

Screening of fixed-point coupling sites of cysteine-modified antibody-toxin conjugate (TDC)

Technical Field

The present invention relates to a compound as well as methods for the preparation and applications thereof, and in particular relates to a class of cysteine-modified antibody-toxin conjugates (TDCs) and to methods for the preparation and applications thereof.

Technical Background:

Antibody drug conjugates (ADCs) represent a currently hot area in the field of targeted therapy, with two drugs already approved in the U.S., Adcetris and Kadcyra, showing good clinical efficacy, and more than 50 ADCs are currently being investigated as part of clinical phase studies. The novel cysteine-modified antibody-toxin conjugate (TDC) disclosed in this patent has the advantage of better drug homogeneity and fewer side effects compared to non-fixed-point coupled ADCs, and have been shown to perform significantly better than non-fixed-point coupled ADCs in preclinical studies.

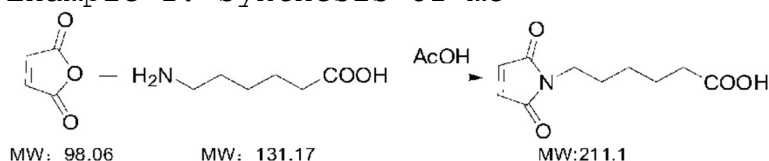
Summary of the Invention:

For the cysteine-modified antibody-toxin conjugate (TDC) constituted by the present invention, corresponding cysteine insertion sites comprise one or more sites selected from the following 15 insertion sites: Light Chain Position 110 (Kabat number, with amino acid sequence EIKRTCVAAPS (SEQ ID NO:45)), Light Chain Position 111 (Kabat number, with amino acid sequence IKRTVCAAPSV (SEQ ID NO:46)), Light Chain Position 142 (Kabat number, with amino acid sequence NNFYPCREAKV (SEQ ID NO:47)), Heavy Chain Position 254 (with amino acid sequence ISRTPCEVTCV (SEQ ID NO:48)), Heavy Chain Position 255 (with amino acid sequence SRTPECVTCVV (SEQ ID NO:49)), Heavy Chain Position 258 (with amino acid sequence PEVTCCVVVDV (SEQ ID NO:50)), Heavy Chain

Position 259 (with amino acid sequence EVTCVCVVDVS (SEQ ID NO:51)), Heavy Chain Position 354 (with amino acid sequence PSRDECLTKNQ (SEQ ID NO:52)), Heavy Chain Position 355 (with amino acid sequence SRDELCTKNQV (SEQ ID NO:53)), Heavy Chain Position 357 (with amino acid sequence DELTKCNQVSL (SEQ ID NO:54)), Heavy Chain Position 378 (with amino acid sequence IAVEWCESNGQ (SEQ ID NO:55)), Heavy Chain Position 379 (with amino acid sequence AVEWECSNGQP (SEQ ID NO:56)), Heavy Chain Position 386 (with amino acid sequence GQPENCNYKTT (SEQ ID NO:57)), Heavy Chain Position 387 (with amino acid sequence QPENNCYKTTP (SEQ ID NO:58)), and heavy chain position 410 (with amino acid sequence KLTVDCKSRWQ (SEQ ID NO:59)). Antibodies that contain one or more of the cysteine insertion mutations described above maintain the ability of their parental antibody to bind to corresponding antigens (affinity). By performing antibody-toxin conjugate (TDCs) fixed-point coupling between a cysteine sulfhydryl group subject to light-chain insertion and/or a cysteine sulfhydryl group subject to heavy-chain insertion, a toxin to antibody ratio (DAR) of 1.6 to 2.0 or 3.2 to 4.0 can be realized.

Examples

Example 1: Synthesis of mc



3.9 g (0.03 mol) of 6-aminocaproic acid and 3.5 g (0.036 mol) of 1.2 eq maleic anhydride were added to 30 ml of glacial acetic acid. The reaction solution was stirred at 120°C for 4 to 6 hours. After the reaction was complete, heating was stopped and the solution was allowed to cool to room temperature naturally. The solution was then concentrated under reduced pressure at 60°C to remove most of the acetic acid. The brownish

Cell-expressed 2A1-HC-Cys357ins antibodies (SEQ ID NO:34) were purified using Mabselect Sure and after elution at low pH, a Tris solution was immediately added to achieve neutralization, and the solution was exchanged to a Tris-HCl buffer of pH 7.5. The compound mc-vc-PAB-MMAE was a white powder and was dissolved in DMA for later use. The antibody needed to be first reduced to remove the barrier present on the mutant cysteine residue. 1M DTT aqueous solution was added at a 40x molecular ratio to the 2A1-HC-Cys357ins antibody solution and the resulting mixture was mixed well and allowed to react at 20°C for two hours. Thereafter, the pH of the sample was adjusted to 5.0, and the DTT and barrier in the sample was removed via SP Sepharose FF cation exchange chromatography. Next, DHAA solution was added to the sample at a molecular ratio of 20x and the reaction was allowed to proceed for four hours at 25°C in the dark to reconnect the disulfide bonds between the antibody chains. Thereafter, mc-vc-PAB-MMAE solution was added to induce coupling between mc-vc-PAB-MMAE and the antibody mutant cysteine, after which thorough mixing was performed and the reaction was allowed to proceed at 25°C for two hours. After the reaction was complete, SP Sepharose FF cation exchange chromatography was used to remove the mc-vc-PAB-MMAE that was not coupled to an antibody molecule, and 2A1-HC-Cys357ins-mc-vc-PAB-MMAE TDC samples were obtained.

