

SPECIFICATION

COMPOUND AND ANTIBODY-DRUG CONJUGATE COMPRISING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is based on Chinese Patent Application No. 202311037231.5 filed on August 16, 2023 and claims priority thereto, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present disclosure belongs to the field of biomedicines, and relates to a compound and an antibody-drug conjugate comprising same. The present disclosure also relates to a pharmaceutical composition comprising the antibody-drug conjugate and use thereof. Specifically, the antibody-drug conjugate is an anti-HER3 antibody-drug conjugate.

BACKGROUND

Cancer is the leading bane threatening human life and health. Traditional treatment methods include surgery, chemotherapy, radiotherapy, etc., but these methods usually exert toxic and side effects on normal cells while killing cancer cells, resulting in a narrow therapeutic window. Immunotherapy is a significant approach in cancer treatment, which typically leverages the body's own immune mechanisms to combat cancer cells, and is a preferred treatment scheme for cancer patients with small early lesions due to its unique advantages of high targeting property and few side effects. However, for advanced solid tumors, immunotherapy has limited efficacy and requires combination with other methods to jointly

combat cancer cells (Li Yunfeng, Xin Jie, Li Jing, et al., Research progress of linkage technology of antibody-drug conjugates[J]. *Chinese Journal of New Drugs and Clinical Remedies*, 2022, 41(12): 10).

Antibody-drug conjugates (ADCs) are an emerging class of macromolecular targeted drugs that combine the powerful killing effect of traditional small-molecule chemotherapy with the tumor-targeting property of antibody drugs. An ADC consists of three main moieties: an antibody responsible for selectively recognizing a cancer cell surface antigen, a payload responsible for killing cancer cells, and a linker linking the antibody and the payload. After the ADC drug enters the blood circulation, its antibody moiety specifically binds to an antigen on the surface of tumor cells, and the complex formed by the binding enters the cells through receptor-mediated endocytosis (which can be classified into four types: clathrin-mediated endocytosis, cave-like invagination, macropinocytosis, and clathrin- and caveolin-independent endocytosis). Cleavable linkers are sensitive to environmental factors in tumor cells and can be cleaved by specific pH environments, proteases, or certain chemical substances. ADC drugs carrying the non-cleavable linkers are digested by lysosomes, leading to the release of the drug. Small-molecule payloads of certain ADC drugs can penetrate cell membranes to further kill surrounding cancer cells, achieving a bystander killing effect (Khongorzul, Puregmaa, et al., “Antibody-Drug Conjugates: A Comprehensive Review”. *Mol Cancer Res* 1(2020); Kostova Vesela, Désos Patrice, Starck Jérôme-Benoît et al., The Chemistry Behind ADCs.[J]. *Pharmaceuticals (Basel)*, 2021, 14: undefined.).

The accumulation of payloads within tumors is limited, thus requiring tumor killing at sub-nanomolar concentrations. In addition, payloads need to have good stability to maintain structural integrity and activity in the circulatory system and lysosomes. Common payloads mainly include

tubulin inhibitors and DNA damaging agents. Tubulin inhibitors achieve the purpose of killing tumor cells and inhibiting the rapid proliferation of tumor cells by inhibiting the generation and aggregation of tubulin. Tubulin inhibitors commonly used for ADC construction include maytansines, auristatins, tubulysins, and the like. DNA damaging agents are classified into three major categories based on different mechanisms of action: DNA double-strand breakers, DNA intercalators, and DNA alkylating agents. DNA is critical in the growth and proliferation process of cells. By disrupting DNA, tumor cells can be efficiently killed and the rapid proliferation of the tumor cells can be inhibited. DNA damaging agents commonly used for ADC construction include pyrrolobenzodiazepines, indolochlorobenzodiazepines (e.g., pyrrolo[2,1-c][1,4]benzodiazepine, PBD), duocarmycins, camptothecin (CPT) and derivatives thereof (e.g., topoisomerase I inhibitors Dxd and SN-38), calicheamicins, and the like (Dan Nirnoy, Setua Saini, Kashyap Vivek K et al., *Antibody-Drug Conjugates for Cancer Therapy: Chemistry to Clinical Implications*. [J]. *Pharmaceuticals(Basel)*, 2018, 11: undefined.).

HER3 is a member of the human epidermal growth factor receptor (HER) family and consists of three moieties: an extracellular ligand-binding region, an α -helical transmembrane region, and an intracellular tyrosine kinase region. HER3 is abnormally overexpressed in a variety of invasive tumors, including breast tumor, non-small cell lung cancer (NSCLC), metastatic colon cancer, head and neck cancer, pancreatic cancer, ovarian cancer, clear cell sarcoma, gastric cancer, skin cancer, and the like. HER3 can bind to the ligand neuregulin (NRG). Although HER3 lacks intrinsic tyrosine kinase activity, it can form a heterodimer with HER2 to induce the crosslinking and phosphorylation of highly conserved intracellular kinase residues, that is, a process where one receptor phosphorylates specific tyrosine residues on the other, thereby recruiting and activating

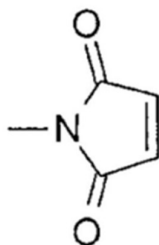
downstream proteins. This process directly activates the PI3K/AKT pathway, triggering a signal cascade that promotes the occurrence and progression of tumor cells.

In summary, the development of HER3-targeted ADC drugs with significant efficacy and low toxic and side effects has broad prospects.

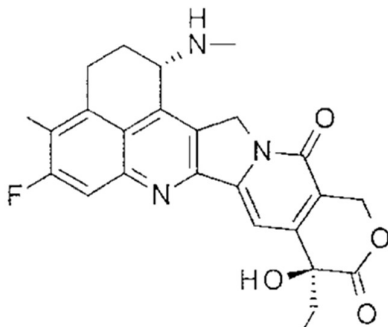
SUMMARY

Through intensive studies and creative efforts, the inventors have prepared a compound capable of serving as a linker and a payload, and have further prepared an antibody-drug conjugate. The prepared antibody-drug conjugate has good stability and/or biological activity. The present disclosure is detailed below:

One aspect of the present disclosure relates to a compound or a pharmaceutically acceptable salt thereof, wherein the compound comprises a compound of Formula III, *n* alanine residues, and a compound of Formula IV that are sequentially linked,



Formula III,



Formula IV,

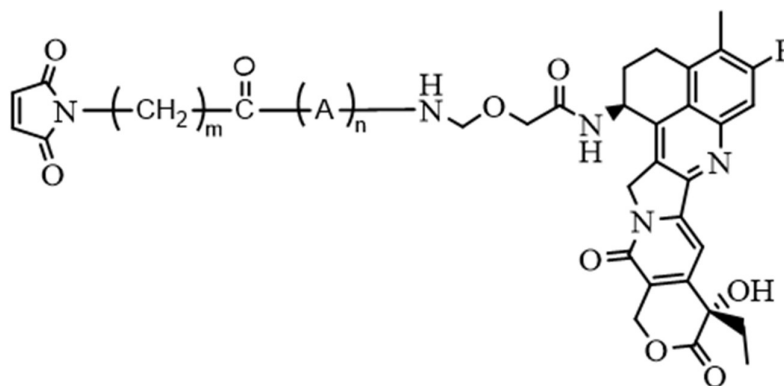
wherein

n is 2, 3, 4, 5, or 6;

any two of the compound of Formula III, the n alanine residues, and the compound of Formula IV are independently either linked directly (e.g., via a chemical bond) or via a chemical group.

The pharmaceutically acceptable salt of the compound of the present disclosure includes conventional salts formed with pharmaceutically acceptable inorganic or organic acids, or inorganic or organic bases. Examples of suitable acid addition salts include salts formed with hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, perchloric acid, fumaric acid, acetic acid, propionic acid, succinic acid, glycolic acid, formic acid, lactic acid, maleic acid, tartaric acid, citric acid, pamoic acid, malonic acid, hydroxymaleic acid, phenylacetic acid, glutamic acid, benzoic acid, salicylic acid, fumaric acid, toluenesulfonic acid, methanesulfonic acid, naphthalene-2-sulfonic acid, benzenesulfonic acid, hydroxynaphthoic acid, hydroiodic acid, malic acid, tannic acid, and the like. Examples of suitable base addition salts include salts formed with sodium, lithium, potassium, magnesium, aluminum, calcium, zinc, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, N-methylglucamine, procaine, and the like.

In some embodiments of the present disclosure, provided is the compound or the pharmaceutically acceptable salt thereof, having a structural formula represented by Formula I below,



Formula I

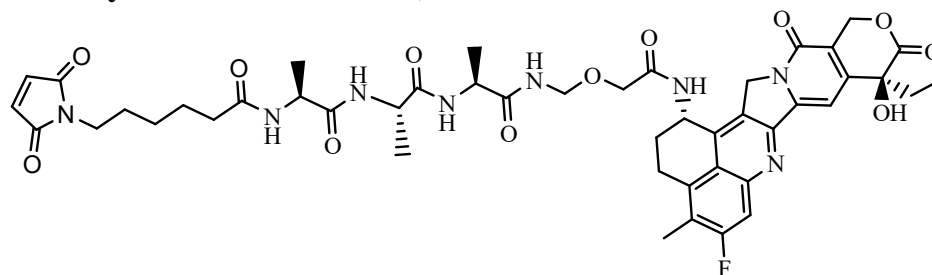
wherein

A represents alanine (alanine residue);

m is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12;

n is 2, 3, 4, 5, or 6.

