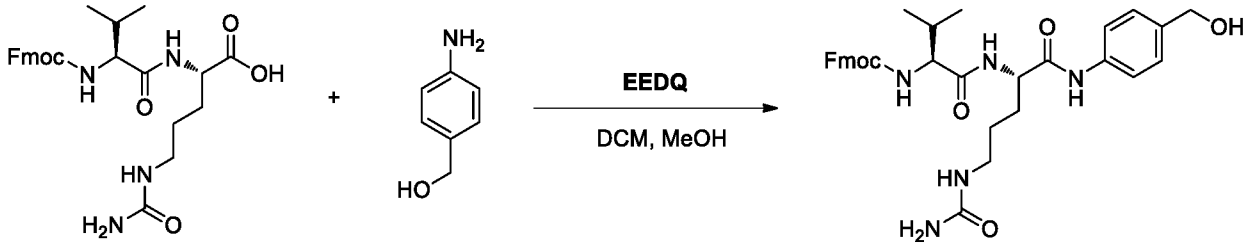
To a solution of a mixture of Fmoc-Val (10 g) and HOSu (3.4 g) in THF (100 mL) at 0°C was slowly added DCC (6 g) dissolved in 50 ml acetonitrile. The reaction solution was stirred at room temperature for 24 hours. Perform filtration, and the filter cake was washed with THF. A transparent oil was obtained by concentrating the filtrates under reduced pressure. The oil was directly used in the next step directly without further purification. m/z：437.4 [M+H]+.

Example 4: Synthesis and preparation of Fmoc-vc



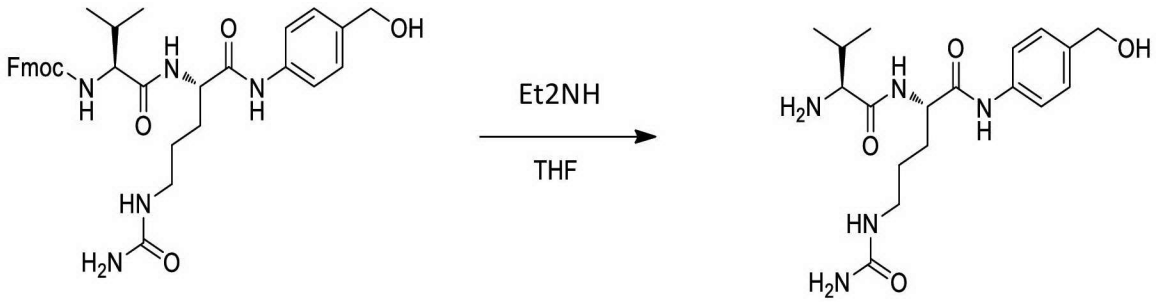
To a solution of Cit (4.0 g) in THF (20 mL) was added a solution of 60 ml aqueous sodium hydrogencarbonate (containing $NaHCO_3$ 2 g, 1.05 eq). Asolution of Fmoc-Val-OSu (22.35 mmol) in DME (60 mL) was added to the mixture. After stirred at room temperature for 24 hours, the reaction was added a solution of 15% aqueous citric acid solution (110 ml), and then extracted with EtOAc twice. The combined organic phases were concentrated in vacuum to get a white solid. 100 ml of methyl tert-butyl ether was added to the white material, the mixture was stirred, filtered, and the filter cake was dried under reduced pressure at 40 ° C for 4 h to obtain the product 4.83 g, and yield 65%. m/z： 497.6 (M+H)+。1HNMR(400Mz,DMSO): 0.92 (6H, m), 1.35 ~ 1.65 (4H, m), 2.10 (1H, m), 3.01(2H, q), 3.99 (1H, t), 4.01 -4.45 (2H, m), 4.45 (2H, t), 5.46 (2H, br), 6.03(1H, t), 7.20-8.02 (8H, m), 8.25 (1H, d).

Example 5: Synthesis and preparation of Fmoc-vc-PABOH



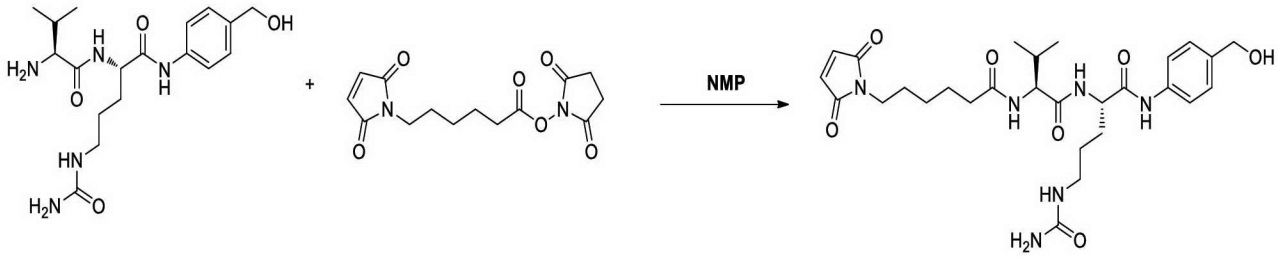
To a solution of Fmoc-vc (2 g, 4.2 mmol) and PABOH (1.04 g, 2 eq) in DCM/MeOH = 2/1 (60 mL) was added EEDQ (2.0 g, 2 eq) at 0 °C. After stirred for 10 min, a solution of (S)-1-phenylethanamine (17.5 g, 144.2 mmol) in MeOH (200 mL) was added slowly to the mixture after partial dissolution. The reaction system was stirred at room temperature for 2 days in the dark. After completion of the reaction, the mixture was concentrated in vacuum at 40 °C to yield a white solid. The white solid was collected, washed with methyl tert-butyl ether (100 ml), and filtered. The filter cake was washed with methyl tert-butyl ether, and the obtained white solid was dried under reduced pressure at 40 °C to give the white solid 2.2 g, and yield 88%. m/z： 602.6 (M+H)+. ^{1}HNMR (400Mz, DMSO): 0.95 (6H,m), 1.45~1.69 (4H, m), 2.10 (1H, m), 3.11(2H, m), 3.99 (1H, m), 4.30 (2H, d), 4.05~-4.66 (2H, m), 4.55 (2H, d), 5.21 (1H, t), 5.51 (2H, br), 6.11(1H, t), 7.09 -8.10 (12H, m), 8.21 (1H, d), 10.51(1H, br).

Example 6: Synthesis and preparation of vc-PABOH



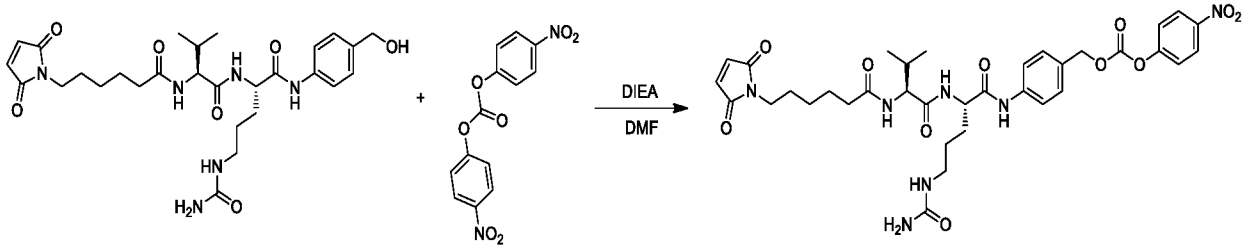
To a solution of Fmoc-vc-PABOH (490 mg, 0.815 mmol) in THF (10 mL) was added diethylamine (2 ml). The reaction mixture was stirred at room temperature for 24 h. 20 ml of DCM was added to the obtained product, the mixture was stirred, and crystalline was precipitated out of reaction solution. Filter the crystalline and the filter cake was washed with DCM, and the obtained solid was dried under reduced pressure to yield 277 mg. The yield was 90%. m/z： 380.2 (M+H)+。1HNMR (400Mz, DMSO): 0.89 (6H, m), 1.31~1.61 (4H, m), 1.82 (1H, m), 2.86 (1H, m), 2.89(2H, d), 4.38 (2H, d), 4.44 (1H, m), 5.01 (1H, br), 5.35 (2H, br), 5.84 (1H, br), 7.14 (2H, d), 7.42 (2H, d), 8.08 (1H, br), 9.88 (1H, br).

Example 7: Synthesis and preparation of mc-vc-PABOH



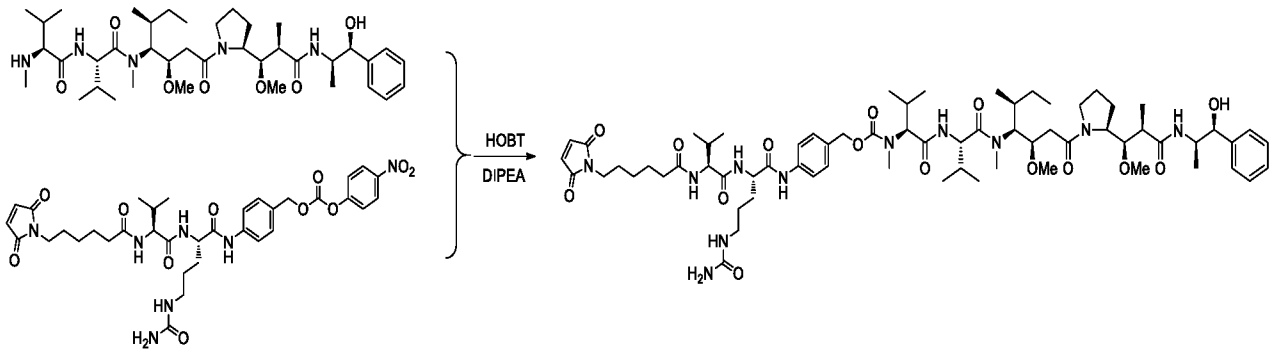
VP-PABOH (205 mg, 0.54 mmol) and MC-OSu (184 mg, 1.1 eq) were added to 10 ml of NMP, and the reaction was stirred at room temperature for 24 h. After completion of the reaction, the mixture was concentrated in vacuo at 40 ° C. Methyl tert-butyl ether (20 ml) was added to the obtained oil and stirred to crystallization. After filtering the crystalline and washing the filter cake with methyl tert-butyl ether, the product was yielded at 310 mg. The yield is 100%. m/z: 573.3 (M+H)+。1HNMR (400Mz, DMSO): 0.89 (6H, m), 1.15-1.99 (10H, m), 2.11(1H, m), 2.31 (2H, t), 3.21(2H, m), 3.53 (2H, t), 4.32 (1H, t), 4.51 (1H, m), 4.59 (2H, br), 5.24 (1H, br), 5.56 (2H, br), 6.20(1H, br), 7.12(2H, s), 7.23(2H, d) ,7.58 (2H, d), 7.94 (1H, d) , 8.1 7 (1H, d), 10.21 (1H, br)

Example 8: Synthesis and preparation of mc-vc-PAB-PNP



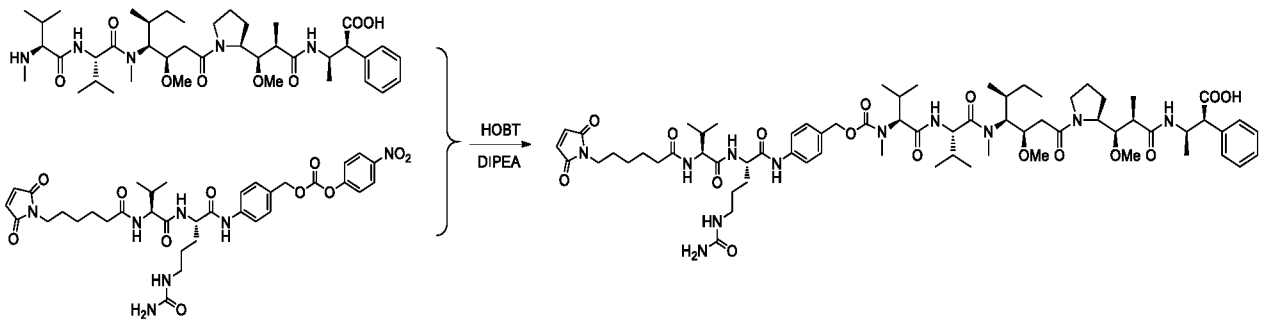
Under nitrogen, to a solution of mc-vc-PABOH (166.0 mg, 0.294 mmol) in anhydrous pyridine (5 ml) was added PNP (179 mg, 3 eq) dissolved in DCM (5 ml) at 0 °C slowly. After stirring at about 0 °C for 10 min, the ice bath was removed, and the reaction was stirred at room temperature for 3 h. After completion of the reaction, EA (70 ml) and a 15% aqueous citric acid solution (100 ml) were added, and the organic layer was separated and recovered. The organic layer was sequentially washed with citric acid, water, brine, dried with anhydrous sodium sulfate, filtered, and the filtrate was concentrated under reduced pressure to yield light yellowish oily product. Adding methyl tert-butyl ether for crystallization resulted in the white-like solid (86 mg). The yield was 40%. m/z: 738(M+H)+。1HNMR (400Mz, CDCl3/CD3OD): 0.84 (6H, m), 1.11-1.84 (10H, m), 2.05 (1H, m), 2.15 (2H, t), 3.09 (2H, m), 3.32 (2H, t), 4.12 (1H, m), 4.38 (1H, m), 5.15 (2H, s), 6.61 (2H, s), 6.84 (1H, d) ,7.61 (1H, d), 7.21 (2H,d), 7.50 (2H,d), 7.61 (2H,d), 8.18 (2H, d), 9.59 (1H, br)

Example 9: Synthesis and preparation of mc-vc-PAB-MMAE



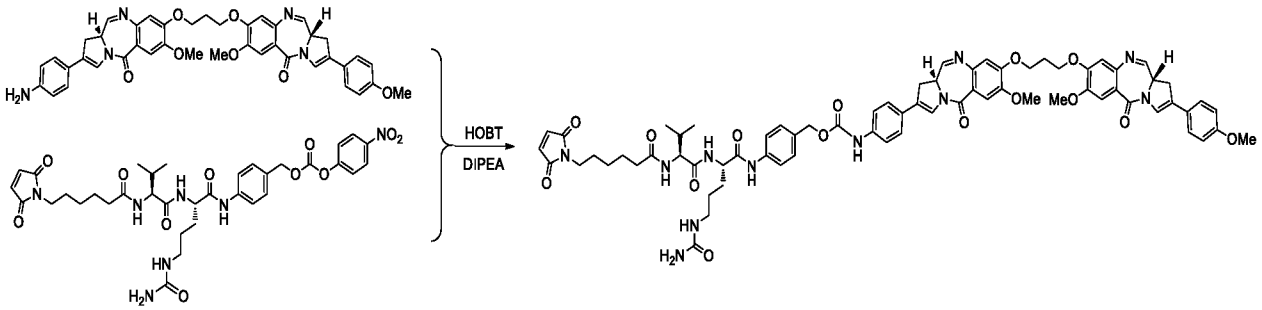
20 mg of mc-vc-PAB-PNP (1.5 eq) and 3 mg of HOBT were added to 2 ml of DMF. After stirring at room temperature for a moment, 13 mg of MMAE, 0.5 ml of pyridine, and 25 ul of DIEA were added. The reaction solution was stirred at room temperature for 2 d. After the reaction is completed, the reaction solution is directly purified by a preparative column, and the desired components are collected, concentrated, and lyophilized to obtain about 10 mg of a product, and the yield is about 42%. m/z: 1317.1 (M+H)+.