Example 16: HIC-HPLC Measurement of Toxin to Antibody Ratio (DAR)

TDC samples were analyzed via high performance liquid chromatography hydrophobic chromatography, and DAR was calculated based on corresponding peak area. The specific method used was as follows:

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

	Antibody Mutation	R ²	EC50 (nM)
Antibody	2A1-LC-Cys110ins	0.9948	0.04039
	2A1-LC-Cys111ins	0.9957	0.04667
	2A1-LC-Cys142ins	0.9951	0.04976
	2A1-HC-Cys254ins	0.9956	0.04556
	2A1-HC-Cys255ins	0.9932	0.03904
	2A1-HC-Cys258ins	0.9955	0.04231
	2A1-HC-Cys259ins	0.9946	0.04308
	2A1-HC-Cys354ins	0.9951	0.04053
	2A1-HC-Cys355ins	0.9954	0.04152
	2A1-HC-Cys357ins	0.9974	0.04031
	2A1-HC-Cys378ins	0.9964	0.04039
	2A1-HC-Cys379ins	0.9952	0.04089
	2A1-HC-Cys386ins	0.9918	0.04346
	2A1-HC-Cys387ins	0.9974	0.04516
	2A1-HC-Cys410ins	0.9978	0.04204

Appended Figure 3 and Appended Table 4: 2A1-LC-Cys110ins (SEQ ID NO:16), 2A1-LC-Cys111ins (SEQ ID NO:18), 2A1-LC-Cys142ins (SEQ ID NO:20), 2A1-HC-Cys254ins (SEQ ID NO:22), 2A1-HC-Cys255ins (SEQ ID NO:24), 2A1-HC-Cys258ins (SEQ ID NO:26), 2A1-HC-Cys259ins (SEQ ID NO:28), 2A1-HC-Cys354ins (SEQ ID NO:30), 2A1-HC-Cys355ins (SEQ ID NO:32), 2A1-HC-Cys357ins (SEQ ID NO:34), 2A1-HC-Cys378ins (SEQ ID NO:36), 2A1-HC-Cys379ins (SEQ ID NO:38), 2A1-HC-Cys386ins (SEQ ID NO:40), 2A1-HC-Cys387ins (SEQ ID NO:42) and 2A1-HC-Cys410ins (SEQ ID NO:44) antibodies maintained 2A1 affinity for the antigen EGFRvIII.

Example 19: Cytotoxicity Testing

TDC cytotoxic activity was determined via the following experimental procedure: TDC was added to EGFR-overexpressing or EGFRvIII-expressing human tumor cell culture medium, and cell viability was determined after

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72 hours of cell culture. Cell-based *in vitro* experiments were used to determine cell viability, cytotoxicity and

Claims

1. A cysteine-modified antibody-toxin conjugate, comprising an antibody and a highly active cytotoxin, wherein the antibody comprises an inserted cysteine at one or more cysteine insertion site, wherein the cysteine insertion site comprises one or more of: kappa/λ light chain constant region light chain positions 110, 111, 142, IgG antibody heavy chain constant region positions 254, 255, 258, 259, 354, 355, 357, 378, 379, 386, 387 or 410.

2. The cysteine-modified antibody-toxin conjugate of claim 1, wherein the amino acid sequence of said cysteine insertion site comprises one or more of:

LC-110ins: EIKRTCVAAPS (SEQ ID NO:45);
LC-111ins: IKRTVCAAPSV (SEQ ID NO:46);
LC-142ins: NNFYPCREAKV (SEQ ID NO:47);
HC-254ins: ISRTPCEVTCV (SEQ ID NO:48);
HC-255ins: SRTPECVTCVV (SEQ ID NO:49);
HC-258ins: PEVTCCVVVDV (SEQ ID NO:50);
HC-259ins: EVTCVCVVDVS (SEQ ID NO:51);
HC-354ins: PSRDECLTKNQ (SEQ ID NO:52);
HC-355ins: SRDELCTKNQV (SEQ ID NO:53);
HC-357ins: DELTKCNQVSL (SEQ ID NO:54);
HC-378ins: IAVEWCESNGQ (SEQ ID NO:55);
HC-379ins: AVEWECSNGQP (SEQ ID NO:56);
HC-386ins: GQPENCNYKTT (SEQ ID NO:57);
HC-387ins: QPENNCYKTP (SEQ ID NO:58);
HC-410ins: KLTVDCKSRWQ (SEQ ID NO:59).