In some embodiments of the present disclosure, provided is the compound or the pharmaceutically acceptable salt, wherein the compound is represented by Formula II below,



Formula II

Another aspect of the present disclosure relates to an antibody-drug conjugate (ADC) comprising an antibody or an antigen-binding fragment thereof, a linker, and a payload,

wherein the linker and the payload are the compound or the pharmaceutically acceptable salt thereof according to any one of the aspects of the present disclosure;

preferably, the linker is linked to the antibody or the antigen-binding

**fragment thereof via one or more thioether bonds;
preferably, the linker forms a thioether bond with a sulfur atom at a
disulfide bond position of the hinge portion of the antibody.**

**Without being bound by theory, the maleimide-N-yl undergoes an
alkylation reaction with the sulfhydryl group in the antibody to form a
stable thioether bond.**

**In some embodiments of the present disclosure, provided is the antibody-
drug conjugate, wherein the antibody is an IgG antibody, preferably an
IgG1 antibody.**

**In some embodiments of the present disclosure, provided is the antibody-
drug conjugate, wherein the antibody is an anti-HER3 antibody.**

**In some embodiments of the present disclosure, provided is the antibody-
drug conjugate, wherein the antibody comprises a heavy chain variable
region and a light chain variable region,
wherein**

**the heavy chain variable region comprises an HCDR1 set forth in SEQ ID
NO: 1, an HCDR2 set forth in SEQ ID NO: 2, and an HCDR3 set forth in
SEQ ID NO: 3;**

**the light chain variable region comprises an LCDR1 set forth in SEQ ID
NO: 4, an LCDR2 set forth in SEQ ID NO: 5, and an LCDR3 set forth in
SEQ ID NO: 6.**

**In some embodiments of the present disclosure, provided is the antibody-
drug conjugate, wherein the heavy chain variable region comprises an
amino acid sequence set forth in SEQ ID NO: 7, and the light chain variable
region comprises an amino acid sequence set forth in SEQ ID NO: 9.**

**In some embodiments of the present disclosure, provided is the antibody-
drug conjugate, wherein a heavy chain constant region of the antibody is
an Ig gamma-1 chain C region or an Ig gamma-4 chain C region, and a
light chain constant region is an Ig kappa chain C region.**

In some embodiments of the present disclosure, provided is the antibody-drug conjugate, wherein the heavy chain constant region of the antibody comprises an amino acid sequence set forth in SEQ ID NO: 11, and the light chain constant region comprises an amino acid sequence set forth in SEQ ID NO: 13.

In some embodiments of the present disclosure, provided is the antibody-drug conjugate, wherein an average number of linker-payloads conjugated to each antibody molecule is 1-8, 2-8, 3-8, 4-8, 5-8, 6-8, or 7-8.

In some embodiments of the present disclosure, provided is the antibody-drug conjugate, wherein the antigen-binding fragment is selected from Fab, Fab', F(ab')₂, Fd, Fv, dAb, a complementarity determining region fragment, a single chain fragment variable, a humanized antibody, a chimeric antibody, and a diabody.

In some embodiments of the present disclosure, provided is the antibody-drug conjugate, wherein, the heavy chain constant region of the antibody is a heavy chain constant region of human IgG1, IgG2, IgG3 or IgG4 or a variant thereof, preferably a heavy chain constant region of human IgG1; the light chain constant region is an Ig kappa chain C region.

In some embodiments of the present disclosure, provided is the antibody-drug conjugate, which has ADCC activity.

In some embodiments of the present disclosure, provided is the antibody-drug conjugate, wherein

“An EC₅₀ for binding of the antibody-drug conjugate to an antigen targeted by the antibody-drug conjugate” is identical to or substantially identical to “an EC₅₀ for binding of an antibody or an antigen-binding fragment thereof comprised in the antibody-drug conjugate to the same antigen”. “Substantially identical” herein may mean, for example:

the difference between “an EC₅₀ for binding of the antibody-drug conjugate to an antigen targeted by the antibody-drug conjugate” and “an

EC₅₀ for binding of an antibody or an antigen-binding fragment thereof comprised in the antibody-drug conjugate to the same antigen” is within $\pm 10\%$, within $\pm 15\%$, within $\pm 20\%$, within $\pm 25\%$, within $\pm 30\%$, within $\pm 35\%$, within $\pm 40\%$, or within $\pm 45\%$.

The antibody-drug conjugate of the present disclosure can be used for treating or preventing a tumor;

preferably, the tumor is an HER3-positive tumor;

preferably, the tumor is selected from one or more of lung cancer, clear cell sarcoma, skin cancer, renal cancer, urothelial cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, gastrointestinal stromal tumor, cervical cancer, head and neck cancer, esophageal cancer, epidermoid carcinoma, peritoneal cancer, adult glioblastoma multiforme, colon cancer, rectal cancer, uterine cancer, salivary gland cancer, vulvar cancer, thyroid cancer, anal cancer, and penile cancer;

preferably, the lung cancer is non-small cell lung cancer (NSCLC);

preferably, the colon cancer is metastatic colon cancer.

An antibody-drug conjugate can be prepared by reacting a compound with an antibody or a reactive derivative thereof and conjugating the compound or the pharmaceutically acceptable salt thereof of the present disclosure to the antibody by forming a thioether bond in a disulfide bond moiety present in the hinge portion of the antibody.

Yet another aspect of the present disclosure relates to a pharmaceutical composition comprising the antibody-drug conjugate according to any one of the aspects of the present disclosure, and one or more pharmaceutically acceptable auxiliary materials.

Yet another aspect of the present disclosure relates to use of the antibody-drug conjugate according to any one of the aspects of the present disclosure in the preparation of a medicament for treating or preventing a

tumor;

preferably, the tumor is an HER3-positive tumor;

preferably, the tumor is selected from one or more of lung cancer, clear cell sarcoma, skin cancer, renal cancer, urothelial cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, gastrointestinal stromal tumor, cervical cancer, head and neck cancer, esophageal cancer, epidermoid carcinoma, peritoneal cancer, adult glioblastoma multiforme, colon cancer, rectal cancer, uterine cancer, salivary gland cancer, vulvar cancer, thyroid cancer, anal cancer, and penile cancer;

preferably, the lung cancer is non-small cell lung cancer (NSCLC);

preferably, the colon cancer is metastatic colon cancer.

Yet another aspect of the present disclosure relates to a method for treating or preventing a tumor, comprising a step of administering to a subject in need an effective amount of the antibody-drug conjugate according to any one of the aspects of the present disclosure;

preferably, the tumor is an HER3-positive tumor;

preferably, the tumor is selected from one or more of lung cancer, clear cell sarcoma, skin cancer, renal cancer, urothelial cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, gastrointestinal stromal tumor, cervical cancer, head and neck cancer, esophageal cancer, epidermoid carcinoma, peritoneal cancer, adult glioblastoma multiforme, colon cancer, rectal cancer, uterine cancer, salivary gland cancer, vulvar cancer, thyroid cancer, anal cancer, and penile cancer;

preferably, the lung cancer is non-small cell lung cancer (NSCLC);

preferably, the colon cancer is metastatic colon cancer.

In the present disclosure, unless otherwise defined, the scientific and

technical terms used herein have the meanings generally understood by those skilled in the art. In addition, the laboratory operations of cell culture, molecular genetics, nucleic acid chemistry, and immunology used herein are the routine procedures widely used in the corresponding fields. Meanwhile, in order to better understand the present disclosure, the definitions and explanations of the related terms are provided below.

As used herein, the term EC₅₀ refers to the concentration for 50% of maximal effect, i.e., the concentration that can cause 50% of the maximal effect.

As used herein, the term “no ADCC activity” refers to the situation where, as detected by existing instruments and equipment or detection methods (such as the method described in Example 9), no ADCC activity is observed, ADCC activity is undetectable, or the activity falls below the detection limit of the instruments, equipment and detection methods.

As used herein, the term “antibody” refers to an immunoglobulin molecule that generally consists of two pairs of polypeptide chains (each pair with one “light” (L) chain and one “heavy” (H) chain). Antibody light chains can be classified into κ and λ light chains. Heavy chains can be classified into μ , δ , γ , α , or ϵ . Isotypes of antibodies are defined as IgM, IgD, IgG, IgA, and IgE. In light chains and heavy chains, the variable region and constant region are linked via a “J” region of about 12 or more amino acids, and the heavy chain further comprises a “D” region of about 3 or more amino acids. Each heavy chain consists of a heavy chain variable region (VH) and a heavy chain constant region (CH). The heavy chain constant region consists of 3 domains (CH1, CH2, and CH3). Each light chain consists of a light chain variable region (VL) and a light chain constant region (CL). The light chain constant region consists of one domain CL. The constant region of the antibody can mediate the binding of immunoglobulins to host tissues or factors, including the binding of

various cells of the immune system (e.g., effector cells) to the first component (C1q) of the classical complement system. The VH and VL regions can be further subdivided into hypervariable regions (called complementarity determining regions (CDRs)), with conservative regions called framework regions (FRs) interspersed therebetween. Each VH and VL consists of 3 CDRs and 4 FRs arranged from amino terminus to carboxyl terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions (VH and VL) of each heavy chain/light chain pair form an antibody-binding site. The assignment of amino acids to the regions or domains is based on Chothia & Lesk *J. Mol. Biol.*, 1987; 196: 901-917; Chothia et al., *Nature*, 1989; 342: 878-883, or the definition of the IMGT numbering system, see the definition in Ehrenmann F, Kaas Q, Lefranc M P., IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MhcSF[J], *Nucleic acids research*, 2009; 38(suppl_1): D301-D307.

The variable regions of the light and heavy chains determine antigen binding; the variable region of each chain contains three hypervariable regions called complementarity determining regions (CDRs), wherein the CDRs of the heavy chain (H) include HCDR1, HCDR2, and HCDR3, and the CDRs of the light chain (L) include LCDR1, LCDR2, and LCDR3. In the present disclosure, the CDRs are defined by the IMGT numbering system. See Ehrenmann F, Kaas Q, Lefranc M P. IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MhcSF [J]. *Nucleic acids research*, 2009; 38(suppl_1):D301-D307.

The term “antibody” is not limited by any specific method for producing the antibody. For example, the antibody includes a recombinant antibody, a monoclonal antibody, and a polyclonal antibody. The antibody may be

antibodies of different isotypes, such as IgG (e.g., subtype IgG1, IgG2, IgG3, or IgG4), IgA1, IgA2, IgD, IgE, or IgM.