Example 10: Synthesis and preparation of mc-vc-PAB-MMAF



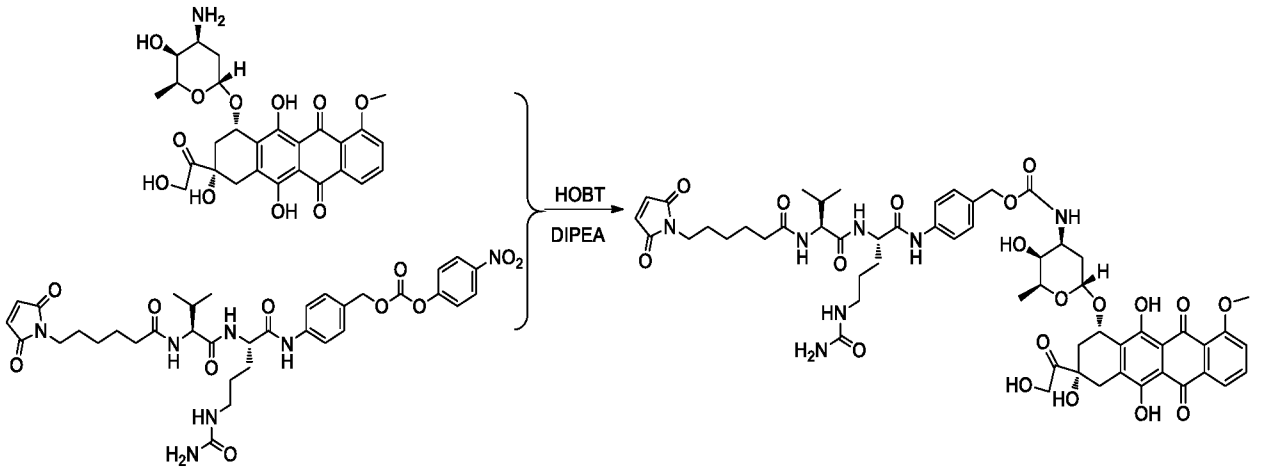
Operate according to the steps of Example 9, about 12.5 mg of mc-vc-PAB-MMAF was obtained, and the yield was 45.2%.

Example 11: Synthesis and preparation of mc-vc-PAB-PBD



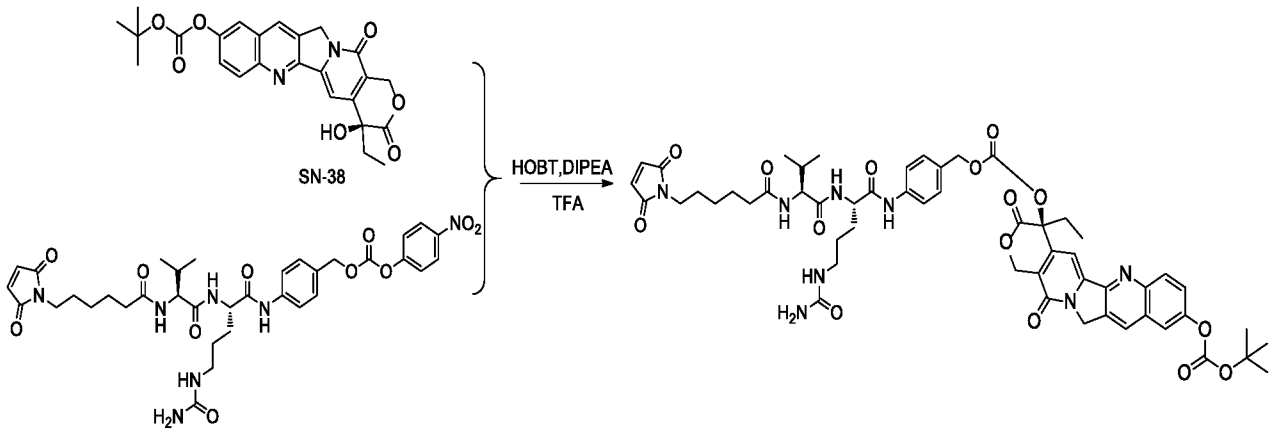
Operate according to the steps of Example 9, about 9.5 mg of mc-vc-PAB-PBD was obtained. The yield was 32.5%. m/z: 1325.4 (M+H)+.

Example 12: Synthesis and preparation of mc-vc-PAB-DOX



Operate according to the steps of Example 9, about 11.2 mg of mc-vc-PAB-DOX was obtained. The yield was 38.9%. m/z: 1143.2 (M+H)+.

Example 14: Synthesis and preparation of mc-vc-PAB-SN-38



100 mg of 10-O-Boc-SN-38 was dissolved in 10 ml of dry dichloromethane, 25.6 mg (1 eq) of DMAP was added to the solvent, and a solution of triphosgene in dichloromethane was added dropwise at 0 °C (62 mg of triphosgene was dissolved in 2 ml of Dichloromethane), and the reaction was continued at 0 °C for 12 h. The dichloromethane was removed under reduced pressure. The crude products were dissolved in 10 ml of dry DMF, 144 mg of mc-vc-PABOH was then added, and the mixture was stirred at room temperature for 24 h. 41 mg of mc-vc-PAB-SN-38 was isolated bypreparation liquid phase separation, and the total yield in two steps was 19.7%. m/z: 1063.2(M+H)+.

Example 15: Target antibody expression and purification

The target antibody was expressed using Freestyle™ 293-F (Invitrogen) suspension cells. One day before transfection, cells were seeded at a density of 6×10^5 cells/mL in a 1 L shake flask containing 300 mL of F17 complete medium (Freestyle™ F17 expression medium, Gibco), grew overnight by shaken at 37 °C, 5% CO_2, 120 rpm at cell incubator. The next day, transfection of the antibody expression plasmid was carried out with PEI, wherein the ratio of plasmid: PEI was 2:1. One day after the transfection, the TN1 feed medium was added at 2.5% (v/v), and the culture was continued for 4 days, and the supernatant was collected by centrifugation.

The collected cell expression supernatant was eluted by a Protein An affinity chromatography column (Mabselect Sure LX, GE) eluting with 0.1 M citric acid (pH 3.0), and the captured antibody was treated with 1 M Tris-HCl (pH 9.0) and adjusted to pH 7.0 at 1/10 (v/v). Remove impurities such as multimers and endotoxin by gel filtration column SEC (Superdex 200, GE), and replace the antibody buffer with PBS (pH 7.4) at the same time, a sample of the target peak of UV280 nm was collected and concentrated to 2 mg/ml through an ultrafiltration centrifuge tube (30 KD, Pall Corporation). The target antibody monomer (POI%) obtained by this method was greater than 90% and was stored for subsequent experiments.

Example 16: Synthesis and preparation of 2A1-HC-Cys439ins-mc-vc-PAB-MMAE TDC by conjugating/coupling 2A1-HC-Cys439ins antibody and mc-vc-PAB-MMAE

The 2A1-HC-Cys439ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 2A1-HC-Cys439ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 2A1-HC-Cys439ins-mc-vc-PAB-MMAE TDC sample.

Example 17: Synthesis and preparation of 2A1-LC-Cys205ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 2A1-LC-Cys205ins antibody and mc-vc-PAB-MMAE

The 2A1-LC-Cys205ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 2A1-LC-Cys205ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 2A1-LC-Cys205ins-mc-vc-PAB-MMAE TDC sample.

Example 18: Synthesis and preparation of 2A1-LC-Cys206ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 2A1-LC-Cys206ins antibody and mc-vc-PAB-MMAE

The 2A1-LC-Cys206ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution

immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 2A1-LC-Cys206ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 2A1-LC-Cys206ins-mc-vc-PAB-MMAE TDC sample.

Example 19: Synthesis and preparation of 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 4D3-HC-Cys439ins antibody and mc-vc-PAB-MMAE

The 4D3-HC-Cys439ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 4D3-HC-Cys439ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin.

Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC sample.

Example 20: Synthesis and preparation of 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 4D3-LC-Cys205ins antibody and mc-vc-PAB-MMAE

The 4D3-LC-Cys205ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer.

The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 4D3-LC-Cys205ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC sample.

Example 21: Synthesis and preparation of 4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 4D3-LC-Cys206ins antibody and mc-vc-PAB-MMAE

The 4D3-LC-Cys206ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 4D3-LC-Cys206ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC sample.

Example 22: Synthesis and preparation of 4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 4E1-HC-Cys439ins antibody and mc-vc-PAB-MMAE

The 4E1-HC-Cys439ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A

1 M aqueous solution of DTT was added to the 4E1-HC-Cys439ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC sample.

Example 23: Synthesis and preparation of 4E1-LC-Cys205ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 4E1-LC-Cys205ins antibody and mc-vc-PAB-MMAE

The 4E1-LC-Cys205ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 4E1-LC-Cys205ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 4E1-LC-Cys205ins-mc-vc-PAB-MMAE TDC sample.

Example 24: Synthesis and preparation of 4E1-LC-Cys206ins-mc-vc-PAB-MMAE TDC sample by conjugating/coupling 4E1-LC-Cys206ins antibody and mc-vc-PAB-MMAE

The 4E1-LC-Cys206ins antibody expressed by the cells was purified by Protein A resin such as Mabselect Sure, eluted with low pH solution and neutralized by adding Tris solution immediately after the low pH elution, and the solution was changed to a pH 7.5 Tris-HCl buffer. The mc-vc-PAB-MMAE compound, being a white powder, was dissolved in DMA for use. In order to remove the masking group on the mutant cysteine residue, the antibody was reduced first. A 1 M aqueous solution of DTT was added to the 4E1-LC-Cys206ins antibody solution at a molecular ratio of 1:40, and the mixture was mixed evenly and reacted at 20 ° C for 2 hours. After the

reaction time was reached, the pH of the sample was adjusted to 5.0, and the DTT and the masking group in the mixture were removed by cation exchange chromatography such as SP Sepharose F.F. resin. Subsequently, a DHAA solution was added to the sample at a molecular ratio of 1:20 and reacted at 25 ° C for 4 hours in the dark to re-connect the interchain disulfide bonds. subsequently, mc-vc-PAB-MMAE solution was added to couple the mc-vc-PAB-MMAE with the inserted or mutant cysteine in the antibody, and the mixture was thoroughly mixed and reacted at 25 ° C for 2 hours. After the end of the reaction, mc-vc-PAB-MMAE to which the antibody was not coupled was removed using cation exchange chromatography such as SP Sepharose F.F. to obtain a 4E1-LC-Cys206ins-mc-vc-PAB-MMAE TDC sample.

Example 25: Measurement of toxin:antibody ratio (DAR, Drug Antibody ratio) by HIC-HPLC

The TDC sample was analyzed by high performance liquid chromatography with hydrophobic chromatography, and drug:antibody ratio (DAR, also known as toxin:antibody ratio) was calculated from the corresponding peak area. One specific method is described in detail as follows:

Column: Proteomix® HICBu-NP5 (5 µm, 4.6 x 35 mm);

Mobile phase: Buffer A: 2M ammonium sulfate, 0.025 M, pH 7 phosphate buffer; Buffer B: 0.025 M, pH 7 phosphate buffer; Buffer C: 100% isopropanol;

Buffer A was used for equilibration, Buffer B and buffer C were used for gradient elution, detection was performed at 25 ° C, 214 nm and 280 wavelengths. Based on data gathered from FIGUREs 1-3, the site-specific coupled DAR is calculated to be between 1.6 and 1.7, showing excellent compound uniformity or homogeneity. Based on data gathered from FIGUREs 4-6, the site-specific coupled DAR is calculated to be between 1.6 and 1.95, showing excellent compound uniformity or homogeneity. Based on data gathered from FIGUREs 7-8, the site-specific coupled DAR is calculated to be between 1.6 and 1.9, showing excellent compound uniformity or homogeneity.

Example 26: Measurement of toxin:antibody ratio (DAR, Drug Antibody ratio) by RP-HPLC

The ratio of toxin to antibody was measured by RP-HPLC. The samples treated with DTT were analyzed by reversed-phase hydrophobic high-performance liquid chromatography, and DAR was calculated from the corresponding peak area. One specific method is described in detail as follows:

Column: Proteomix RP-1000 (5µm, 4.6×100mm)

Mobile phase: Buffer A: 0.1% TFA aqueous solution; Buffer B: 0.1% acetonitrile solution.

Mobile phase A and mobile phase B were used to elute in a proportional gradient at 80 °C, measurement was performed at 214 nm and 280 wavelengths. Based on data gathered in FIGURE 9, the site-specific coupled DAR was calculated to be 1.82, showing excellent compound uniformity or homogeneity.

TABLE I: Coupling Efficiency DAR List for ADRs: 2A1-LC-V205C-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys206ins--mc-vc-PAB-MMAE TDC, 2A1-HC-Cys439ins-mc-vc-PAB-MMAE, 4E1-LC-Cys205ins-mc-vc-PAB-MMAE, 4E1-LC-Cys206ins-mc-vc-PAB-MMAE, 4E1-HC-Cys439ins-mc- vc-PAB-MMAE TDC, 4D3-LC-Cys205ins-mc-vc-PAB-MMAE, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE

	Compounds	DAR
Site-specific coupling (TDC)	2A1-LC-V205C-mc-vc-PAB-MMAE TDC	1.81
	2A1-LC-Cys205ins-mc-vc-PAB-MMAE TDC	1.72
	2A1-LC-Cys206ins-mc-vc-PAB-MMAE TDC	1.65
	2A1-HC-Cys439ins-mc-vc-PAB-MMAE TDC	1.74
	4E1-LC-Cys205ins-mc-vc-PAB-MMAE TDC	1.92
	4E1-LC-Cys206ins-mc-vc-PAB-MMAE TDC	1.64
	4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC	1.75
	4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC	1.81
	4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC	1.74
	4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC	1.82

TABLE 1 shows that the coupling efficiency of site-directed TDC compounds by cysteine insertion mutation modification is uniformly high (theoretical maximum is 2.0), with DAR ≥ 1.6.

Example 27: Measurement of TDC antibody skeleton aggregation by SEC-HPLC

TDC antibody skeleton samples were stored at 37 ° C, and their aggregation was analyzed by SEC-HPLC on days 0, 7, 21, and 29, respectively. One specific method is described in detail as follows:

Chromatography columns: TSKgel SuperSW mAb HR (7.8mm × 30cm),

Mobile phase: 0.1 M sodium sulfate, 0.1 M, pH 6.7 phosphate buffer,

Measurements were performed at 25 ° C, 280 nm.

As shown in FIGUREs 10-13, SEC-HPLC was used to detect and measure the aggregation of TDC antibody skeleton 4D3, 4D3-LC-Cys205ins, 4D3-LC-Cys206ins and 4D3-HC-Cys439ins. The samples were stored at 37 ° C for 4 weeks, and the aggregate content remained essentially unchanged.