3. The cysteine-modified antibody-toxin conjugate of claim 1, wherein the highly active cytotoxin is conjugated to the inserted cysteine by being coupled through its sulfhydryl

group via a linker wherein the linker and the antibody light chain have an amino acid sequence corresponds to: EIKRTCVAAAPS (SEQ ID NO:45), IKRTVCAAPSV (SEQ ID NO:46) and NNFYPCREAKV (SEQ ID NO:47) and wherein the linker and the antibody heavy chain have an amino acid sequence corresponds to: ISRTPCEVTCV (SEQ ID NO:48), SRTPECVTCVV (SEQ ID NO:49), PEVTCCVVVDV (SEQ ID NO:50), EVTCVCVVDVS (SEQ ID NO:51), PSRDECLTKNQ (SEQ ID NO:52), SRDELCTKNQV (SEQ ID NO:53), DELTKCNQVSL (SEQ ID NO:54), IAVEWCESNGQ (SEQ ID NO:55), AVEWECSNGQP (SEQ ID NO:56), GQPENCNYKTT (SEQ ID NO:57), QPENNCYKTTT (SEQ ID NO:58) or KLTVDCKSRWQ (SEQ ID NO:59); wherein C corresponds to the inserted cysteine in the light or heavy chain of the cysteine-modified antibody.

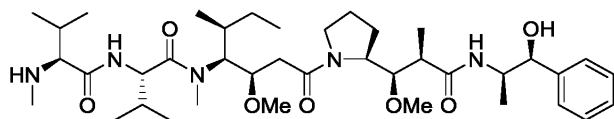
4. The cysteine-modified antibody-toxin conjugate of claim 1, wherein said antibody light chain comprises kappa or lambda isotypes.

5. The cysteine-modified antibody-toxin conjugate of claim 1, wherein said antibody heavy chain comprises IgG1, IgG2, IgG3 or IgG4 isotypes.

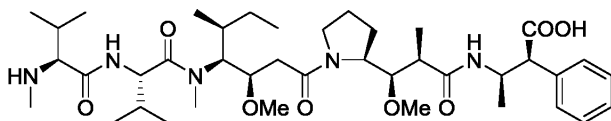
6. The cysteine-modified antibody-toxin conjugate of claim 1, wherein said inserted cysteine comprises a sulfhydryl group before being coupled to a highly active cytotoxin.

7. The cysteine-modified antibody-toxin conjugate of claim 6, wherein said sulfhydryl group is capable of chemical coupling.

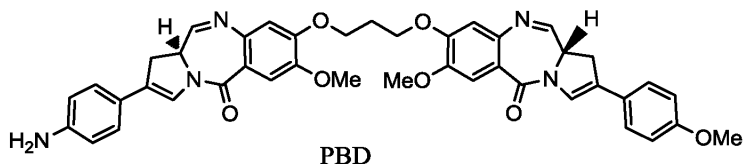
8. The cysteine-modified antibody-toxin conjugate of claim 3, wherein said highly active cytotoxin is selected from a set comprising of MMAE, MMAF, PBD, SN-38, Dox, Amanitin having the following molecular formulas, and their derivatives thereof:



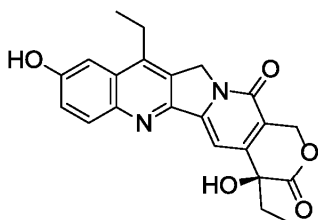
MMAE



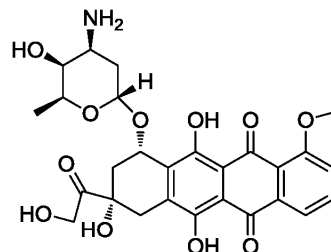
MMAF



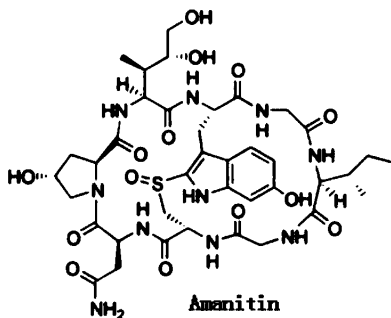
PBD



SN-38



DOX



Amanitin

9. The cysteine-modified antibody-toxin conjugate of claim 1, wherein said highly active cytotoxin to the antibody ratio ranges from 1.6 to 2.0 or from 3.2 to 4.0.

10. The cysteine-modified antibody-toxin conjugate of claim 1, prepared by,
reducing a cysteine-modified antibody comprising a shielded cysteine having a shielded sulfhydryl group and a shielding group,
using a reducing agent to remove the shielding group from the shielded cysteine to provide a free shielding group and a reduced antibody comprising a de-shielded cysteine having a sulfhydryl group,
removing the free shielding group and any excess reducing agent using a method comprising cation exchange chromatography or ultrafiltration,
oxidizing the reduced antibody using an oxidizing agent to reattach the interchain disulfide bonds of the antibody,
coupling the active cytotoxin with the sulfhydryl group on the inserted cysteine to provide the cysteine-modified antibody-toxin conjugate, and
removing any uncoupled highly active cytotoxin using a method comprising cation exchange chromatography or ultrafiltration.