As used herein, the terms “mAb” and “monoclonal antibody” refer to an antibody or a fragment of an antibody that is derived from a group of highly homologous antibodies, i.e., from a group of identical antibody molecules, except for natural mutations that may occur spontaneously. The monoclonal antibody is highly specific for a single epitope on an antigen. The polyclonal antibody, relative to the monoclonal antibody, generally comprises at least two or more different antibodies which generally recognize different epitopes on an antigen. Monoclonal antibodies can generally be obtained using hybridoma technology first reported by Kohler et al. (Köhler G, Milstein C., Continuous cultures of fused cells secreting antibody of predefined specificity [J]. *Nature*, 1975; 256(5517):495), but can also be obtained using recombinant DNA technology (see, e.g., U.S. Patent 4,816,567).

As used herein, the term “humanized antibody” refers to an antibody or an antibody fragment obtained when all or a part of the CDR regions of a human immunoglobulin (receptor antibody) are replaced by the CDR regions of a non-human antibody (donor antibody), wherein the donor antibody may be a non-human (e.g., mouse, rat, or rabbit) antibody having expected specificity, affinity, or reactivity. In addition, some amino acid residues in the framework regions (FRs) of the receptor antibody can also be replaced by the amino acid residues of corresponding non-human antibodies or by the amino acid residues of other antibodies to further improve or optimize the performance of the antibody. For more details on humanized antibodies, see, e.g., Jones et al., *Nature*, 1986; 321: 522-525; Reichmann et al., *Nature*, 1988; 332: 323-329; Presta, *Curr. Op. Struct. Biol.*, 1992; 2: 593-596; and Clark, *Immunol. Today*, 2000; 21: 397-402. In some cases, the antigen-binding fragments of the antibodies are diabodies, in

which the VH and VL domains are expressed on a single polypeptide chain. However, the linker used is too short to allow the pairing of the two domains on the same chain. Thus, the domains are forced to pair with the complementary domains on the other chain, and two antigen-binding sites are generated (see, e.g., Holliger P. et al., *Proc. Natl. Acad. Sci. USA*, 1993; 90:6444-6448 and Poljak R.J. et al., *Structure*, 1994; 2:1121-1123).

As used herein, the term “single chain fragment variable (ScFv)” refers to a molecule comprising an antibody heavy chain variable region (VH) and an antibody light chain variable region (VL) linked via a linker. The VL and VH domains are paired to form a monovalent molecule via a linker enabling production as a single polypeptide chain (see, e.g., Bird et al., *Science*, 1988; 242:423-426 and Huston et al., *Proc. Natl. Acad. Sci. USA*, 1988; 85:5879-5883). Such scFv molecules may have the following general structures: NH₂-VL-linker fragment-VH-COOH or NH₂-VH-linker fragment-VL-COOH. A suitable linker in the prior art consists of a repeated GGGGS (SEQ ID NO: 15) amino acid sequence or a variant thereof. For example, a linker having the amino acid sequence (GGGGS)₄ (SEQ ID NO: 16) may be used, but variants thereof may also be used (Holliger et al., *Proc. Natl. Acad. Sci. USA*, 1993; 90: 6444-6448). Other linkers that can be used in the present disclosure are described in Alfthan et al., *Protein Eng.*, 1995; 8: 725-731; Choi et al., *Eur. J. Immunol.*, 2001; 31: 94-106; Hu et al., *Cancer Res.*, 1996; 56: 3055-3061; Kipriyanov et al., *J. Mol. Biol.*, 1999; 293: 41-56; and Roovers et al., *Cancer Immunology, Immunotherapy*, 2001, 50(1): 51-59.

As used herein, the term “antibody moiety” refers to an antibody moiety in an antibody-drug conjugate, which, in certain specific embodiments, is linked to an intermediate linker moiety via a specific functional group, and can specifically bind to an antigen.

As used herein, the term “linker” refers to a moiety that links an antibody

to a cytotoxin, and is classified into cleavable and non-cleavable forms. Cleavable linkers mainly include sensitive chemical bonds, and can facilitate the cleavage between the linker and the drug according to specific chemical species (such as glutathione and acidity or basicity) or different enzyme concentrations in the internal environment of the body, and are mainly linked in the form of hydrazone bonds, disulfide bonds, or polypeptide linkages. Non-cleavable linkers have no built-in chemical bond that can trigger cleavage, and thus antibodies need to be converted into amino acids by proteolysis in cancer cells to release cytotoxic drugs carrying the linkers and amino acid fragments, which are primarily linked in the form of thioethers.

As used herein, the term “payload” refers to a cytotoxin molecule mainly responsible for performing a cell killing function in an antibody-drug conjugate. The payload is linked to an intermediate linker moiety by chemical synthesis, enters tumor cells through internalization, and releases the cytotoxin molecule under the action of lysosomes, thereby exerting an anti-tumor effect. Common targets are DNA in the nucleus and tubulin in the cytoplasm.

As used herein, the term “DAR (drug-to-antibody ratio)” refers to an average number of small-molecule drugs conjugated to each antibody molecule, which is one of the important quality attributes of ADCs. According to the chemical properties and conjugation mode (lysine conjugation, cysteine conjugation, glycosyl conjugation, etc.) of the linker and the small-molecule cytotoxin, the common characterization and analysis methods include ultraviolet-visible spectrophotometry, hydrophobic chromatography, reversed-phase chromatography, and mass spectrometry (MS).

As used herein, the term “isolated” refers to obtaining by artificial means from a natural state. If a certain “isolated” substance or component is

present in nature, it may be the case that a change occurs in its natural environment, or that it is isolated from the natural environment, or both. For example, a certain non-isolated polynucleotide or polypeptide naturally occurs in a certain living animal, and the same polynucleotide or polypeptide with high purity isolated from such a natural state is referred to as an isolated polynucleotide or polypeptide. The term “isolated” does not exclude the existence of artificial or synthetic substances or other impurities that do not affect the activity of the substance.

As used herein, the term “vector” refers to a nucleic acid vehicle into which a polynucleotide can be inserted. When a vector allows the expression of the protein encoded by the inserted polynucleotide, the vector is referred to as an expression vector. The vector can be introduced into a host cell by transformation, transduction, or transfection, such that the genetic substance elements carried by the vector can be expressed in the host cell. Vectors are well known to those skilled in the art, including but not limited to: plasmids; phagemids; cosmids; artificial chromosomes, such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC); phages such as λ phages or M13 phages; and animal viruses. Animal viruses that can be used as vectors include, but are not limited to, retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses, herpes viruses (such as herpes simplex virus), poxviruses, baculoviruses, papillomaviruses, and papovaviruses (such as SV40). A vector may comprise a variety of elements that control expression, including, but not limited to, promoter sequences, transcription initiation sequences, enhancer sequences, selection elements, and reporter genes. In addition, the vector may further comprise a replication initiation site.

As used herein, the term “host cell” refers to cells to which vectors can be introduced, including but not limited to, prokaryotic cells such as *E. coli*

or *bacillus subtilis*, fungal cells such as yeast cells or *aspergillus*, insect cells such as S2 drosophila cells or Sf9, or animal cells such as fibroblasts, CHO cells, GS cells, COS cells, NS0 cells, HeLa cells, BHK cells, HEK293 cells, or human cells.

As used herein, the term “specifically bind” refers to a non-random binding reaction between two molecules, such as a reaction between an antibody and an antigen it targets. In some embodiments, an antibody specifically binding to an antigen (or an antibody specific to an antigen) means that the antibody binds to the antigen with an affinity (KD) of less than about 10^{-5} M, such as less than about 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, or 10^{-10} M, or less.

As used herein, the term “KD” refers to a dissociation equilibrium constant for a specific antibody-antigen interaction and is used to describe the binding affinity between the antibody and the antigen. A smaller dissociation equilibrium constant indicates a stronger antibody-antigen binding and a higher affinity between the antibody and the antigen. Generally, antibodies bind to antigens with a dissociation equilibrium constant (KD) of less than about 10^{-5} M, such as less than about 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M or 10^{-10} M or less. KD can be determined using methods known to those skilled in the art, e.g., using a Fortebio molecular interaction instrument.

As used herein, the terms “monoclonal antibody” and “mAb” have the same meaning and are used interchangeably; the terms “polyclonal antibody” and “pAb” have the same meaning and are used interchangeably. Besides, in the present disclosure, amino acids are generally represented by single-letter and three-letter abbreviations known in the art. For example, alanine can be represented by A or Ala.

As used herein, the term “pharmaceutically acceptable auxiliary material” refers to a carrier and/or excipient that is pharmacologically and/or

physiologically compatible with the subject and the active ingredient, which is well known in the art (see, e.g., *Remington's Pharmaceutical Sciences*. Edited by Gennaro AR, 19th ed. Pennsylvania: Mack Publishing Company, 1995), and includes, but is not limited to, pH regulators, surfactants, adjuvants, and ionic strength enhancers. For example, the pH regulators include, but are not limited to, phosphate buffer; the surfactants include, but are not limited to, cationic, anionic, or non-ionic surfactants, such as Tween-80; the ionic strength enhancers include, but are not limited to, sodium chloride.

As used herein, the term “effective amount” refers to an amount sufficient to obtain or at least partially obtain a desired effect. For example, a prophylactically effective amount against a disease (e.g., a tumor) refers to an amount sufficient to prevent, stop, or delay the development of the disease (e.g., a tumor); a therapeutically effective amount refers to an amount sufficient to cure or at least partially stop the disease and complications thereof in patients suffering from the disease. It is undoubtedly within the ability of those skilled in the art to determine such an effective amount. For example, the amount effective for therapeutic purpose will depend on the severity of the disease to be treated, the overall state of the patient's own immune system, the general condition of the patient such as age, body weight and gender, the route of administration, and other treatments given concurrently, etc.

Beneficial Effects of the Present Disclosure

The present disclosure achieves one or more of the following technical effects:

- (1) The antibody-drug conjugates, such as anti-HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure have good biological activity.
- (2) The antibody-drug conjugates, such as anti-HER3 antibody-drug

conjugate KA0013, prepared using the compound of the present disclosure can effectively release the payload moiety.