Using the same detecting and measurement method, the aggregations of the following TDCs are measured: 2A1-LC-V205C-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys206ins--mc-vc-PAB-MMAE TDC, 2A1-HC-Cys439ins-mc-vc-PAB-MMAE, 4E1-LC-

Cys205ins-mc-vc-PAB-MMAE, 4E1-LC-Cys206ins-mc-vc-PAB-MMAE 4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC, 4D3-LC-Cys205ins-mc-vc-PAB-MMAE, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC. The results are shown in TABLE II

TABLE II: TDS target monomer content list for 2A1-LC-V205C-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys206ins--mc-vc-PAB-MMAE TDC and 2A1-HC-Cys439ins-mc-vc-PAB-MMAE, 4E1-LC-Cys205ins-mc-vc-PAB-MMAE, 4E1-LC-Cys206ins-mc-vc-PAB-MMAE, 4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC, 4D3-LC-Cys205ins-mc-vc-PAB-MMAE, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE

	Compound	POI%
Site-specific coupling (TDC)	2A1-LC-V205C-mc-vc-PAB-MMAE TDC	96.0%
	2A1-LC-Cys205ins-mc-vc-PAB-MMAE TDC	90.0%
	2A1-LC-Cys206ins-mc-vc-PAB-MMAE TDC	90.4%
	2A1-HC-Cys439ins-mc-vc-PAB-MMAE TDC	90.0%
	4E1-LC-Cys205ins-mc-vc-PAB-MMAE TDC	98.18%
	4E1-LC-Cys206ins-mc-vc-PAB-MMAE TDC	94.34%
	4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC	95.77%
	4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC	97.27%
	4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC	96.06%
	4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC	96.98%

As shown by TABLE II, the target monomer content of the TDC compound coupled by the inserted cysteine is above 90%.

Example 28: Measurement of affinities between skeletal antibodies undergoing cysteine site-directed mutagenesis and insertional mutagenesis and parental antibodies and EGFRvIII, affinities between 4E1 antibodies and c-met, affinities between 4D3 antibodies and Trop2

The relative affinities of 2A1-LC-V205C, 2A1-LC-Cys205ins, 2A1-LC-Cys206ins, 2A1-HC-Cys439ins and 2A1 for EGFRvIII were compared by indirect ELISA. The specific steps are as follows: Recombinant EGFRvIII-His*6 antigen-coated plate was blocked by fish skin gelatin; Antibodies 2A1, 2A1-LC-V205C, 2A1-LC-Cys205ins, 2A1-LC-Cys206ins and 2A1-HC-Cys439ins were respectively diluted by 4 folds gradient with a total of 11 concentrations with the highest concentration being 10ug/ml; HRP-labeled secondary antibody incubation were performed; after TMB coloration, absorption was detected and measured at 450 nm. The absorption measurement results at A450 were plotted against concentration. The cysteine site-directed mutagenesis or insertion of the

mutant antibodies 2A1-LC-V205C, 2A1-LC-Cys205ins, 2A1-LC-Cys206ins and 2A1-HC-Cys439ins retained affinities to EGFRvIII similar to 2A1, as shown by the close EC_{50} values; these results indicate that the site-directed mutagenesis of the light chain V205C on 2A1 antibody, the insertional mutation at position 205 of the light chain of the antibody, the insertional mutation at position 206 of the light chain of the antibody, or the insertion mutation at position 439 of the heavy chain of the antibody does not affect their affinity for the EGFRvIII antigen.

As shown in the FIGURE 14, the 2A1-LC-V205C, 2A1-LC-Cys205ins, 2A1-LC-Cys206ins, 2A1-HC-Cys439ins antibodies maintain the affinity of 2A1 to antigen EGFRvIII.

Example 29: Measurement of affinities of skeletal antibodies undergoing cysteine site-directed mutagenesis and insertional mutagenesis and linked to toxin/drug towards connate antigens, affinities of 4E1 antibodies to c-met, affinities of 4D3 antibodies to Trop2

The relative affinities of 4E1-LC-Cys205ins-MVPM, 4E1-LC-Cys206ins-MVPM, 4E1-HC-Cys439ins-MVPM and 4E1 for C-met were compared by indirect ELISA. The specific steps are as follows:

Recombinant C-met-His*6 antigen-coated plate was blocked by fish skin gelatin; TDC 4E1-LC-Cys205ins-MVPM, 4E1-LC-Cys206ins-MVPM, 4E1-HC-Cys439ins-MVPM and antibody 4E1 were respectively diluted by 4 folds gradient with a total of 11 concentrations with the highest concentration being 10ug/ml; HRP-labeled secondary antibody incubation were performed; after TMB coloration, absorption was detected and measured at 450 nm. The absorption measurements at A450 were plotted against concentration, and the result shows that the antibodies harboring cysteine site-directed insertion mutation, TDC 4E1-LC-Cys205ins-MVPM、4E1-LC-Cys206ins-MVPM、and 4E1-HC-Cys439ins-MVPM, retained their binding affinities to C-met similar to 4E1, as indicated by the close EC_{50} values; which indicates that the insertional mutation at the position 205 or 206 of 4E1 light chain or at the position 439 of 4E1 heavy chain does not affect the binding affinity of the corresponding TDC to the c-met antigen.

The relative affinities of 4D3-LC-Cys205ins-MVPM、4D3-LC-Cys206ins-MVPM、4D3-HC-Cys439ins-MVPM and 4D3 for Trop2 were compared by indirect ELISA. The specific steps are as follows:

Recombinant Trop2-His*6 antigen-coated plate was blocked by fish skin gelatin; TDC 4D3-LC-Cys205ins-MVPM、4D3-LC-Cys206ins-MVPM、4D3-HC-Cys439ins-MVPM and antibody 4D3 were respectively diluted by 4 folds gradient with a total of 11 concentrations with the highest concentration being 10ug/ml; HRP-labeled secondary antibody incubation were performed; after TMB coloration, absorption was detected and measured at 450 nm. The absorption measurements at A450 were plotted against concentration. TDC 4D3-LC-Cys205ins-MVPM、4D3-LC-Cys206ins-MVPM、and 4D3-HC-Cys439ins-MVPM retained their binding affinities to Trop2 similar to that of 4D3, as shown by the close EC_{50} values; which indicates that the insertional

mutation at the position 205 or 204 of 4D3 light chain or at the position 439 of 4D3 heavy chain does not affect the binding affinity of the corresponding TDC to the Trop2 antigen.

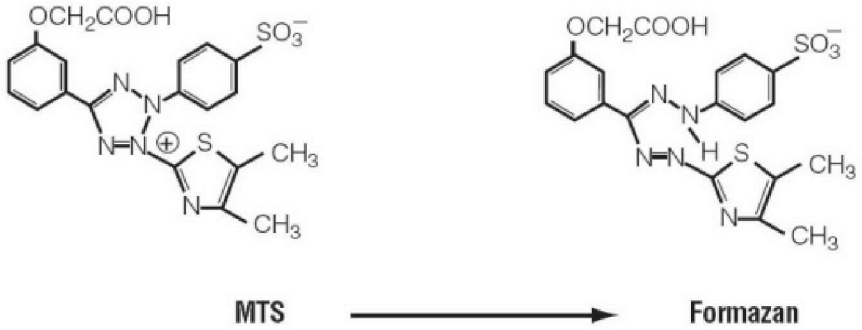
As shown in FIGURE 15, 4E1-LC-Cys205ins-MVPM, 4E1-LC-Cys206ins-MVPM, 4E1-HC-Cys439ins-MVPM antibodies retained the affinity of 4E1 for antigen c-met.

As shown in FIGURE 16, 4D3-LC-Cys205ins-MVPM, 4D3-LC-Cys206ins-MVPM, 4D3-HC-Cys439ins-MVPM antibodies retained the affinity of 4D3 for the antigen Trop2.

Example 30: Cytotoxicity pharmaceutical efficacy test

TDC cytotoxic activity was determined by the following experimental procedures: TDC was separately added to culture media of human tumor cells in which EGFR was overexpressed or EGFRVIII was expressed, and cell viability was measured after 72 hours of cell culture. Cell-based in vitro assays were used to determine cell viability, cytotoxicity, and TDC-induced apoptosis in the present disclosure.

The in vitro efficacy of the antibody-cytotoxin conjugate was determined by a cell proliferation assay. In one embodiment, the CellTiter 96® Aqueous One Solution Cell Proliferation Assay is commercially available (Promega Corp., Madison, WI). The Cell Proliferation Assay (a) is a detection reagent that uses colorimetry to detect the number of viable cells in cell proliferation and cytotoxicity experiments. This reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electronic coupling agent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be mixed with MTS to form a stable solution. This convenient "single solution" mode is based on the first generation CellTiter 96® AQueous Assay, in which the electronic coupling agent PMS and MTS solution are supplied separately. MTS (Owen's reagent) is biologically reduced by cells to a colored formazan product that is directly soluble in the medium (FIGURE 1). This transformation is most likely accomplished by the action of NADPH or NADH produced by dehydrogenase in metabolically active cells. For detection, simply add a small amount of CellTiter 96® AQueous One Solution Reagent directly to the culture medium well, incubate for 1–4 hours, and then read the absorbance at 490 nm with a microplate reader.



The amount of formazan product detected at 490 nm is directly proportional to the number of viable cells in the culture. Since the MTS hyperthyroid product is soluble in tissue culture media, the CellTiter 96® AQueous One Solution Assay has fewer steps than the MTT or INT method.

In the present disclosure, A431 (EGFR overexpressing cells), U87-EGFRVIII (EGFR mutant stable cell line), U87-MG (glioblastoma cell line with highly expressed c-Met) and BXPC-3 (pancreatic cancer cell line with high expression of Trop2) are used as research systems for in vitro drug efficacy testing. In a 96-well plate, cell plating was performed at a concentration of 6000/well, and after 24 hours, antibody administration was performed. The initial concentrations of various TDCs corresponding to A431, U87-EGFRVIII cell lines were 10 μM, which were sequentially diluted according to a 5-fold gradient. The initial concentrations of various TDCs corresponding to U87-MG and BXPC-3 cell lines were 1 μM, which were sequentially diluted according to a 5-fold gradient. MTS assay for cell viability were performed after 72 hours of treatment

TABLE III: Cytotoxicity IC_{50} detection results of TDC, ADC on EGFRwt overexpressing cell line A431 and EGFRvIII expression stable strain U87-EGFRVIII

	Compounds	MTS			
		A431	U87MG-EGFRvIII	U87-MG	BXPC-3
Antibody 2A1	2A1	>10μM	>10μM	/	/
	2A1-LC-V205C	>10μM	>10μM	/	/
	2A1-LC-Cys205ins	>10μM	>10μM	/	/
	2A1-LC-Cys206ins	>10μM	>10μM	/	/
	2A1-HC-Cys439ins	>10μM	>10μM	/	/
Site-directed coupling (TDC)	2A1-LC-V205C-mc-vc-PAB-MMAE	92.16 nM	296.11 nM	/	/
	2A1-LC-Cys205ins-mc-vc-PAB-MMAE	133.67 nM	492.14 nM	/	/
	2A1-LC-Cys206ins-mc-vc-PAB-MMAE	179.26 nM	457.48 nM	/	/
	2A1-HC-Cys439ins-mc-vc-PAB-MMAE	26.62 nM	118.52 nM	/	/

Antibody 4E1	4E1	/	/	>1μM	/
Site-directed coupling (TDC)	4E1-LC-Cys205ins-mc-vc-PAB-MMAE	/	/	120.50nM	/
	4E1-LC-Cys206ins-mc-vc-PAB-MMAE	/	/	79.57nM	/
	4E1-HC-Cys439ins-mc-vc-PAB-MMAE	/	/	7.41nM	/
Antibody 4D3	4D3	/	/	/	>1μM
Site-directed coupling (TDC)	4D3-LC-Cys205ins-mc-vc-PAB-MMAE	/	/	/	0.38nM
	4D3-LC-Cys206ins-mc-vc-PAB-MMAE	/	/	/	0.45nM
	4D3-HC-Cys439ins-mc-vc-PAB-MMAE	/	/	/	0.23nM

The data from the TABLE III shows that, 2A1-LC-V205C-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys205Cins-mc-vc-PAB-MMAE TDC, 2A1-LC-Cys206ins-mc-vc-PAB-MMAE TDC, and 2A1-HC-Cys439ins -mc-vc-PAB-MMAE TDC have comparable cytotoxic activity to EGFRwt overexpressing cell line A431 and EGFRvIII expression stable strain U87-EGFRVIII, and 439 inserted mutant TDC's activity is slightly higher than 205 and 206 insertion mutant TDC.

There was a certain correlation between cytotoxic activity and coupling position for the 4E1-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 4E1-LC-Cys206ins-mc-vc-PAB-MMAE TDC and 4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC in U87-MG cells. The TDC activity of the 439 inserted mutant was slightly better than those of the 205 and 206 insertion mutants. The activity of TDC was significantly better than that of the parental antibody.

The cytotoxic activity of 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC and 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC in pancreatic cancer cell line BXPC-3 was comparable or similar to each other, and the TDC activity of the 439 inserted mutant was slightly better than those of the 205 and 206 insertion mutant TDCs, and the activity of TDC was significantly better than that of the parental antibody.

Example 31: Plasma Stability test

Take a certain amount of ADC sample, add it to human plasma from which human IgG has been removed, repeat 2 tubes for each ADC, incubate in a 37 °C water bath, incubate for 0h, 72h, take ADC samples, add 100 μl ProteinA (MabSelect SuReTM LX Lot: #10221479 GE washed with

PBS), shaken for 2 h with a vertical mixer, and subjected to a washing and elution step to obtain an ADC after incubation. The samples, which had undergone incubation for a certain time, were subjected to HIC-HPLC and RP-HPLC to determine the plasma stability of the samples.

FIGUREs 21-23 shows the result of the test for in human plasma stability for the 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC and 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC. The detection method for 4D3-HC-Cys439ins-mc-vc-PAB-MMAE was RP-HPLC; The detection method for 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC and 4D3-LC-Cys206ins-mc-vc is HIC-HPLC.

FIGUREs 24-26 shows the result of the test for in human plasma stability for the 4E1-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 4E1-LC-Cys206ins-mc-vc-PAB-MMAE TDC and 4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC. The detection method is HIC-HPLC

TABLE IV: TDC plasma stability test result (calculated by the change of DAR)

	Compound	DAR	
		37℃ 0h	37℃ 72h
Site-directed coupling (TDC)	4E1-LC-Cys205ins-mc-vc-PAB-MMAE TDC	1.89	1.77
	4E1-LC-Cys206ins-mc-vc-PAB-MMAE TDC	1.81	1.62
	4E1-HC-Cys439ins-mc-vc-PAB-MMAE TDC	1.85	1.83
	4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC	1.86	1.71
	4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC	1.76	1.52
	4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC	1.81	1.80

The above TDCs were stable after being incubated at 37 ° C for 72 hours in human plasma samples and had good drug-forming properties. In comparison, TDC with 439 insertion mutations had the best stability, followed by TDC with 205 and 206 insertion mutations.

Example 32: Tumor-bearing mice pharmaceutical efficacy test

In the present disclosure, a BXPC-3 tumor-bearing mouse model was established to evaluate the in vivo efficacy of TDC and parental antibodies. In one embodiment, 3×10^6 BXPC-3 cells were subcutaneously injected into the back side of 4-8 weeks old BALB/c nude mice, and the average tumor size of the mice was grown to 400-500 mm^3, the mice were randomly grouped, 5 mice in each group. On Day 0 and Day 7, 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC and 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC were

administered in a single intravenous dose at a dose of 5 mg/kg, and the parental antibody 4D3 was administered at a dose of 5 mg/kg. Data A shows the mean tumor volume ± SE at the time of measurement, and data B shows the average body weight of the mouse at the time of measurement ± SE.

FIGURE 27 shows the results of the test on the efficacy in the tumor-bearing mice for 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC, and 4D3. TDC showed significant anti-tumor effect in vivo compared to the parental antibodies.

FIGURE 28 shows the results of the test on the efficacy in the tumor-bearing mice for 4D3-LC-Cys205ins-mc-vc-PAB-MMAE TDC, 4D3-LC-Cys206ins-mc-vc-PAB-MMAE TDC, 4D3-HC-Cys439ins-mc-vc-PAB-MMAE TDC and 4D3 parental antibody. There was no significant change in the body weight of the mice, which proved that the TDCs have no or minor toxicity in vivo.

The disclosure is not limited to the scope of the specific embodiments disclosed in the embodiments, which are intended to illustrate several aspects of the disclosure, and any embodiments that are functionally equivalent are within the scope of the disclosure. In fact, various modifications of the disclosure are obvious to those skilled in the art and are in the scope of the appended claims.

SEQUENCE LISTING

TABLE V: amino acids

English Name	Symbol or Abbreviation
Alanine	A or Ala
Arginine	R or Arg
Asparagine	N or Asn
Aspartic acid	D or Asp
Cysteine	C or Cys
Glutamine	Q or Gln
Glutamic acid	E or Glu
Glycine	G or Gly
Histidine	H or His
Isoleucine	I or Ile
Leucine	L or Leu
Lysine	K or Lys
Methionine	M or Met
Phenylalanine	F or Phe
Proline	P or Pro
Serine	S or Ser
Threonine	T or Thr
Tryptophan	W or Trp
Tyrosine	Y or Tyr
Valine	V or Val

SEQ ID NO:1 heavy chain constant region (Fc) DNA sequence

>IgG1-Fc

GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGG
CCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGAC
CAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCG
TGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGT
GGACAAGAGAGTTGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTC
CTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGA
GGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGG
CGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAG
CGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCC

CTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCC
TGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCC
AGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGT
GCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGG
GAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGT
CTCCGGGTAAA

SEQ ID NO:2 heavy chain constant region (Fc) amino acid sequence
>IgG1-Fc
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:3 light chain constant region (Kappa) DNA sequence
>LC-Kappa
ACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTT
GTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATC
GGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCT
GACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAG
CTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAG

SEQ ID NO:4 light chain constant region (Kappa) amino acid sequence
>LC-Kappa
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO:5 2A1-LC-Cys205ins light chain constant region (Kappa) DNA sequence
>LC-Cys205ins-Kappa
ACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTT
GTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATC
GGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCT
GACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAG
CTCGCCC**TGC**GTCACAAAGAGCTTCAACAGGGGAGAGTGTTAG

SEQ ID NO:6 LC-Cys205ins light chain constant region (Kappa) amino acid sequence
>LC-Cys205ins-Kappa

TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSP**C**VTKSFNRGEC

wherein, the C in the GLSSP**C**VTKSFN is the site-specific conjugation position. In one embodiment, the cysteine is conjugated with mc-vc-PAB-payload site-specifically.

SEQ ID NO:7 LC-Cys206ins light chain constant region (Kappa) DNA sequence
>LC-Cys206ins-Kappa
ACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTT
GTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATC
GGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCT
GACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAG
CTCGCCCGTC**TGC**ACAAAGAGCTTCAACAGGGGAGAGTGTTAG

SEQ ID NO:8 LC-Cys206ins light chain constant region (Kappa) amino acid sequence
>LC-Cys206ins-Kappa
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSPV**C**TKSFNRGEC
wherein, the C in the GLSSPV**C**TKSFN is the site-specific conjugation position. In one embodiment, the cysteine is conjugated with mc-vc-PAB-payload site-specifically.

SEQ ID NO:9 IgG1-Fc-Cys439ins heavy chain constant region (Fc) DNA sequence
>IgG1-Fc-Cys439ins
GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGG
CCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGAC
CAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCG
TGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGT
GGACAAGAGAGTTGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTC
CTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGA
GGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGG
CGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAG
CGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCC
CTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCC
TGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCC
AGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGT
GCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGG
GAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCC**TGC**C
TGTCTCCGGGTAAA

SEQ ID NO: 10 IgG1-Fc-Cys439ins heavy chain constant region (Fc) amino acid sequence
>IgG1-Fc-Cys439ins
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEALHNHYTQKSLS**C**LSPGK
Wherein the **C** in the TQKSLS**C**LSPGK sequence is the site-specific conjugation/coupling position, and undergoes site-specific conjugation with mc-vc-PAB-payload.

SEQ ID NO:11 LC-V205C light chain constant region（Kappa）DNA sequence
>LC-V205C-Kappa
ACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTT
GTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATC
GGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCT
GACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAG
CTCGCCC**TGC**ACAAAGAGCTTCAACAGGGGAGAGTGTTAG

SEQ ID NO:12 LC-V205C light chain constant region (Kappa) amino acid sequence
>LC-V205C-Kappa
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEKHKVYACEVTHQGLSSP**C**TKSFNRGEC
Wherein the C in the GLSSP**C**TKSFN sequence is the site-specific conjugation/coupling position, and undergoes site-specific conjugation with mc-vc-PAB-payload.

SEQ ID NO:13 LC-V205C light chain constant region (Kappa) amino acid sequence
GLSSPCVTKSF

SEQ ID NO:14 LC-V206C light chain constant region (Kappa) amino acid sequence
GLSSPVCTKS

SEQ ID NO:15 Heavy chain amino acid sequence
TQKSLSCLSPGK

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CLAIMS

What is claimed is:

1. A cysteine modified antibody-cytotoxin conjugate, comprising an antibody and a cytotoxin, wherein the antibody comprises an inserted cysteine at a cysteine insertion site, wherein the cysteine insertion site is kappa/λ light chain constant region position 205 in Kabat numbering scheme, light chain constant region position 206 in Kabat numbering scheme, or IgG heavy chain constant region position 439 (474 in Kabat numbering scheme).

2. The cysteine modified antibody-cytotoxin conjugate of Claim 1, wherein the cysteine insertion site comprises an amino acid sequence selected from LC-205ins: GLSSPCVTKSF, LC-206ins: GLSSPVCTKSF, and HC-439ins: TQKSLSCLSPGK, and wherein the "C" is the inserted cysteine at the light chain constant region position 205, the light chain constant region position 206, or the heavy chain constant region position 439.

3. The cysteine modified antibody-cytotoxin conjugate of Claim 1, wherein the inserted cysteine comprises a free thiol group, wherein the cytotoxin is conjugated to the free thiol group through a linker, wherein the antibody comprises a light chain having an amino acid sequence selected from GLSSPCVTKSF and GLSSPVCTKSF, wherein the antibody comprises a heavy chain having an amino acid sequence of TQKSLSCLSPGK, and wherein the C is the inserted cysteine at the light chain position 205, the light chain position 206, or heavy chain position 439 of the antibody.

4. The cysteine modified antibody-cytotoxin conjugate of any one of Claims 1 to 3, wherein the antibody comprises a light chain, wherein the light chain comprises a kappa (κ) or lambda (λ) isotype.

5. The cysteine modified antibody-cytotoxin conjugate of any one of Claims 1 to 4, wherein the antibody comprises a heavy chain, wherein the heavy chain comprises IgG1, IgG2, IgG3 or IgG4.

6. The cysteine modified antibody-cytotoxin conjugate of Claim 1 or 2, wherein the inserted cysteine comprises a thiol group (-SH).

7. The cysteine modified antibody-cytotoxin conjugate of Claim 6, wherein the thiol group is configurated for chemical conjugation.

8. The cysteine modified antibody-cytotoxin conjugate of any one of Claims 1 to 7, wherein the cytotoxin is selected from MMAE, MMAF, PBD, SN-38, Dox, or a derivative thereof.

9. A method for preparing the cysteine modified antibody-cytotoxin conjugate of any one of Claims 1 to 8, comprising:

- reducing the antibody with a reducing agent to provide a reduced antibody, wherein the antibody comprises the inserted cysteine having its thiol group shielded with a shielding group,
- removing the shielding group and the reducing agent through cation exchange

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chromatography or ultrafiltration, wherein the reduced antibody having the shielding group removed comprises the inserted cysteine with a free thiol group,

- oxidizing the reduced antibody to provide an oxidized antibody having an inter-chain disulfide bond,
- conjugating a linker-cytotoxin with the free thiol group on the inserted cysteine to provide the cysteine modified antibody-cytotoxin conjugate, and
- removing unconjugated linker-cytotoxin by cation exchange chromatography or ultrafiltration.

10. A method of treating cancer in a subject in need thereof comprising administering the cysteine modified antibody-cytotoxin conjugate according to any one of claims 1 to 8 to the subject in need thereof.

11. Use of the cysteine modified antibody-cytotoxin conjugate according to any one of claims 1 to 8 in the preparation of a medicament for the treatment of cancer in a subject in need thereof.

12. The method of claim 10 or the use of claim 11, wherein the cancer is squamous cell carcinoma, glioma, malignant glioma or pancreatic cancer.

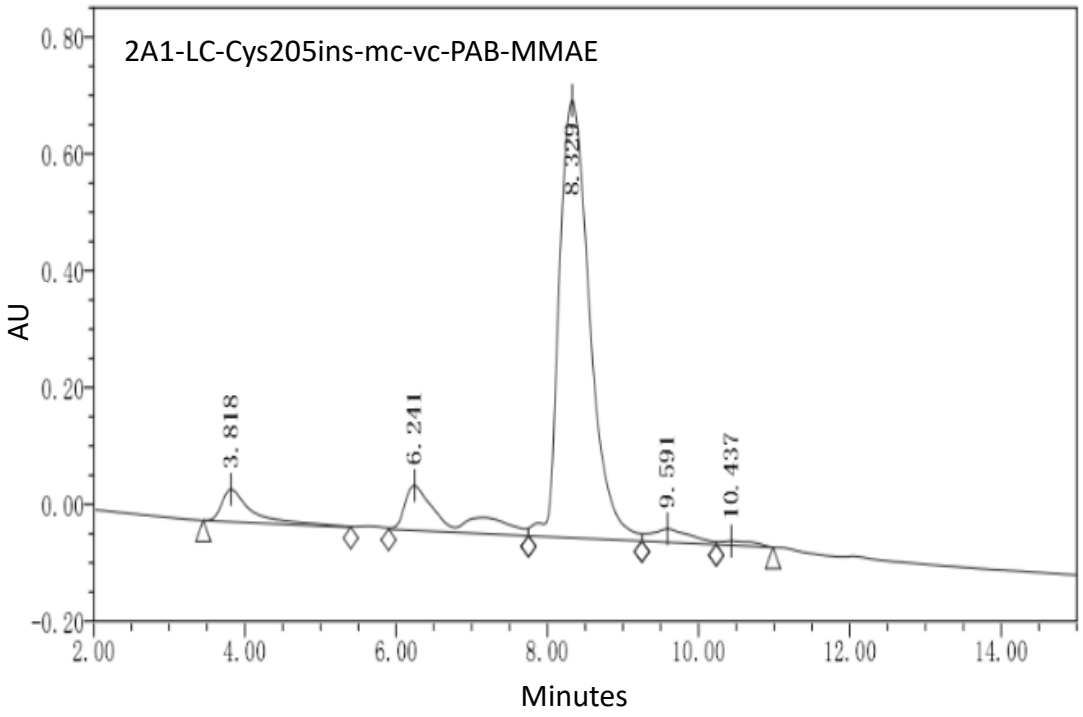

2A1-LC-Cys205ins-mc-vc-PAB-MMAE
AU
Minutes
3.818
6.241
8.329
9.591
10.437

FIG.1

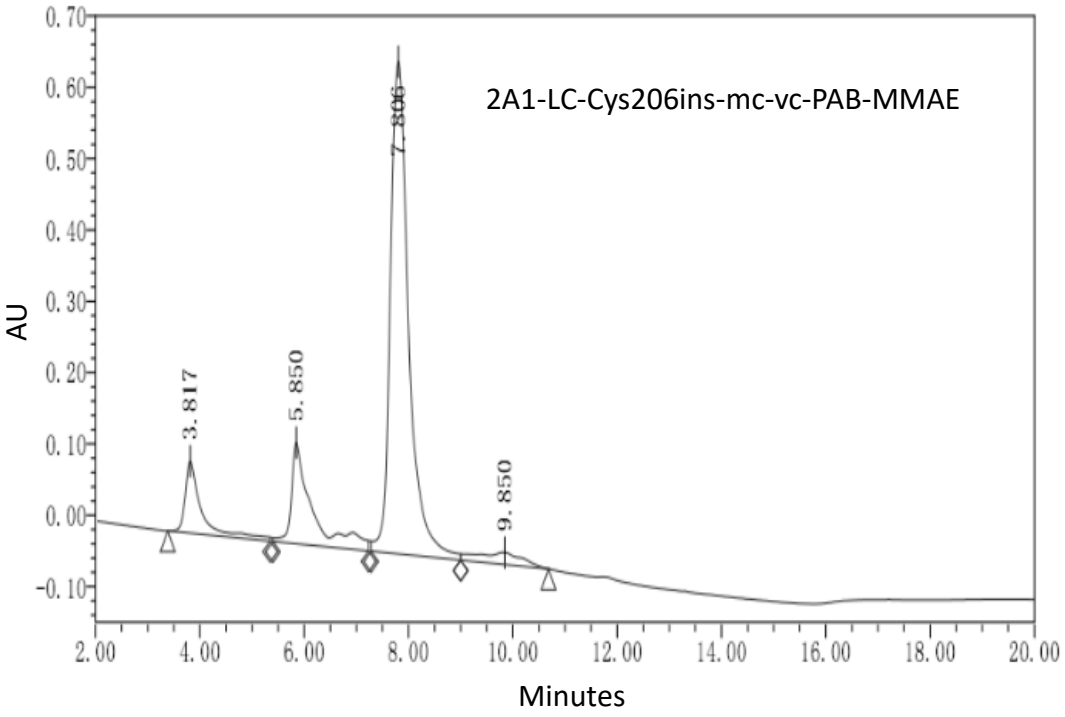

2A1-LC-Cys206ins-mc-vc-PAB-MMAE
AU
Minutes
3.817
5.850
7.806
9.850

FIG.2

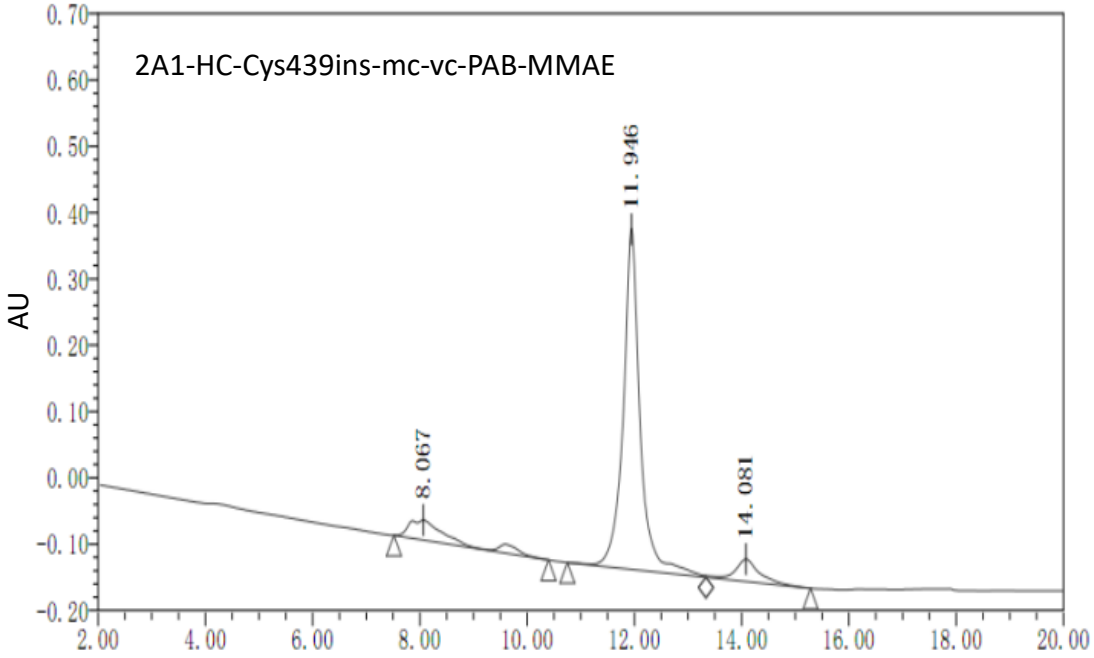

2A1-HC-Cys439ins-mc-vc-PAB-MMAE
AU
Minutes
8.067
11.946
14.081
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
-0.20
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