(3) The anti-HER3 antibody-drug conjugate KA0013 of the present disclosure can effectively bind to HER3.

(4) The antibody-drug conjugates, such as anti-HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure have internalization activity.

(5) The antibody-drug conjugates, such as anti-HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure have good stability in plasma.

(6) The antibody-drug conjugates, such as anti-HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure can specifically kill tumor cells.

(7) The antibody-drug conjugates, such as HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure have ADCC activity.

(8) The antibody-drug conjugates, such as anti-HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure can effectively inhibit tumor growth *in vivo*.

(9) The antibody-drug conjugates, such as HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure have good safety.

(10) The antibody-drug conjugates, such as HER3 antibody-drug conjugate KA0013, prepared using the compound of the present disclosure have a bystander killing effect.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Assay results of binding activity of anti-HER3 antibody-drug conjugate KA0013 for surface HER3 of SK-BR-3 cells expressing HER3

on the cell surface.

FIG. 2: Assay results of internalization activity of anti-HER3 antibody-drug conjugate KA0013 in MDA-MB-453 cells expressing HER3 on the cell surface.

FIG. 3: Assay results of stability for non-specific killing caused by the cleavage of the linker-payload moiety of anti-HER3 antibody-drug conjugate KA0013.

FIG. 4: Assay results of plasma stability of anti-HER3 antibody-drug conjugates KA0013 and KA0011.

FIG. 5: *In vitro* assay of the cleavage efficiency of the linker-payload moieties of KA0013 and KA0011 mediated by cathepsin B.

FIG. 6: Assay results of binding activity of anti-HER3 antibody-drug conjugates KA0013 and KA0011 for antigen human HER3-his by indirect ELISA.

FIG. 7: Assay results of kinetic characteristic parameters of anti-HER3 antibody-drug conjugate KA0013.

FIG. 8: Assay results of kinetic characteristic parameters of anti-HER3 antibody-drug conjugate KA0011.

FIG. 9: Assay results of specific cell killing activity of anti-HER3 antibody-drug conjugates.

FIG. 10: Assay results of ADCC activity of anti-HER3 antibody-drug conjugates.

FIG. 11: *In vivo* efficacy results of anti-HER3 antibody-drug conjugates in CB-17 SCID mouse model of HCC827 human lung cancer cell xenograft tumor.

FIG. 12: Effect of anti-HER3 antibody-drug conjugates on body weight of CB-17 SCID mouse model of HCC827 human lung cancer cell xenograft tumor.

DETAILED DESCRIPTION

Embodiments of the present disclosure will be described in detail below with reference to examples, but those skilled in the art will understand that the following examples are only for illustrating the present disclosure and should not be construed as limitations to the scope of the present disclosure. The experimental procedures without specified conditions in the examples are conducted under conventional conditions or conditions recommended by the manufacturer. The used reagents or instruments without specified manufacturers are all commercially available conventional products.

In the following experimental examples of the present disclosure, the positive control ADC drug KA0011 used is the Her3 antibody-conjugate drug U3-1402 developed by Daiichi Sankyo Pharmaceutical Co., Ltd., Japan. For the sequence information and preparation method therefor, refer to Chinese Patent No. CN 106163559 B.

In the following experimental examples of the present disclosure, the isotype control antibodies used, i.e., the IgG (i.e., anti-HEL) components in IgG-Dxd, IgG-KA0012, and IgG-KA0010, and hIgG1 and anti-HEL, are all antibodies targeting human anti-hen egg lysozyme (HEL). The variable region sequences of these antibodies are derived from the study reported by Acierno et al., entitled “Affinity maturation increases the stability and plasticity of the Fv domain of anti-protein antibodies” (Acierno et al., *J Mol Biol.*, 2007; 374(1): 130-46). For the constant region fragments, an Ig gamma-1 chain C region (ACCESSION: P01857) is used as the heavy chain constant region and an Ig kappa chain C region (ACCESSION: P01834) is used as the light chain constant region. IgG-DXd or IgG-KA0012, hIgG1, and anti-HEL are all prepared in the laboratory of Akeso Biopharma Inc.

Preparation Example 1: Preparation of Anti-HER3 Antibody Patritumab

Amino acid sequence of the heavy chain variable region of patritumab:

**QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKG
LEWIGEINHSGSTNYPNPSLKSRVTISVETSKNQFSLKLSSVTAADTA
VYYCARDKWTWYFDLWGRGTLVTVSS (SEQ ID NO: 7)**

**Nucleotide sequence encoding the heavy chain variable region of
patritumab:**

**CAGGTGCAGCTGCAGCAGTGGGGCGCCGGACTGCTGAAGCCA
TCCGAGACCCTGTCTCTGACATGTGCCGTGTACGGCGGCTCCT
TTTCTGGCTACTATTGGAGCTGGATCAGGCAGCCCCCTGGCAA
GGGACTGGAGTGGATCGGCGAGATCAACCACAGCGGCTCCAC
CAACTATAATCCCTCTCTGAAGAGCCGGGTGACCATCAGCGTG
GAGACATCCAAGAATCAGTTCTCCCTGAAGCTGAGCTCCGTGA
CCGCAGCAGACACAGCCGTGTACTATTGCGCCCCGGGACAAGT
GGACCTGGTACTTTGATCTGTGGGGCAGAGGCACCCTGGTGA
CAGTGTCTAGC (SEQ ID NO: 8)**

Amino acid sequence of the light chain variable region of patritumab:

**DIEMTQSPDSLAVSLGERATINCRSSQSVLYSSSNRNYLAWYQQNP
GQPPKLLIYWASTRESGVPPDRFSGSGSGTDFTLTISLQAEDVAVY
YCQQYYSTPRTFGQGTKVEIK (SEQ ID NO: 9)**

**Nucleotide sequence encoding the light chain variable region of
patritumab:**

**GACATCGAGATGACCCAGTCCCCTGATTCTCTGGCCGTGAGCC
TGGGAGAGAGGGCAACAATCAACTGTAGAAGCTCCCAGTCCG
TGCTGTACTCTAGCTCCAACCGGAATTACCTGGCCTGGTATCA
GCAGAATCCAGGCCAGCCCCCTAAGCTGCTGATCTATTGGGCC
AGCACCAGGGAGTCCGGAGTGCCAGACCGCTTCTCCGGCTCT
GGCAGCGGCACAGACTTCACCCTGACAATCTCTAGCCTGCAGG
CCGAGGACGTGGCCGTGTACTATTGCCAGCAGTACTATAGCAC**

**CCCCAGGACATTTGGCCAGGGCACCAAGGTGGAGATCAAG
(SEQ ID NO: 10)**

The heavy chain constant region of patritumab was an Ig gamma-1 chain C region, and the light chain constant region was an Ig kappa chain C region.

**Amino acid sequence of the heavy chain constant region of patritumab:
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT
KVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPGK (SEQ ID NO: 11)**

Nucleotide sequence encoding the heavy chain constant region of patritumab:

**GCCTCTACAAAGGGGCCCAGCGTGTTTCCTCTGGCCCCTAGCA
GCAAGTCTACAAGCGGAGGAACAGCAGCTCTGGGCTGCCTGG
TGAAGGACTACTTCCCAGAGCCCGTGACCGTGTCTTGGAATC
AGGAGCCCTGACCAGCGGAGTGCACACATTTCCAGCCGTGCT
GCAGAGCAGCGGACTGTATAGCCTGAGCAGCGTGGTGACCGT
GCCTTCTTCTAGCCTGGGCACCCAGACCTACATCTGCAACGTG
AACCACAAGCCCAGCAACACCAAGGTGGACAAGAGGGTGGAG
CCCAAGTCTTGCGACAAGACCCACACTTGTCCTCCTTGTCCAG
CCCCAGAGCTGCTGGGAGGACCAAGCGTGTTTCCTGTTCCCTCC
CAAGCCCAAGGACACCCTGATGATCAGCAGGACCCCAGAAGT
GACTTGCGTGGTGGTGGACGTGTCTCACGAGGACCCCGAAGT
GAAGTTCAATTGGTACGTGGACGGCGTGGAGGTGCACAACGC
TAAGACCAAGCCCAGGGAGGAGCAGTACAACAGCACCTACCG**

**GGTGGTGTCCGTGCTGACAGTGCTGCACCAGGATTGGCTGAA
CGGCAAGGAGTACAAGTGCAAGGTGTCCAATAAGGCCCTGCC
AGCCCCCTATCGAGAAGACCATCAGCAAGGCCAAGGGCCAGCC
TAGAGAGCCTCAGGTGTACACCCTGCCTCCTTCTCGGGAGGAG
ATGACCAAGAACCAGGTGTCCCTGACTTGCCTCGTGAAGGGCT
TCTACCCCAGCGATATTGCCGTCGAGTGGGAGTCTAACGGCCA
GCCCCGAGAACAACCTACAAGACCACACCTCCAGTGCTGGATAGC
GACGGCAGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAAA
GCCGCTGGCAGCAGGGCAACGTGTTTTCTTGCAGCGTGATGCA
CGAAGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTG
TCTCCAGGCAAG (SEQ ID NO: 12)**

Amino acid sequence of the light chain constant region of patritumab:

**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKSTYSLSSTLTLSKADYEKHKVYACEVT
HQGLSSPVTKSFNRGEC (SEQ ID NO: 13)**

**Nucleotide sequence encoding the light chain constant region of
patritumab:**

**CGTACGGTGGCAGCCCCATCTGTCTTCATTTTTCCCCCTAGTG
ACGAGCAGCTGAAATCCGGAACAGCCTCTGTGGTCTGTCTGCT
GAACAATTTCTACCCTCGCGAAGCCAAGGTGCAGTGGAAAGTC
GATAACGCTCTGCAGAGTGGCAATTCACAGGAGAGCGTGACT
GAACAGGACTCCAAGGATTCTACCTATAGTCTGAGCTCCACTC
TGACCCTGTCCAAAGCAGATTACGAAAAGCACAAAGTGTATGC
CTGTGAGGTCACCCACCAGGGGCTGAGTTCTCCAGTCACCAAA
TCCTTCAACAGAGGCGAATGT (SEQ ID NO: 14)**

**The heavy chain cDNA and light chain cDNA of patritumab were
separately cloned into a pcDNA3.1 vector to obtain a recombinant
expression plasmid of the antibody patritumab. The recombinant plasmid
was transfected into 293F cells. The 293F cell culture was purified and then**

detected.