FIG.3

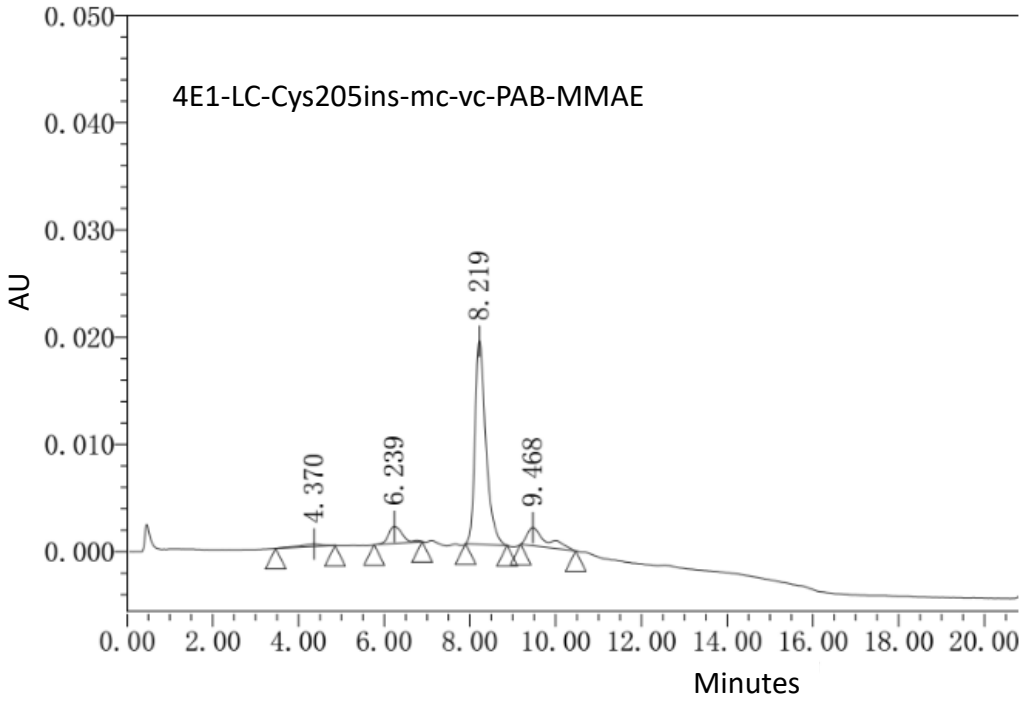

4E1-LC-Cys205ins-mc-vc-PAB-MMAE
AU
Minutes
4.370
6.239
8.219
9.468
0.050
0.040
0.030
0.020
0.010
0.000
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

FIG.4

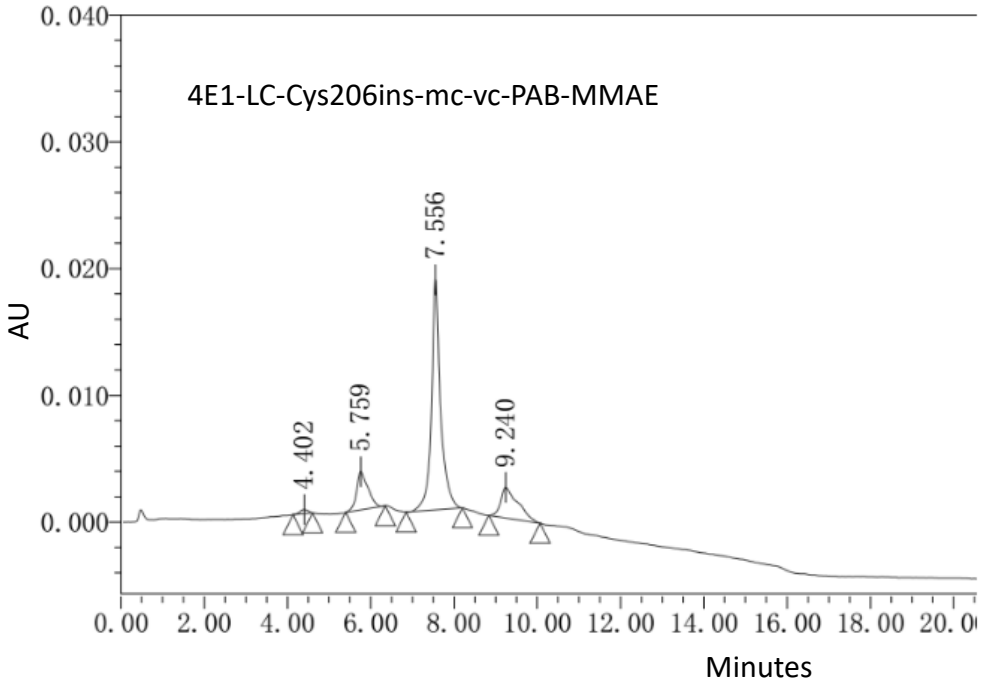

4E1-LC-Cys206ins-mc-vc-PAB-MMAE
AU
Minutes
4.402
5.759
7.556
9.240

FIG.5

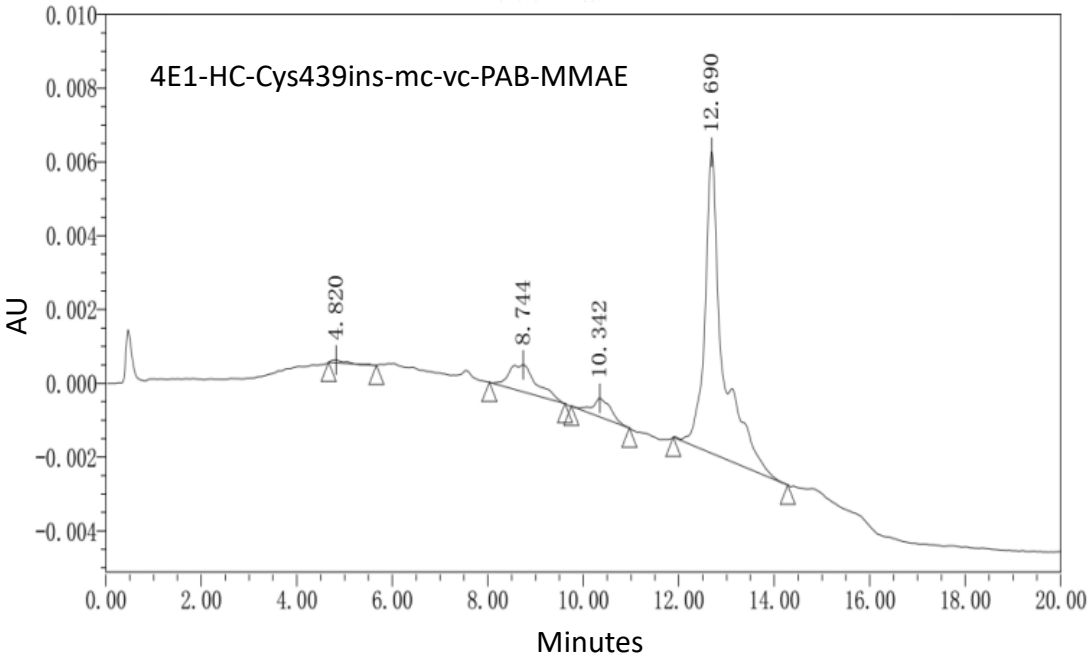

4E1-HC-Cys439ins-mc-vc-PAB-MMAE
AU
Minutes
4.820
8.744
10.342
12.690

FIG.6

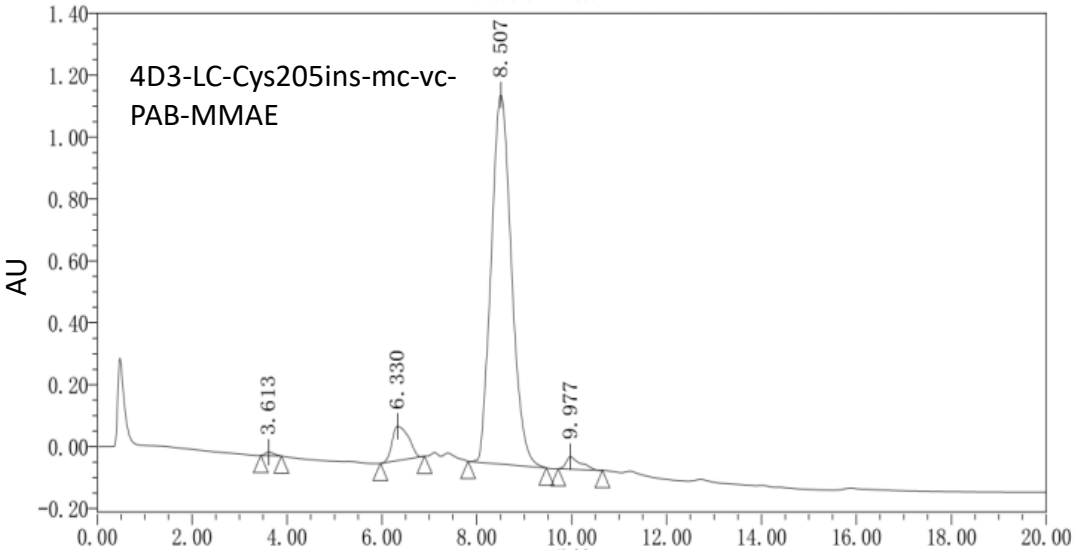

4D3-LC-Cys205ins-mc-vc-PAB-MMAE
AU
Minutes
3.613
6.330
8.507
9.977

FIG.7

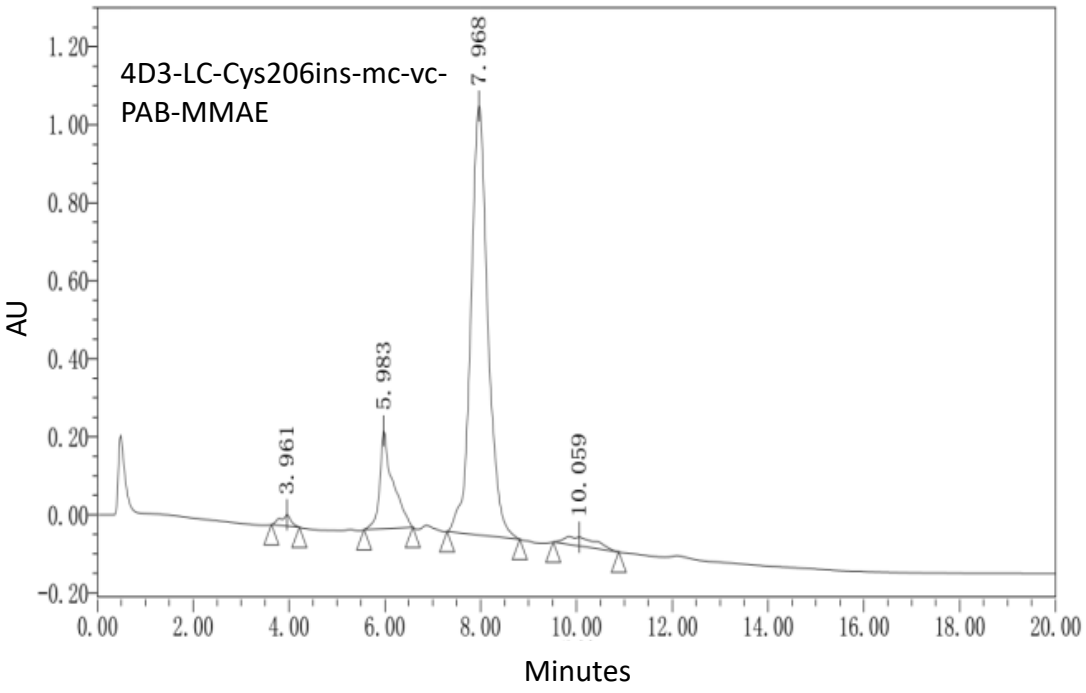

4D3-LC-Cys206ins-mc-vc-PAB-MMAE
AU
Minutes
3.961
5.983
7.968
10.059

FIG.8

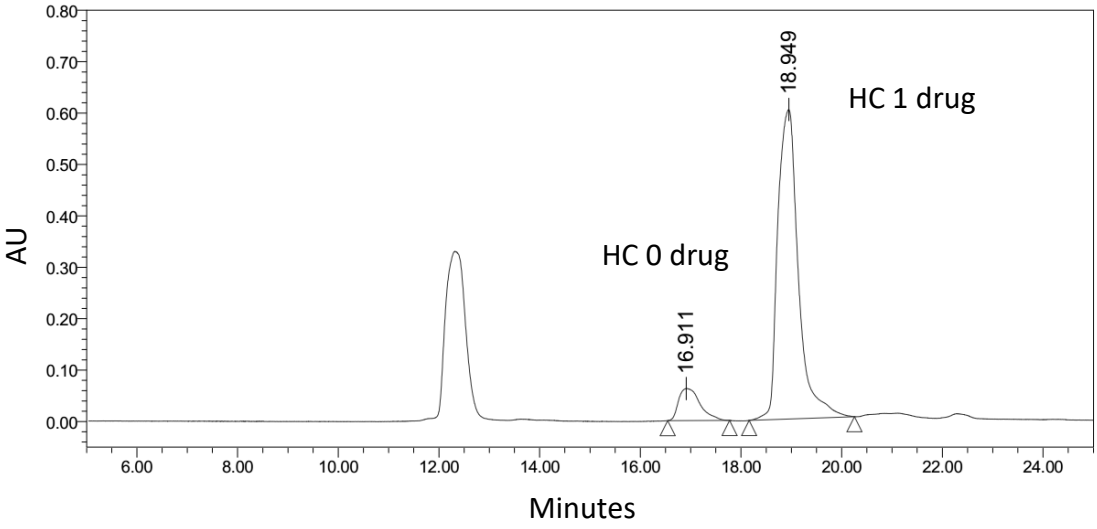

AU
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
18.949
HC 1 drug
HC 0 drug
16.911
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
Minutes

FIG.9

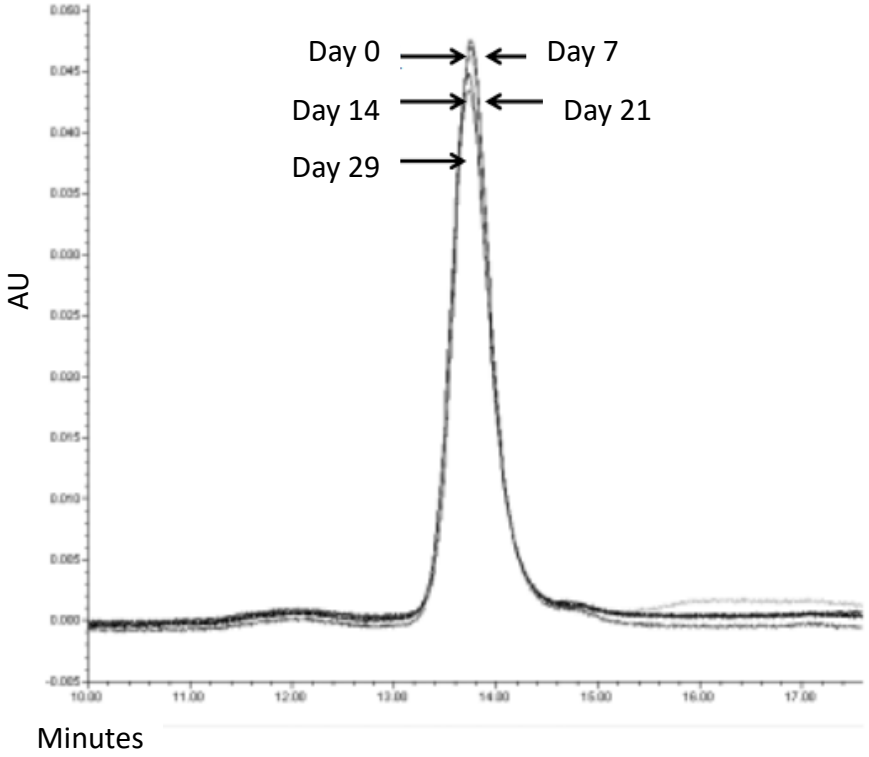

Day 0
Day 7
Day 14
Day 21
Day 29
AU
Minutes

FIG.10

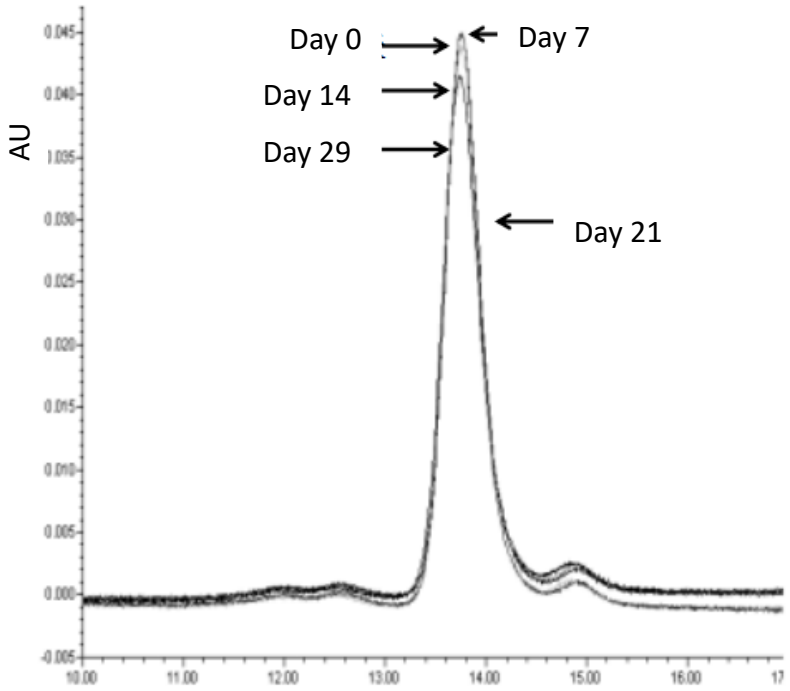

Day 0
Day 7
Day 14
Day 29
Day 21
AU

FIG.11

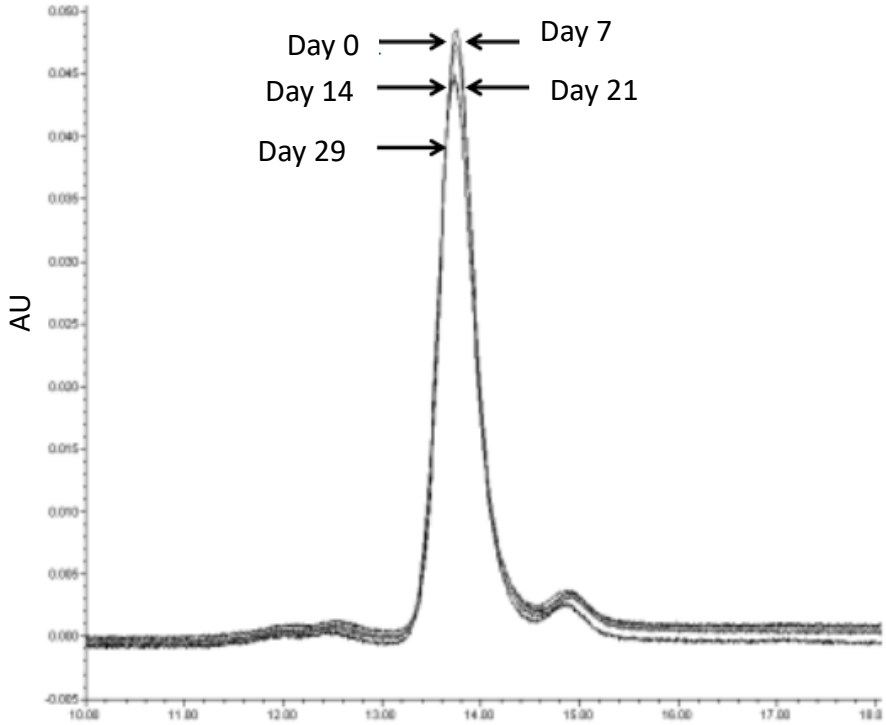

Day 0
Day 7
Day 14
Day 21
Day 29
AU

FIG.12

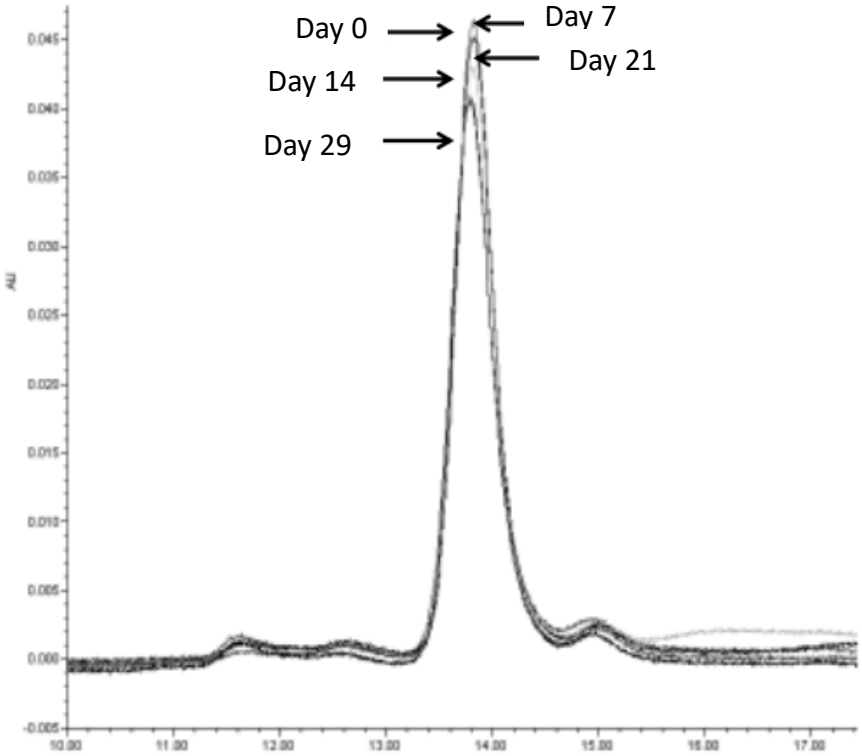

Day 0
Day 7
Day 21
Day 14
Day 29
AU

FIG.13

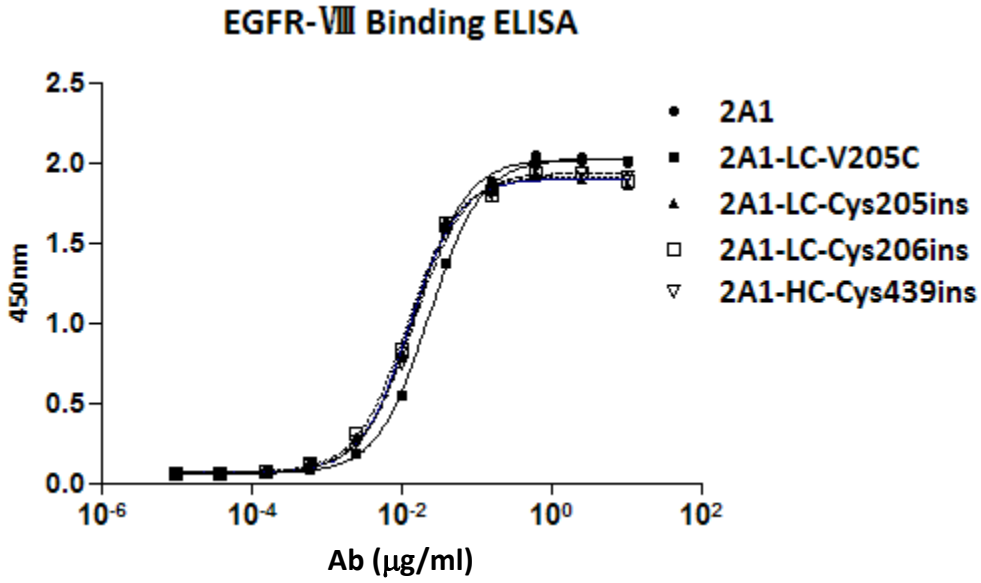

EGFR-Ⅷ Binding ELISA
2A1
2A1-LC-V205C
2A1-LC-Cys205ins
2A1-LC-Cys206ins
2A1-HC-Cys439ins
450nm
Ab (μg/ml)

FIG.14

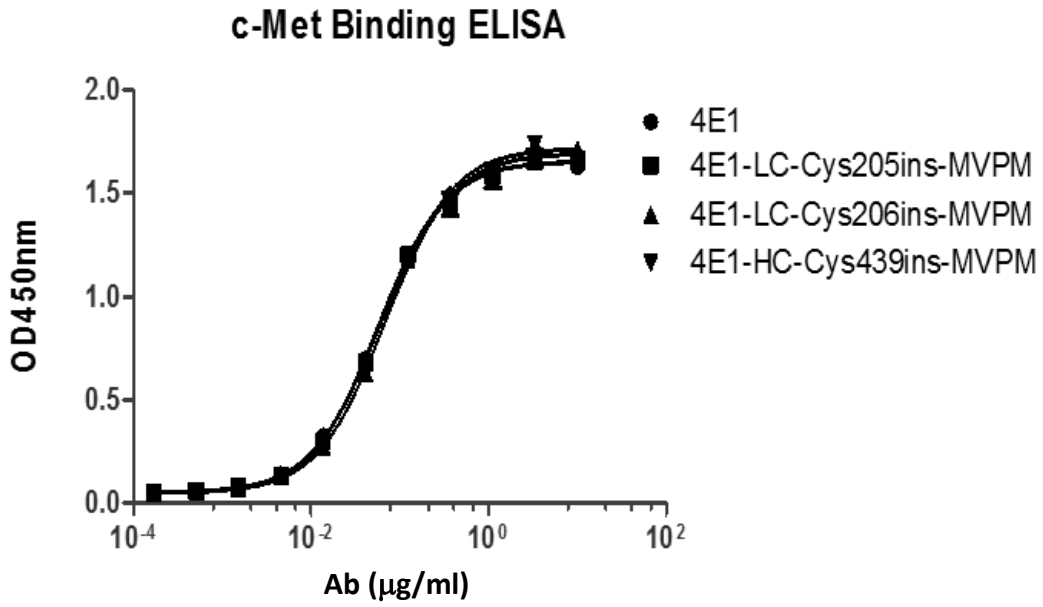

c-Met Binding ELISA
OD450nm
2.0
1.5
1.0
0.5
0.0
10-4
10-2
100
102
Ab (μg/ml)
4E1
4E1-LC-Cys205ins-MVPM
4E1-LC-Cys206ins-MVPM
4E1-HC-Cys439ins-MVPM

FIG.15

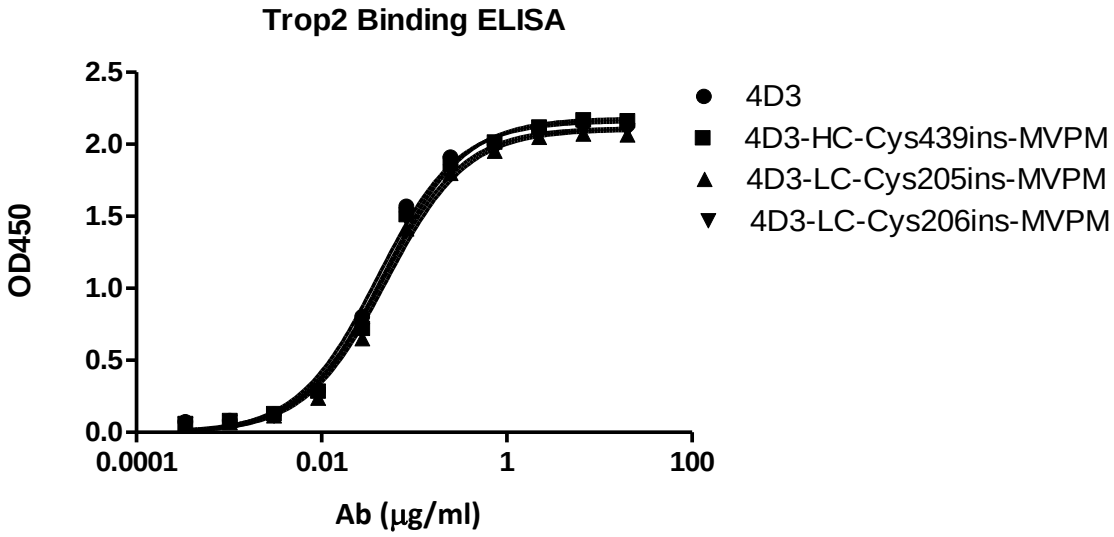

Trop2 Binding ELISA
OD450
2.5
2.0
1.5
1.0
0.5
0.0
0.0001
0.01
1
100
Ab (μg/ml)
4D3
4D3-HC-Cys439ins-MVPM
4D3-LC-Cys205ins-MVPM
4D3-LC-Cys206ins-MVPM