The anti-HER3 monoclonal antibody patritumab was thus obtained.

According to the IMGT numbering system, six CDRs of the anti-HER3 monoclonal antibody patritumab are as follows:

HCDR1: GGSFSGYY (SEQ ID NO: 1)

HCDR2: INHSGST (SEQ ID NO: 2)

HCDR3: ARDKWTWYFDL (SEQ ID NO: 3)

LCDR1: QSVLYSSSNRNY (SEQ ID NO: 4)

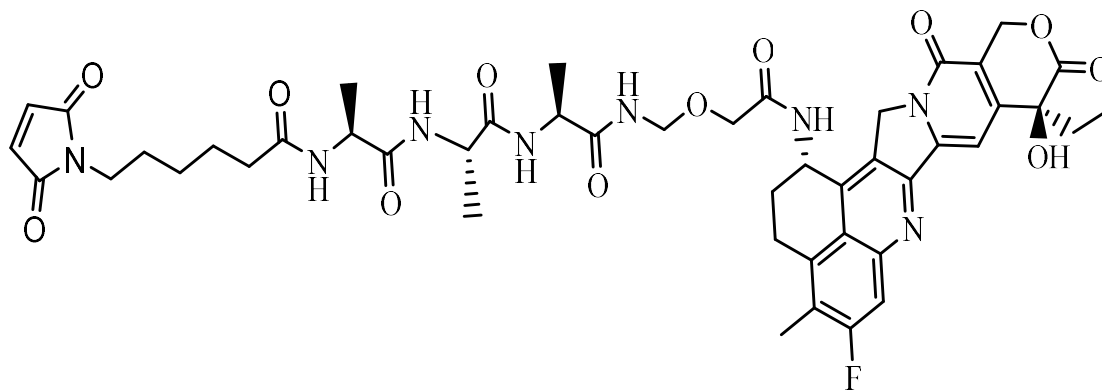
LCDR2: WAS (SEQ ID NO: 5)

LCDR3: QQYYSTPRT (SEQ ID NO: 6)

Preparation Example 2: Preparation of Mc-AAA-Dxd (KA0012)

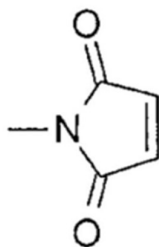
Mc-AAA-Dxd is (maleimide-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-AAA-NH-CH₂-O-CH₂-C(=O)-(NH-DX). The CAS number of Dxd is: 1599440-33-1.

Mc-AAA-Dxd is named KA0012 in the present disclosure, the structure of which is represented by Formula II below:



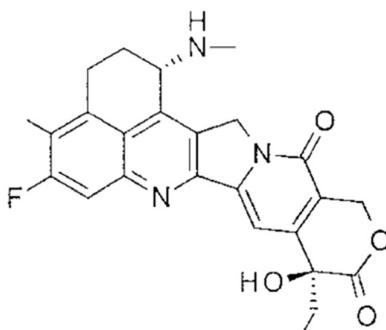
Formula II

wherein Mc is maleimide-N-yl, the structure of which is represented by Formula III below:



Formula III

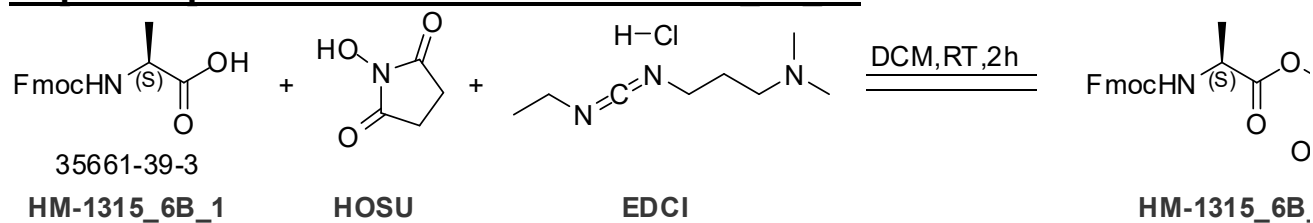
wherein the structural formula of **-(NH-DX)** is represented by **Formula IV** below:



Formula IV

The synthetic steps of KA0012 are shown as step 1 to step 10 below.

Step 1: Preparation of intermediate HM-1315 6B 2

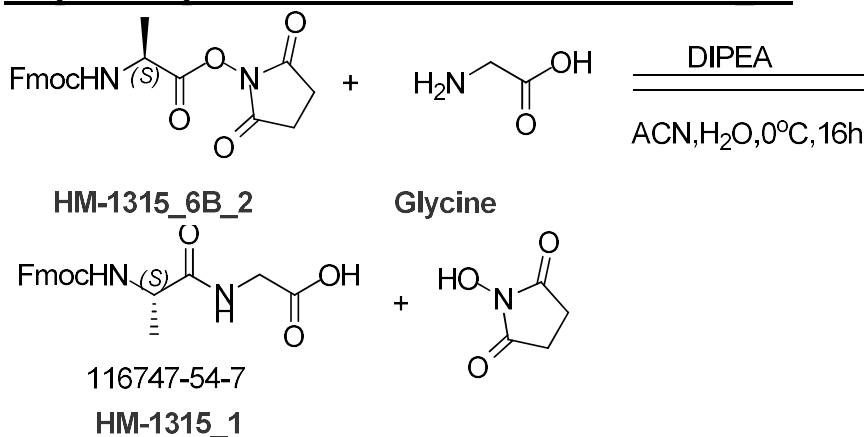


The starting material HM-1315_6B_1 (450.0 g, 1.44 mol, 1 eq) was suspended in DCM (dichloromethane) (4.5 L, 10 V), and HOSU (182.9 g, 1.59 mol, 1.1 eq) and EDCI (304.8 g, 1.59 mol, 1.1 eq) were sequentially

added with the temperature controlled at 0-5 °C. After the addition, the reaction mixture was dissolved until a clear solution was obtained. The solution was reacted at 10-20 °C for 3 h. Semi-saturated brine (1.0 L) was added to the reaction mixture, and the resulting mixture was stirred and left to stand for liquid separation. Then the organic phase was washed once with semi-saturated brine (1.0 L), washed twice with saturated brine (1.0 L × 2), dried over anhydrous sodium sulfate, and concentrated to give a white solid (579.0 g, yield: 98%).

¹H NMR (400 MHz, DMSO) δ 8.13 (d, J = 7.4 Hz, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.71 (t, J = 6.7 Hz, 2H), 7.42 (t, J = 7.3 Hz, 2H), 7.33 (t, J = 7.4 Hz, 2H), 4.52 (t, J=7.4 Hz, 1H), 4.41-4.17 (m, 3H), 2.79 (d, J=15.5 Hz, 4H), 2.59 (s, 1H), 1.46 (d, J =7.4 Hz, 3H). LCMS [M+23]: 431.1.

Step 2: Preparation of intermediate HM-1315_1

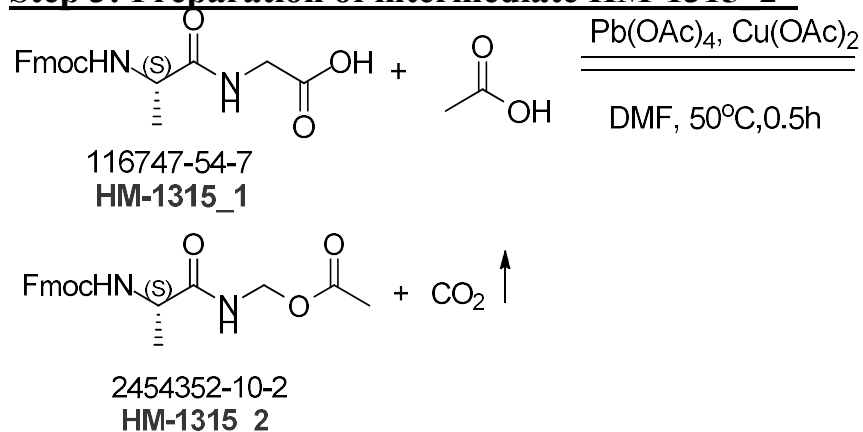


The starting material HM-1315_6B_2 (579.0 g, 1.42 mol, 1.0 eq) was dissolved in acetonitrile (2895 mL, 5 v), and glycine (117.2 g, 1.56 mol, 1.1 eq) and water (2895 mL, 5 v) were added. The mixture was cooled to -5-0 °C, and DIPEA (N,N-diisopropylethylamine) (275.3 g, 2.13 mol, 1.5 eq) was slowly added. After the addition, the mixture was reacted at 20-25 °C for 3 h. The reaction mixture was diluted with ethyl acetate (1.0 L) and adjusted to pH 3-4 with diluted hydrochloric acid (0.5 N). Ethyl acetate

(500 mL) was added for extraction and liquid separation. The aqueous phase was extracted twice with ethyl acetate (1.0 L $\times$ 2). The organic phases were combined, washed once with saturated brine (1.5 L), dried over anhydrous sodium sulfate, filtered, and concentrated. The crude product was slurried with methyl tert-butyl ether/ethyl acetate (2895 mL/289.5 mL, 5 v/0.5 v) for 2 h and filtered, and the filter cake was dried with an oil pump to give a white powdery solid (447.2 g, yield: 85%).