FIG.16

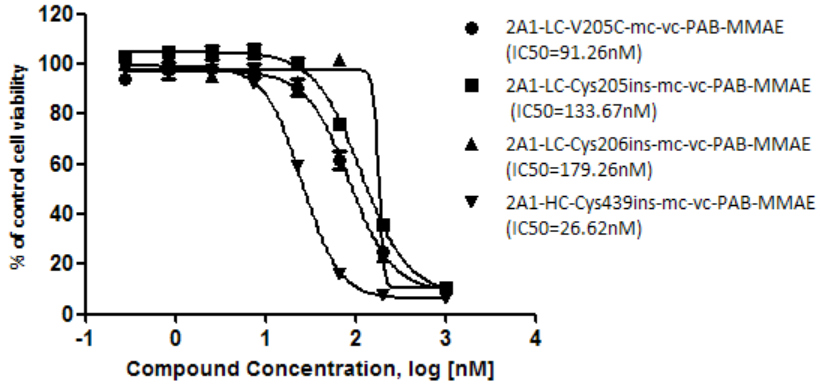


FIG.17

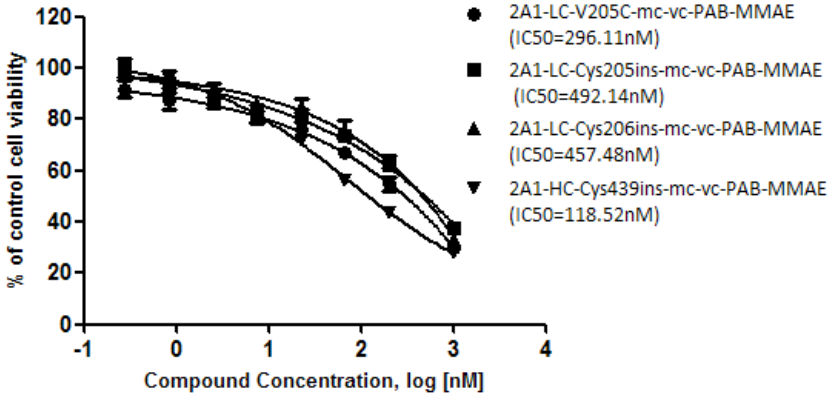


FIG.18

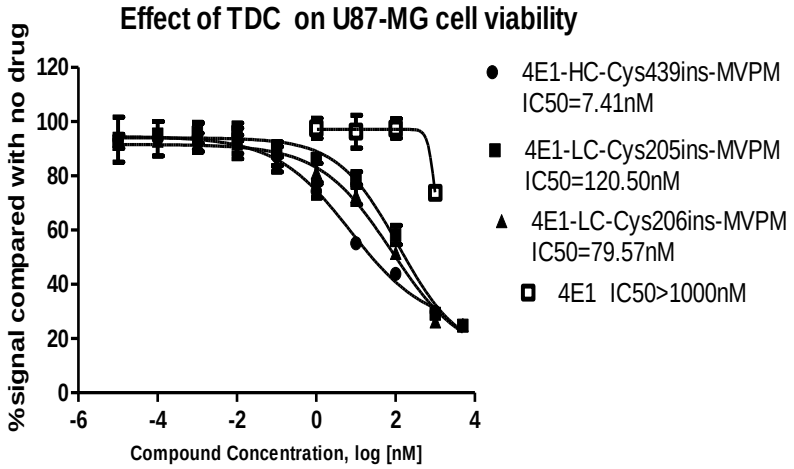


FIG.19

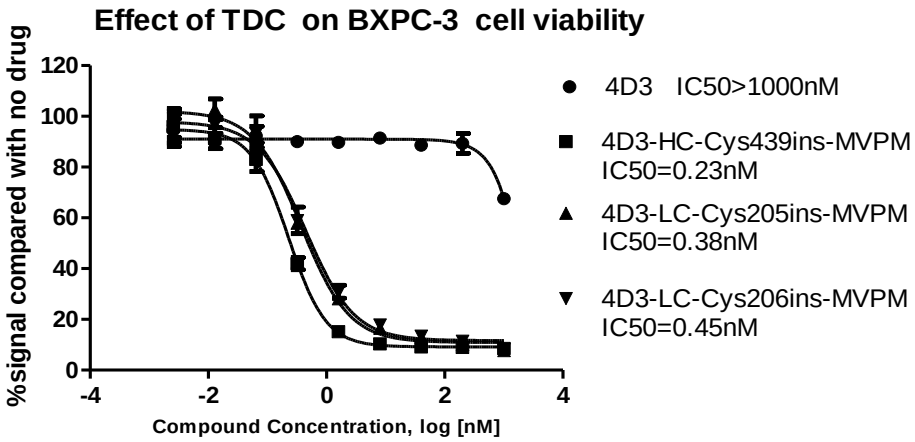

Effect of TDC on BXPC-3 cell viability
%signal compared with no drug
120
100
80
60
40
20
0
-4
-2
0
2
4
Compound Concentration, log [nM]
4D3 IC50>1000nM
4D3-HC-Cys439ins-MVPM
IC50=0.23nM
4D3-LC-Cys205ins-MVPM
IC50=0.38nM
4D3-LC-Cys206ins-MVPM
IC50=0.45nM

FIG.20

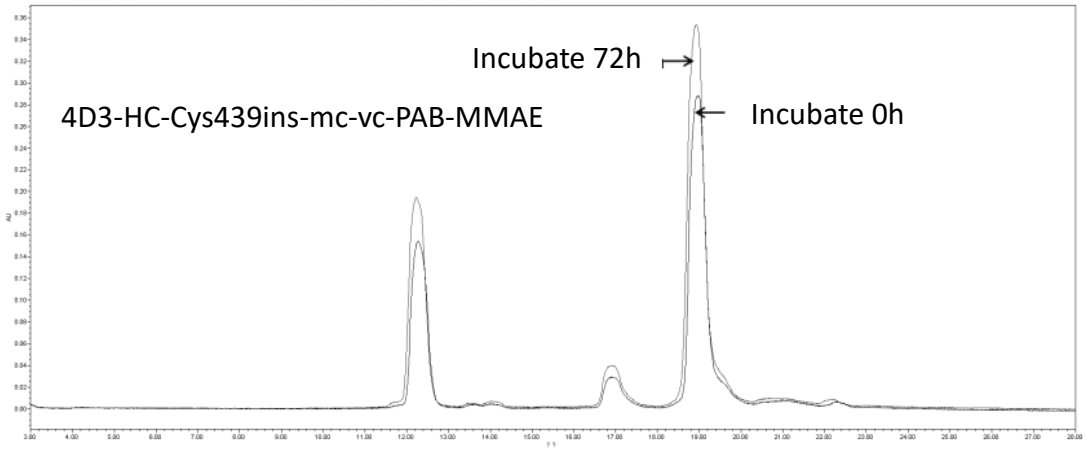

Incubate 72h
4D3-HC-Cys439ins-mc-vc-PAB-MMAE
Incubate 0h

FIG.21

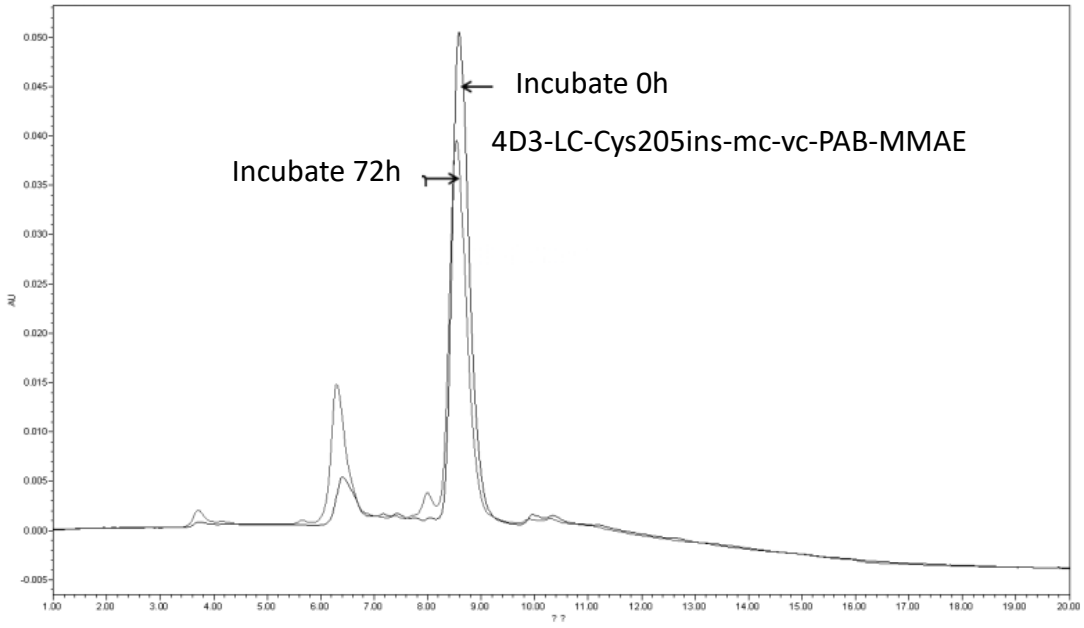

Incubate 0h
4D3-LC-Cys205ins-mc-vc-PAB-MMAE
Incubate 72h
AU
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00

FIG.22

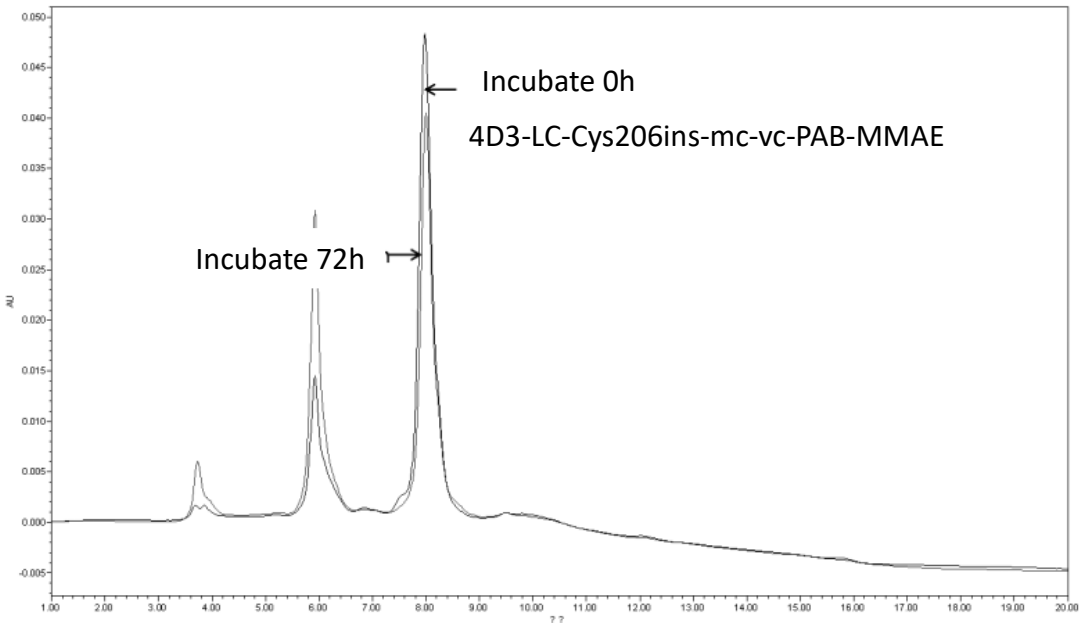
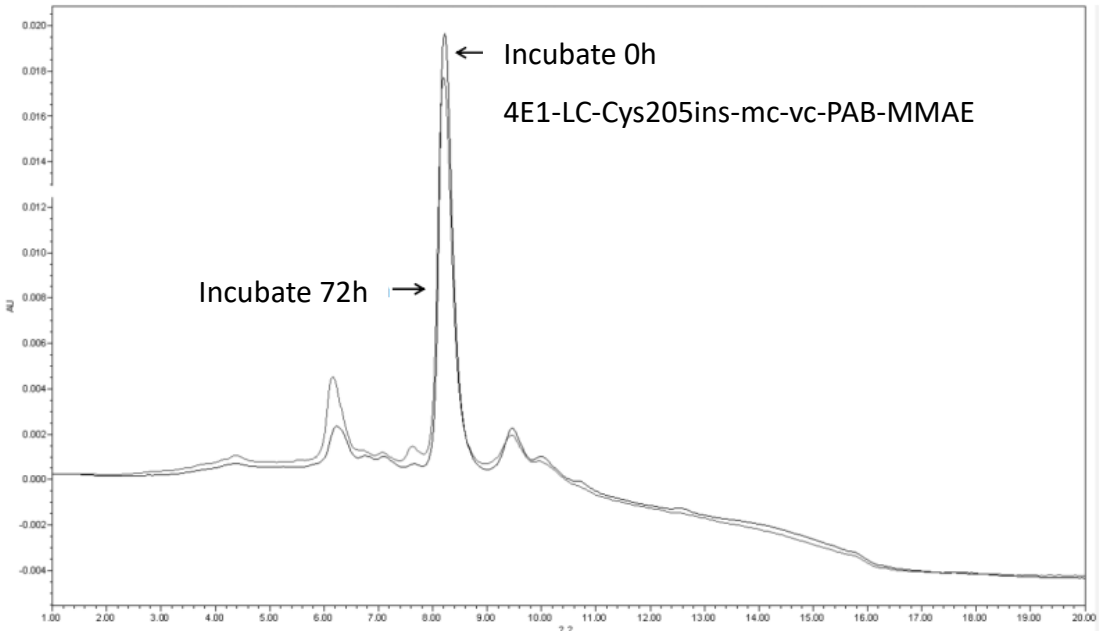

Incubate 0h
4D3-LC-Cys206ins-mc-vc-PAB-MMAE
Incubate 72h
AU
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00

FIG.23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4E1-LC-Cys205ins-mc-vc-PAB-MMAE
Incubate 72h
AU

FIG.24

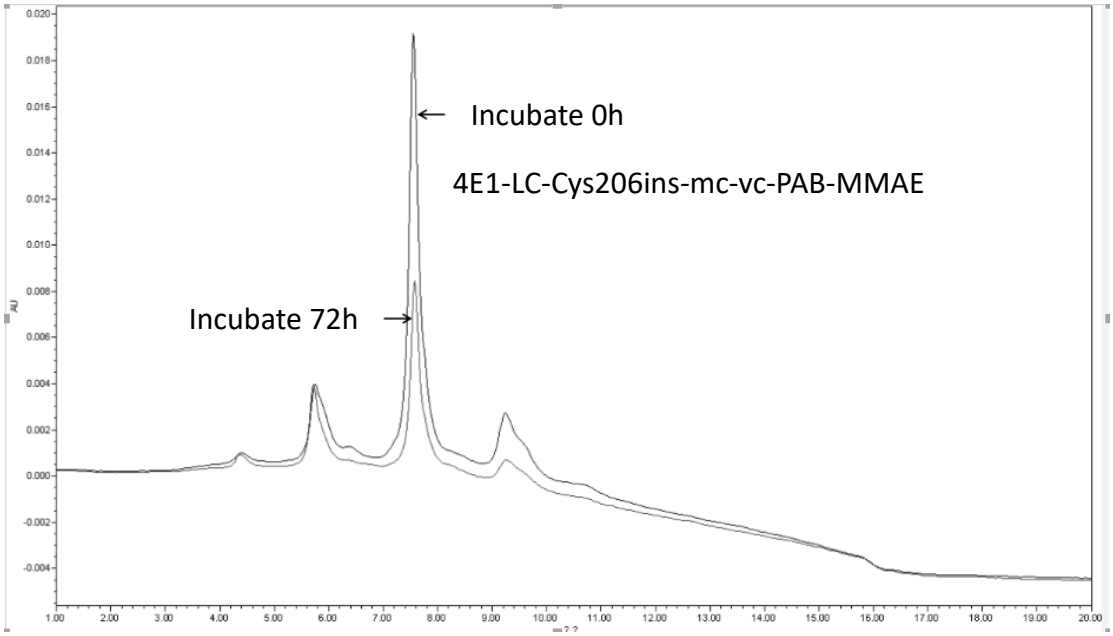
Incubate 0h
4E1-LC-Cys206ins-mc-vc-PAB-MMAE
Incubate 72h
AU