^1H NMR (400 MHz, DMSO) δ 8.12 (t, $J=5.5$ Hz, 1H), 7.89 (d, $J = 7.5$ Hz, 2H), 7.73 (t, $J = 7.1$ Hz, 2H), 7.54 (d, $J=7.8$ Hz, 1H), 7.37 (dt, $J = 34.6, 7.1$ Hz, 4H), 4.24 (dd, $J=15.6, 5.9$ Hz, 3H), 4.13- 4.05 (m, 1H), 3.75 (dd, $J=14.9, 5.7$ Hz, 3H), 1.23 (d, $J=7.2$ Hz, 3H). LCMS $[\text{M}+1]$: 369.1, $[\text{M}+23]$: 392.1.

Step 3: Preparation of intermediate HM-1315_2

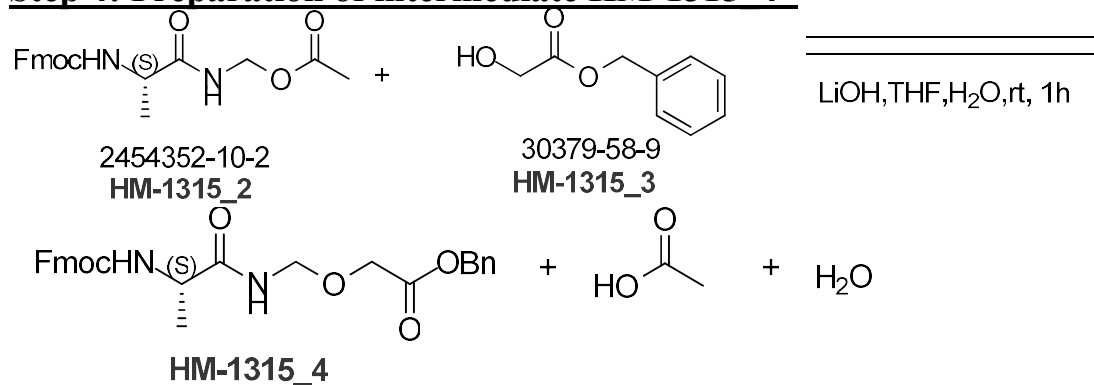


The starting material HM-1315_1 (474.0 g, 1.29 mol, 1.0 eq) was dissolved in ultra-dry DMF (2370 mL, 5 v). Under nitrogen atmosphere, lead tetraacetate (1143.9 g, 2.58 mol, 2.0 eq), anhydrous copper acetate (234.3 g, 1.29 mol, 1.0 eq), and acetic acid (170.5 g, 2.84 mol, 2.2 eq) were added. After the addition, the mixture was heated to about 50 °C. The reaction mixture was reacted under an oil bath at 50-55 °C for 0.5 h. The reaction mixture was cooled, and ethyl acetate (2370 mL, 5 v) was added, followed by the addition of ice water (2370 mL, 5 v). A black viscous solid was precipitated. The reaction mixture was filtered through a Buchner funnel

to remove black insoluble substances, and the filtrate was subjected to liquid separation using a separatory funnel. The aqueous layer was extracted twice with ethyl acetate (2.0 L $\times$ 2). The organic phases were combined, washed twice with water (1.0 L $\times$ 2), washed three times with saturated brine (1.0 L $\times$ 3), dried over anhydrous sodium sulfate, filtered, and concentrated. The resulting crude product was slurried with methyl tert-butyl ether (2370 mL) at room temperature for 2 h and filtered, and the filter cake was dried with an oil pump to give a white solid product (367.7 g, yield: 74%).

^1H NMR (400 MHz, DMSO) δ 8.90 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.73 (t, J = 6.6 Hz, 2H), 7.59 (d, J = 7.6 Hz, 1H), 7.42 (t, J = 7.4 Hz, 2H), 7.33 (t, J = 7.4 Hz, 2H), 5.10 (d, J = 6.7 Hz, 2H), 4.25 (dd, J = 20.3, 6.5 Hz, 3H), 4.12-4.01 (m, 1H), 1.99 (s, 3H), 1.22 (d, J = 7.2 Hz, 3H). LCMS $[\text{M}+23]$: 405.0.

Step 4: Preparation of intermediate HM-1315_4

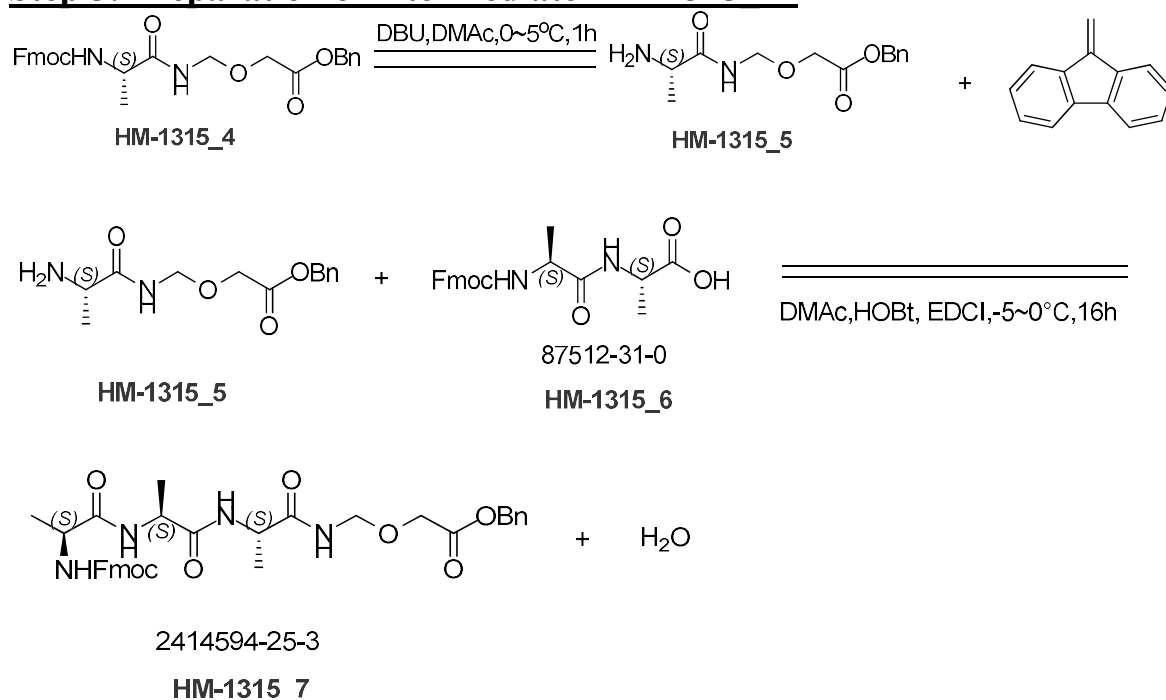


The starting material HM-1315_2 (326.0 g, 0.85 mol, 1.0 eq) was dissolved in THF (3260.0 mL, 10 v), and HM-1315_3 (211.9 g, 1.27 mol, 1.5 eq) was added. After the addition, the reaction was cooled to 0-10 °C, and an aqueous solution (163.0 mL, 0.5 v) of lithium hydroxide (24.4 g, 1.02 mol, 1.2 eq) was slowly added. After the addition, the mixture was naturally warmed to room temperature (15-25 °C) and reacted for 1 h. Ethyl acetate (1.5 L) and water (1.5 L) were added to the reaction mixture, followed by

liquid separation. The aqueous layer was extracted once with ethyl acetate (1.0 L). The organic phases were combined, washed twice with saturated brine (1.0 L $\times$ 2), dried over anhydrous sodium sulfate, filtered, and concentrated. The resulting crude product was purified by column chromatography (elution: petroleum ether/ethyl acetate = 1/1). The product was collected and concentrated, and the residue was dried with an oil pump to give a white solid product (311.8 g, yield: 74%).

^1H NMR (400 MHz, DMSO) δ 8.74 (s, 1H), 7.89 (d, $J=7.5$ Hz, 2H), 7.72 (t, $J=7.9$ Hz, 2H), 7.59 (d, $J=7.3$ Hz, 1H), 7.47-7.25 (m, 9H), 5.13 (s, 2H), 4.64 (d, $J=6.7$ Hz, 2H), 4.31-4.18 (m, 3H), 4.15 (s, 2H), 4.07-3.95 (m, 1H), 1.23 (d, $J=7.2$ Hz, 3H). LCMS $[\text{M}+23]$: 511.1.

Step 5: Preparation of intermediate HM-1315 7

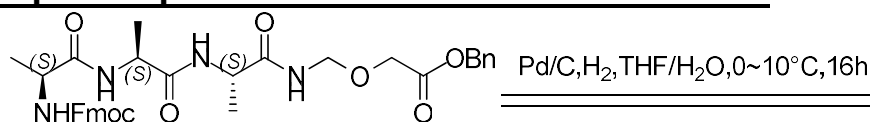


The starting material HM-1315_4 (60.00 g, 122.82 mmol, 1.0 eq) was dissolved in DMAc (N,N-dimethylacetamide) (600.0 mL, 10 v). The solution was cooled to 0-5 $^\circ\text{C}$, and DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) (18.70 g, 122.82 mmol, 1.0 eq) was slowly added. After the addition,

the mixture was reacted at 0-5 °C for 1 h. The reaction mixture was cooled to -5-0 °C, and PPTS (pyridinium 4-methylbenzenesulfonate) (30.86 g, 122.82 mmol, 1.0 eq), EDCI (23.54 g, 122.82 mmol, 1.0 eq), HOBT (1-hydroxybenzotriazole) (16.59 g, 122.82 mmol, 1.0 eq), and HM-1315_6 (42.27 g, 110.54 mmol, 0.9 eq) were sequentially added to the reaction mixture. After the addition, the mixture was reacted at -5-0 °C for 16 h. n-Hexane (1200 mL, 20 v) was added to the reaction mixture, and the resulting mixture was left to stand for liquid separation. The DMAc layer was washed four times with n-hexane (1.2 L $\times$ 4) and then poured into water (1800 mL, 30 v), and a large amount of white solid precipitated. The mixture was filtered through a Buchner funnel, and the filter cake was dried to give a crude product as a white solid. Acetonitrile (300.0 mL, 5 v) was added to a reaction flask, and the white crude product obtained in the previous step was added. The mixture was slurried for 1 h and filtered, and the filter cake was dried with an oil pump to give a white solid product (56.2 g, yield: 80%).