FIG.25

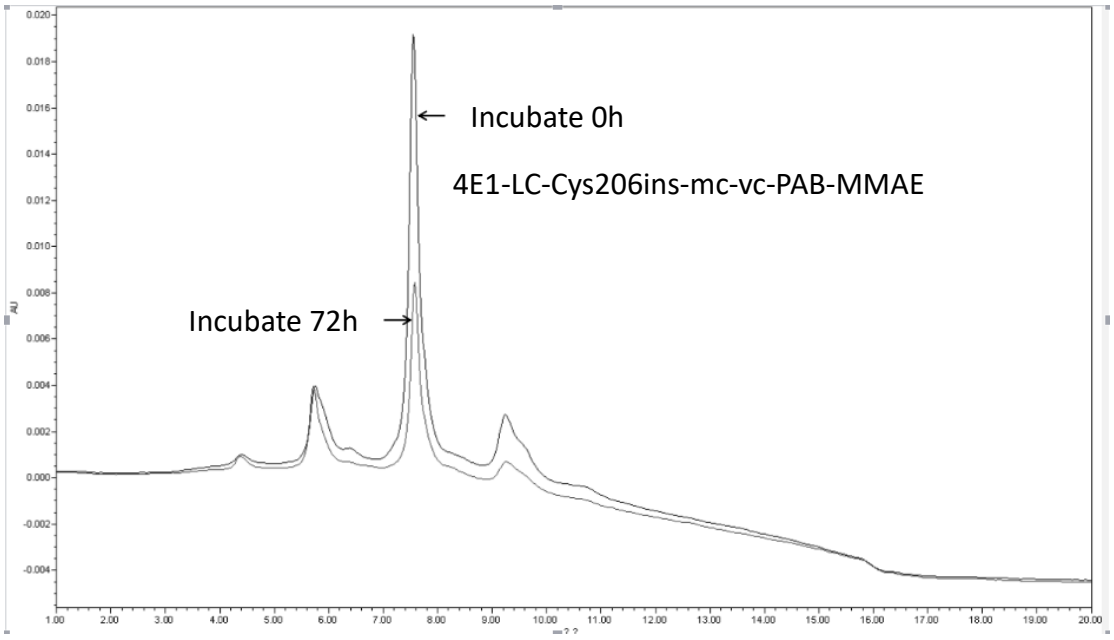

FIG.26

A . Antitumor Acitivity of Trop2 ADC as Single Agents in the Treatment of BXPC-3 Human Pancreatic Cancer Xenograft Model

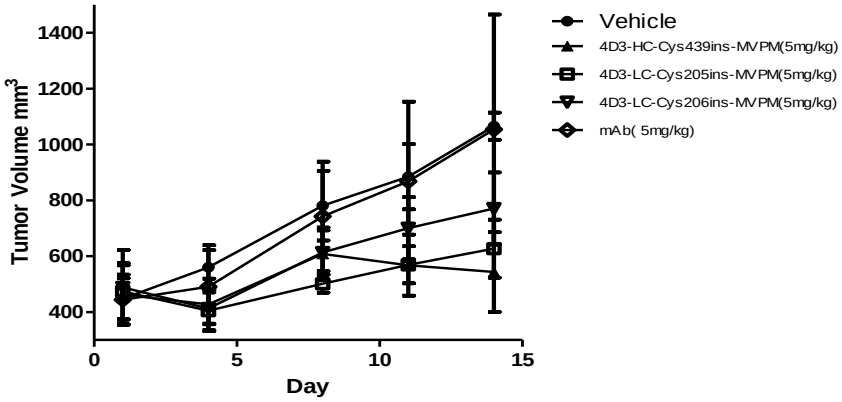

FIG.27

B .The Body Weight Changes of the Mice in the Different Groups

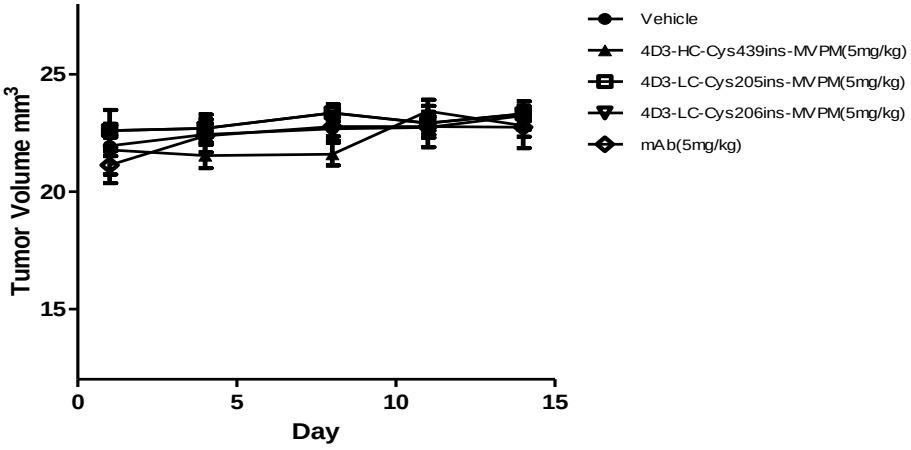

FIG.28

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<151> 2016-10-08
<160> 12

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<212> DNA
<213> [illegible]Fc[illegible]DNA[illegible]

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tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 180
ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 240
tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagag agttgagccc 300
aaatcttgtg acaaaactca cacatgccca ccgtgcccag cacctgaact cctgggggga 360
ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 420
gaggtcacat gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg 480
tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 540
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gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 660
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ctgaccaaga accaggtcag cctgacctgc ctggtcaaag gcttctatcc cagcgacatc 780
gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 840
ctggactccg acggctcctt cttcctctat agcaagctca ccgtggacaa gagcaggtgg 900
cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 960
cagaagagcc tctccctgtc tccgggtaaa 990

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Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Leu Ala Thr
20 25 30
Pro Pro Gly Pro Val Thr Val Ser Thr Ala Ser Gly Ala Leu Thr Ser
35 40 45
Gly Val His Thr Pro Pro Ala Val Leu Gly Ser Ser Gly Leu Thr Ser
50 55 60
Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gly Thr
65 70 75 80
Thr Ile Cys Ala Val Ala His Leu Pro Ser Ala Thr Leu Val Ala Leu
85 90 95
Ala Val Gly Pro Leu Ser Cys Ala Leu Thr His Thr Cys Pro Pro Cys
100 105 110
Pro Ala Pro Gly Leu Leu Gly Gly Pro Ser Val Pro Leu Pro Pro Pro
115 120 125

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Leu Pro Leu Ala Thr Leu Met Ile Ser Ala Thr Pro Gly Val Thr Cys
    130                 135                 140
Val Val Val Ala Val Ser His Gly Ala Pro Gly Val Leu Pro Ala Thr
145                 150                 155                 160
Thr Val Ala Gly Val Gly Val His Ala Ala Leu Thr Leu Pro Ala Gly
                165                 170                 175
Gly Gly Thr Ala Ser Thr Thr Ala Val Val Ser Val Leu Thr Val Leu
            180                 185                 190
His Gly Ala Thr Leu Ala Gly Leu Gly Thr Leu Cys Leu Val Ser Ala
        195                 200                 205
Leu Ala Leu Pro Ala Pro Ile Gly Leu Thr Ile Ser Leu Ala Leu Gly
    210                 215                 220
Gly Pro Ala Gly Pro Gly Val Thr Thr Leu Pro Pro Ser Ala Ala Gly
225                 230                 235                 240
Leu Thr Leu Ala Gly Val Ser Leu Thr Cys Leu Val Leu Gly Pro Thr
                245                 250                 255
Pro Ser Ala Ile Ala Val Gly Thr Gly Ser Ala Gly Gly Pro Gly Ala
            260                 265                 270
Ala Thr Leu Thr Thr Pro Pro Val Leu Ala Ser Ala Gly Ser Pro Pro
        275                 280                 285
Leu Thr Ser Leu Leu Thr Val Ala Leu Ser Ala Thr Gly Gly Gly Ala
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Val Pro Ser Cys Ser Val Met His Gly Ala Leu His Ala His Thr Thr
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Gly Leu Ser Leu Ser Leu Ser Pro Gly Leu
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aaggtggata acgccctcca atcgggtaac tcccaggaga gtgtcacaga gcaggacagc 180
aaggacagca cctacagcct cagcagcacc ctgacgctga gcaaagcaga ctacgagaaa 240
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ttcaacaggg gagagtgtta g 321

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<212> PRT
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Leu Leu Ser Gly Thr Ala Ser Val Val Cys Leu Leu Ala Ala Pro Thr
            20                  25                  30
Pro Ala Gly Ala Leu Val Gly Thr Leu Val Ala Ala Ala Leu Gly Ser
        35                  40                  45
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Gly Ala Ser Gly Gly Ser Val Thr Gly Gly Ala Ser Leu Ala Ser Thr
50 55 60
Thr Ser Leu Ser Ser Thr Leu Thr Leu Ser Leu Ala Ala Thr Gly Leu
65 70 75 80
His Leu Val Thr Ala Cys Gly Val Thr His Gly Gly Leu Ser Ser Pro
85 90 95
Val Thr Leu Ser Pro Ala Ala Gly Gly Cys
100 105

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actgcctctg ttgtgtgcct gctgaataac ttctatccca gagaggccaa agtacagtgg 120
aaggtggata acgccctcca atcgggtaac tcccaggaga gtgtcacaga gcaggacagc 180
aaggacagca cctacagcct cagcagcacc ctgacgctga gcaaagcaga ctacgagaaa 240
cacaaagtct acgcctgcga agtcacccat cagggcctga gctcgccctg cgtcacaaag 300
agcttcaaca ggggagagtg ttag 324

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Leu Leu Ser Gly Thr Ala Ser Val Val Cys Leu Leu Ala Ala Pro Thr
20 25 30
Pro Ala Gly Ala Leu Val Gly Thr Leu Val Ala Ala Ala Leu Gly Ser
35 40 45
Gly Ala Ser Gly Gly Ser Val Thr Gly Gly Ala Ser Leu Ala Ser Thr
50 55 60
Thr Ser Leu Ser Ser Thr Leu Thr Leu Ser Leu Ala Ala Thr Gly Leu
65 70 75 80
His Leu Val Thr Ala Cys Gly Val Thr His Gly Gly Leu Ser Ser Pro
85 90 95
Cys Val Thr Leu Ser Pro Ala Ala Gly Gly Cys
100 105

<210> 7
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aaggtggata acgccctcca atcgggtaac tcccaggaga gtgtcacaga gcaggacagc 180

aaggacagca cctacagcct cagcagcacc ctgacgctga gcaaagcaga ctacgagaaa 240
cacaaagtct acgcctgcga agtcacccat cagggcctga gctcgcccgt ctgcacaaag 300
agcttcaaca ggggagagtg ttag 324

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Leu Leu Ser Gly Thr Ala Ser Val Val Cys Leu Leu Ala Ala Pro Thr
20 25 30
Pro Ala Gly Ala Leu Val Gly Thr Leu Val Ala Ala Ala Leu Gly Ser
35 40 45
Gly Ala Ser Gly Gly Ser Val Thr Gly Gly Ala Ser Leu Ala Ser Thr
50 55 60
Thr Ser Leu Ser Ser Thr Leu Thr Leu Ser Leu Ala Ala Thr Gly Leu
65 70 75 80
His Leu Val Thr Ala Cys Gly Val Thr His Gly Gly Leu Ser Ser Pro
85 90 95
Val Cys Thr Leu Ser Pro Ala Ala Gly Gly Cys
100 105

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tggaactcag gcgccctgac cagcggcgtg cacaccttcc cggctgtcct acagtcctca 180
ggactctact ccctcagcag cgtggtgacc gtgccctcca gcagcttggg cacccagacc 240
tacatctgca acgtgaatca caagcccagc aacaccaagg tggacaagag agttgagccc 300
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ccgtcagtct tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct 420
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tacgtggacg gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac 540
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gagtacaagt gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc 660
aaagccaaag ggcagccccg agaaccacag gtgtacaccc tgccccatc ccgggatgag 720
ctgaccaaga accaggtcag cctgacctgc ctggtcaaag gcttctatcc cagcgacatc 780
gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gcctcccgtg 840
ctggactccg acggctcctt cttcctctat agcaagctca ccgtggacaa gagcaggtgg 900
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cagaagagcc tctcctgcct gtctccgggt aaa 993

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20 25 30
Pro Pro Gly Pro Val Thr Val Ser Thr Ala Ser Gly Ala Leu Thr Ser
35 40 45
Gly Val His Thr Pro Pro Ala Val Leu Gly Ser Ser Gly Leu Thr Ser
50 55 60
Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gly Thr
65 70 75 80
Thr Ile Cys Ala Val Ala His Leu Pro Ser Ala Thr Leu Val Ala Leu
85 90 95
Ala Val Gly Pro Leu Ser Cys Ala Leu Thr His Thr Cys Pro Pro Cys
100 105 110
Pro Ala Pro Gly Leu Leu Gly Gly Pro Ser Val Pro Leu Pro Pro Pro
115 120 125
Leu Pro Leu Ala Thr Leu Met Ile Ser Ala Thr Pro Gly Val Thr Cys
130 135 140
Val Val Val Ala Val Ser His Gly Ala Pro Gly Val Leu Pro Ala Thr
145 150 155 160
Thr Val Ala Gly Val Gly Val His Ala Ala Leu Thr Leu Pro Ala Gly
165 170 175
Gly Gly Thr Ala Ser Thr Thr Ala Val Val Ser Val Leu Thr Val Leu
180 185 190
His Gly Ala Thr Leu Ala Gly Leu Gly Thr Leu Cys Leu Val Ser Ala
195 200 205
Leu Ala Leu Pro Ala Pro Ile Gly Leu Thr Ile Ser Leu Ala Leu Gly
210 215 220
Gly Pro Ala Gly Pro Gly Val Thr Thr Leu Pro Pro Ser Ala Ala Gly
225 230 235 240
Leu Thr Leu Ala Gly Val Ser Leu Thr Cys Leu Val Leu Gly Pro Thr
245 250 255
Pro Ser Ala Ile Ala Val Gly Thr Gly Ser Ala Gly Gly Pro Gly Ala
260 265 270
Ala Thr Leu Thr Thr Pro Pro Val Leu Ala Ser Ala Gly Ser Pro Pro
275 280 285
Leu Thr Ser Leu Leu Thr Val Ala Leu Ser Ala Thr Gly Gly Gly Ala
290 295 300
Val Pro Ser Cys Ser Val Met His Gly Ala Leu His Ala His Thr Thr
305 310 315 320
Gly Leu Ser Leu Ser Cys Leu Ser Pro Gly Leu
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actgcctctg ttgtgtgcct gctgaataac ttctatccca gagaggccaa agtacagtgg       120
aaggtggata acgccctcca atcgggtaac tcccaggaga gtgtcacaga gcaggacagc       180
aaggacagca cctacagcct cagcagcacc ctgacgctga gcaaagcaga ctacgagaaa       240
cacaaagtct acgcctgcga agtcacccat cagggcctga gctcgccctg cacaaagagc       300
ttcaacaggg gagagtgtta g                                                 321

<210> 12
<211> 106
<212> PRT
<213> LC-V205C[illegible]Kappa[illegible]

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Leu Leu Ser Gly Thr Ala Ser Val Val Cys Leu Leu Ala Ala Pro Thr
            20                  25                  30
Pro Ala Gly Ala Leu Val Gly Thr Leu Val Ala Ala Ala Leu Gly Ser
        35                  40                  45
Gly Ala Ser Gly Gly Ser Val Thr Gly Gly Ala Ser Leu Ala Ser Thr
    50                  55                  60
Thr Ser Leu Ser Ser Thr Leu Thr Leu Ser Leu Ala Ala Thr Gly Leu
65                  70                  75                  80
His Leu Val Thr Ala Cys Gly Val Thr His Gly Gly Leu Ser Ser Pro
                85                  90                  95
Cys Thr Leu Ser Pro Ala Ala Gly Gly Cys
            100                 105
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