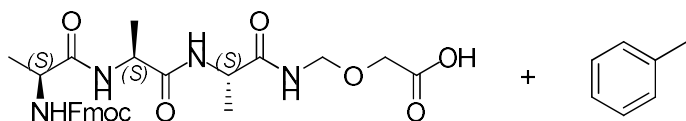
^1H NMR (400 MHz, DMSO) δ 8.68 (t, J = 6.7 Hz, 1H), 7.99 (dd, J = 15.4, 7.2 Hz, 2H), 7.89 (d, J =7.5 Hz, 2H), 7.72 (t, J =7.0 Hz, 2H), 7.54 (d, J =7.4 Hz, 1H), 7.46-7.28 (m, 9H), 5.14 (s, 2H), 4.63 (d, J = 7.7 Hz, 2H), 4.32-4.16 (m, 5H), 4.13 (s, 2H), 4.09-4.01 (m, 1H), 1.21 (dd, J = 7.0, 2.6 Hz, 9H). LCMS $[\text{M}+23]$: 654.3.

Step 6: Preparation of intermediate HM-1315 8

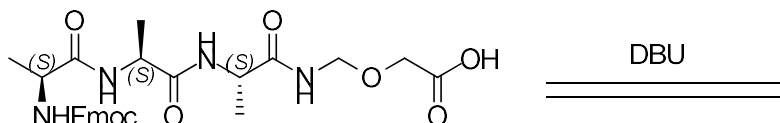


2414594-25-3

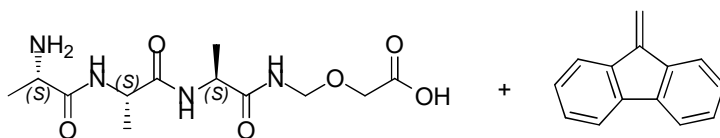
HM-1315_7



HM-1315_7_2



HM-1315_7_2



2414594-27-5

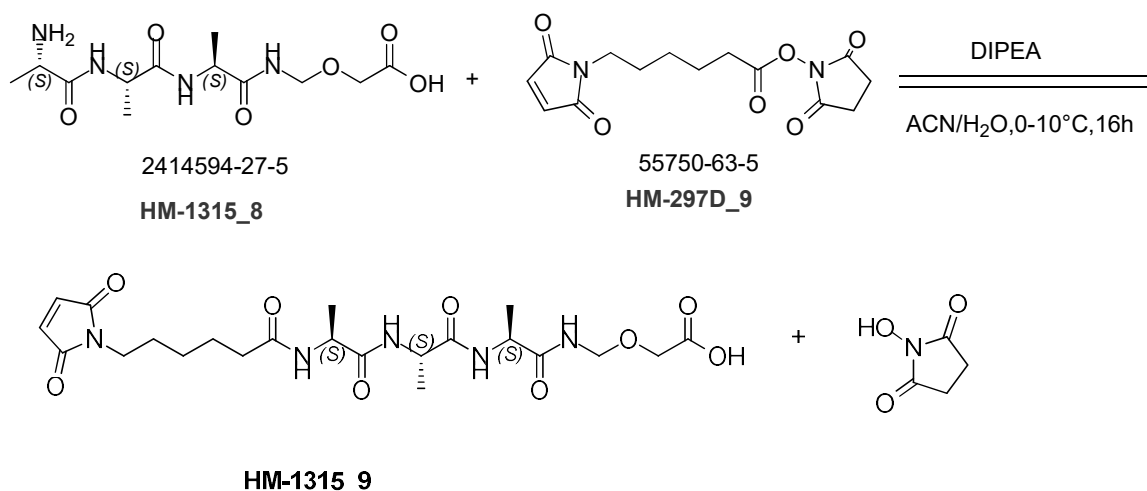
HM-1315_8

The starting material HM-1315_7 (2.59 g, 4.10 mmol, 1.0 eq) was suspended in THF (tetrahydrofuran) (51.8 mL, 20 v) and water (5.2 mL, 2 v). The suspension was cooled to 0-10 °C, and 10% palladium on carbon (0.39 g, 0.15 w/w) was added. The mixture was purged three times with hydrogen and reacted at 0-10 °C for 16 h. The reaction mixture was filtered to remove palladium on carbon, and the filter cake was rinsed with acetonitrile/water (10 mL/10 mL). The filtrate was extracted with 2-methyltetrahydrofuran (50 mL). The organic phase was washed once with water (50 mL) and saturated brine (50 mL), dried over anhydrous sodium sulfate, filtered, and concentrated to give a crude product as a white solid (2.0 g). The crude product (2.0 g, 3.70 mmol, 1.0 eq) was suspended in dichloromethane (20.0 mL, 10 v), and DBU (0.56 g, 3.70 mmol, 1.0 eq) was slowly added dropwise with the temperature controlled at 20-25 °C. After the addition, the reaction mixture was partially dissolved. The mixture was reacted at 20-25 °C for 16 h, during which a white solid was precipitated.

Methyl tert-butyl ether (40.0 mL, 20 v) was added to the reaction mixture, and a greater amount of white solid was precipitated. The mixture was filtered, and the filter cake was transferred to a single-necked flask. Acetonitrile (20.0 mL) was added, and the mixture was slurried for 2 h and filtered. The filter cake was dried with an oil pump to give a white solid product (0.5 g, yield: 38%).

¹H NMR (400 MHz, D₂O) δ 4.73 (q, J=10.8 Hz, 1H), 4.43- 4.21 (m, 1H), 4.04-3.82 (m, 1H), 1.47 (d, J=7.0 Hz, 2H), 1.42 (d, J=7.2 Hz, 3H). LCMS [M+1]: 319.1.

Step 7: Preparation of intermediate HM-1315_9

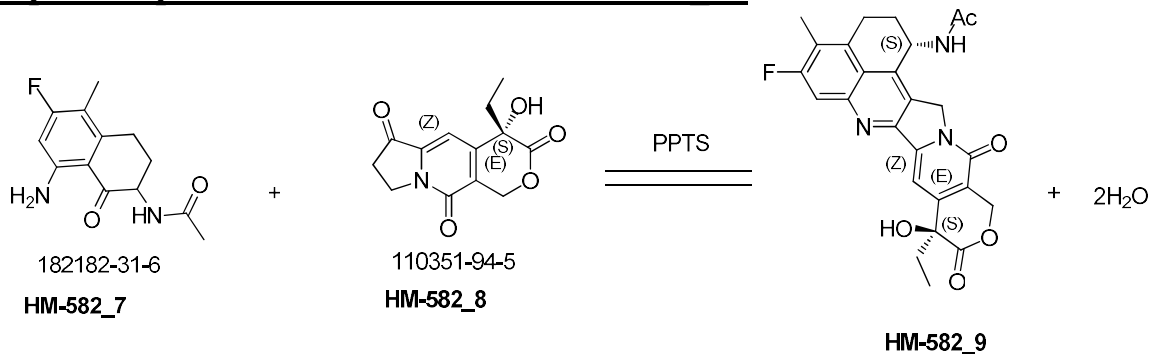


The starting material HM-1315_8 (300 mg, 0.94 mmol, 1.0 eq) was dissolved in acetonitrile (3.0 mL, 10 v) and water (4.5 mL, 15 v), and HM-297D_9 (290 mg, 0.94 mmol, 1.0 eq) was added. The mixture was cooled to 0-10 °C, and DIPEA (121 mg, 0.94 mmol, 1.0 eq) was added. The mixture was reacted at 0-10 °C for 16 h and then separated by preparative chromatography to give a product (290 mg, yield: 60%) as a white solid.

¹H NMR (400 MHz, DMSO) δ 8.61 (t, J=6.7 Hz, 1H), 7.97 (dd, J = 7.1, 3.4 Hz, 2H), 7.91 (d, J=7.3 Hz, 1H), 7.00 (s, 2H), 4.66-4.52 (m, 2H), 4.29-4.12 (m, 3H), 3.95 (s, 2H), 3.44-3.35 (m, 5H), 2.08 (t, J=7.4 Hz, 2H), 1.54-1.40 (m,

4H), 1.28-1.10 (m, 11H). LCMS [M+23]: 534.3.

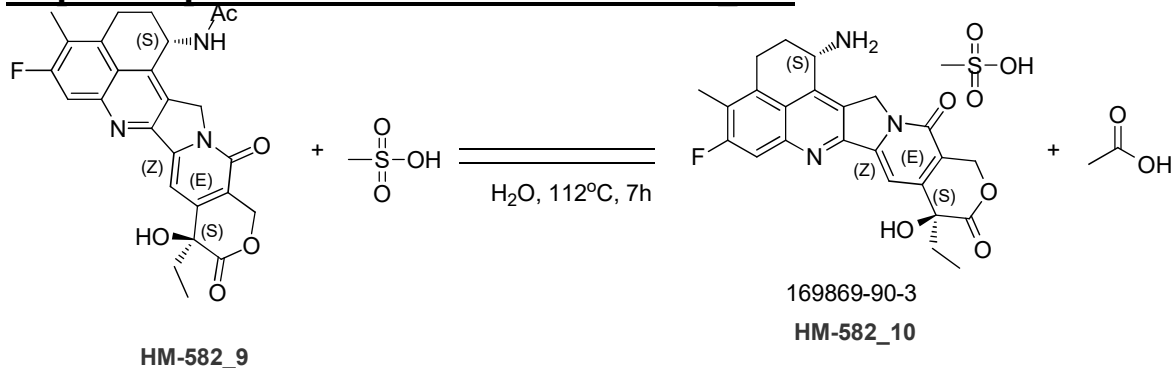
Step 8: Preparation of intermediate HM-582_9



Compound HM-582_7 (10 g, 39.98 mmol, 1.0 eq), compound HM-582_8 (10 g, 38.01 mmol, 0.95 eq), and pyridinium p-toluenesulfonate (6 g, 23.88 mmol, 0.60 eq) were added to a three-necked flask, and toluene (500 mL) was added. The mixture was reacted at 130-140°C with water separation for 48 h. The reaction mixture was cooled, filtered, and washed with methyl tert-butyl ether (100 mL). The solid was collected and dried to give the product (18.4 g, 96.5%).

¹H NMR (DMSO) δ: 8.43-8.55 (m, 1H), 7.80 (d, J = 10.9 Hz, 1H), 7.31 (d, J = 3.8 Hz, 1H), 6.55 (brs, 1H), 5.52-5.57 (m, 1H), 5.42 (s, 2H), 5.16-5.20 (m, 2H), 3.14-3.18 (m, 2H), 2.39 (s, 3H), 2.11-2.16 (m, 2H), 1.80-1.92 (m, 5H), 0.86-0.89 (m, 3H). LCMS [M+1] 478.2.

Step 9: Preparation of intermediate HM-582_10

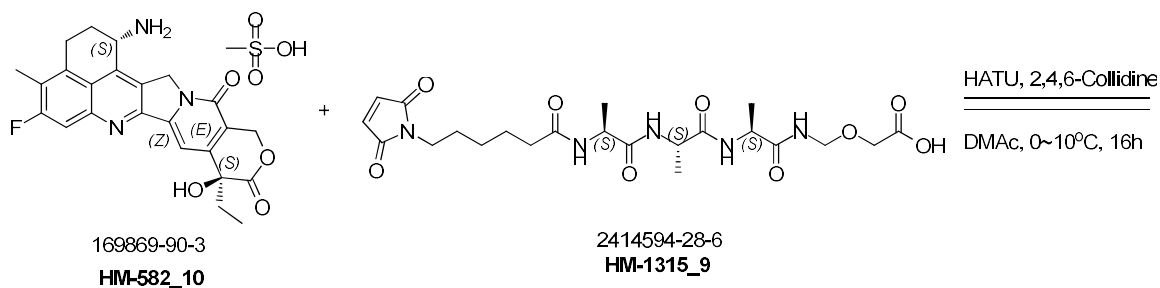


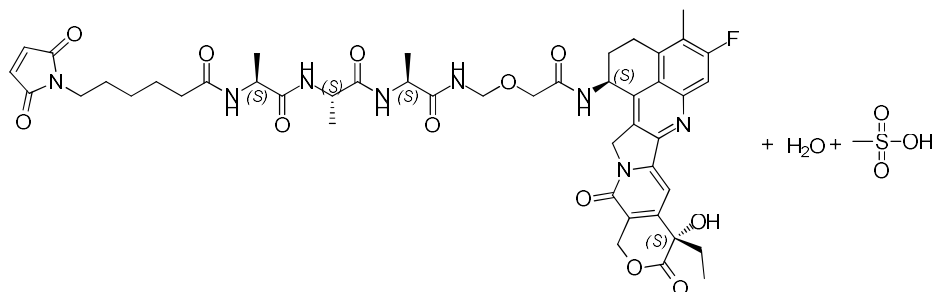
Compound HM-582_9 (30 g) was suspended in water (600 mL), and

methanesulfonic acid (300 mL) was slowly added. The solid was dissolved with an exothermic effect. After purging with nitrogen, the mixture was heated to 112 °C and reacted for 7 h. The reaction mixture was cooled to room temperature and filtered, and the filter cake was washed with water (100 mL). The filtrate was diluted with ethanol (4 L), and a solid was precipitated. The mixture was stirred at room temperature for 20 min, filtered, and dried under reduced pressure. The crude product was suspended in ethanol/water (4:1, 1 L), heated at reflux for 2 h, cooled to room temperature, and filtered. The solid was washed with a small amount of ethanol, dried under reduced pressure, and lyophilized to obtain exatecan mesylate (16.7 g, 50%).

¹H NMR (DMSO) δ 8.41-8.50 (m, 3H), 7.86 (d, J = 10.8 Hz, 1H), 7.33 (s, 1H), 5.66-5.74 (m, 1H), 5.37-5.44 (m, 2H), 5.10 (s, 1H), 3.27-3.32 (m, 1H), 3.08-3.17 (m, 1H), 2.41 (s, 3H), 2.31 (s, 3H), 2.17-2.24 (m, 1H), 1.80-1.95 (m, 2H), 0.88 (t, J = 7.3 Hz, 3H). LCMS [M+1] 436.1.

Step 10: Preparation of HM-1315 10 (KA0012)





HM-1315_10

The starting material HM-1315_9 (150 mg, 0.29 mmol, 1.0 eq) was dissolved in DMAc (3.0 mL, 20 v), and HM-582_10 (156 mg, 0.29 mmol, 1.0 eq) was added. The mixture was cooled to 0-10 °C, and HATU (2-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) (121 mg, 0.32 mmol, 1.0 eq) was added, followed by dropwise addition of 2,4,6-trimethylpyridine (70 mg, 0.58 mmol, 2.0 eq). The mixture was reacted at 0-10 °C for 16 h. After the starting material was completely converted as shown by LCMS, the mixture was separated by preparative chromatography to give a product (135 mg, yield: 50%) as a pale yellow solid product.

¹H NMR (400 MHz, DMSO) δ 8.64 (t, J = 6.6 Hz, 1H), 8.47 (d, J = 8.8 Hz, 1H), 7.95 (dd, J = 9.3, 7.3 Hz, 2H), 7.87 (d, J = 7.2 Hz, 1H), 7.77 (d, J = 11.0 Hz, 1H), 7.30 (s, 1H), 6.99 (s, 2H), 6.51 (s, 1H), 5.60 (d, J = 8.0 Hz, 1H), 5.42 (s, 2H), 5.18 (s, 2H), 4.61 (dt, J = 10.1, 3.3 Hz, 2H), 4.17 (dt, J = 14.1, 7.1 Hz, 3H), 3.98 (s, 2H), 3.38 (s, 1H), 3.16 (dd, J = 14.1, 6.4 Hz, 2H), 2.38 (s, 3H), 2.18 (d, J = 5.4 Hz, 2H), 2.07 (t, J = 7.3 Hz, 2H), 1.85 (dd, J = 15.0, 7.4 Hz, 2H), 1.51 – 1.39 (m, 4H), 1.17 (dt, J = 14.5, 7.4 Hz, 11H), 0.86 (t, J = 7.3 Hz, 3H). LCMS [M+1]:929.3, [M+23]:952.3.

Preparation Example 3: Preparation of Anti-HER3 Antibody-Drug Conjugate KA0013

The structures of KA0013 and KA0011 are shown in Table 1 below.

Table 1

Name	Structure
KA0013	Patritumab -Mc-AAA-Dxd
KA0011	Patritumab -Mc-GGFG-Dxd

An antibody-drug conjugate is obtained by forming a thioether bond in a disulfide bond site present in a hinge portion of an anti-HER3 antibody, wherein the anti-HER3 antibody is treated under reducing conditions and then reacted with a compound (such as Formula II) to give the antibody-drug conjugate. Specifically, an antibody-drug conjugate can be produced by reacting the compound with an antibody having a sulfhydryl group.

Antibodies having a sulfhydryl group can be obtained using methods well known in the art (Hermanson, G. T, Bioconjugate Techniques, pp. 56-136, pp. 456-493, Academic Press (1996)). Examples include, but are not limited to: reacting Traut's reagent with an amino group of the antibody; reacting N-succinimidyl S-acetylthioalkanoate with an amino group of the antibody followed by reaction with hydroxylamine; reacting the antibody with N-succinimidyl 3-(pyridyldithio)propionate followed by reaction with a reducing agent; and reacting the antibody with a reducing agent such as dithiothreitol, 2-mercaptoethanol, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to reduce the disulfide bond in the hinge portion within the antibody to form sulfhydryl.

Specifically, an antibody in which a disulfide bond in a hinge portion in the antibody is partially or completely reduced can be obtained by using 0.3 to 10 molar equivalents of TCEP as a reducing agent per disulfide bond in the hinge portion in the antibody and reacting the reducing agent with the antibody in a buffer containing a chelating agent. Examples of the chelating agent include ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA).

The chelating agent may be at a concentration of 1 mM to 20 mM. Examples of buffers that can be used include sodium phosphate, sodium

borate, and sodium acetate solutions. Specifically, an antibody having a partially or completely reduced sulfhydryl group can be obtained by reacting the antibody with TCEP at 4-37 °C for 1-4 h.

Meanwhile, by performing a reaction of adding a sulfhydryl group to the drug-linker moiety, the drug-linker moiety can be conjugated via a thioether bond.

Using 2-20 molar equivalents of the compound per antibody having a sulfhydryl group, antibody-drug conjugates in which 2-8 drug molecules are conjugated per antibody can be produced. Specifically, it is sufficient to add a solution in which the compound is dissolved in a buffer containing the antibody having a sulfhydryl group to carry out the reaction. Here, examples of buffers that can be used include sodium acetate solution, sodium phosphate, and sodium borate. The reaction is performed at pH 5-9, and more preferably, the reaction is performed at about pH 7. Examples of solvents for dissolving compound (1) include organic solvents such as dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide (DMA), and N-methyl-2-pyrrolidone (NMP).

The reaction may be performed by adding an organic solvent solution in which the compound is dissolved to a buffer containing the antibody having a sulfhydryl group at 1-20% v/v. The reaction temperature is 0-37°C, more preferably 10-25°C, and the reaction time is 0.5-2 h.

After conjugation, the manufactured antibody-drug conjugate can be subjected to buffer exchange, determination of antibody concentration, and determination of the average number of conjugated drugs per antibody molecule, so as to identify the antibody-drug conjugate.

Common procedure A: determination of antibody concentration

The antibody concentration was determined using a UV detector (Nanodrop 1000, Thermo Fisher Scientific Inc.) according to the method specified by the manufacturer. In this case, the extinction coefficient at 280

nm could be deduced from the amino acid sequence of the antibody using a known calculation method (Protein Science, 1995, vol. 4, 2411-2423), and absorbance coefficients at 280 nm (1.3 mL mg⁻¹cm⁻¹ to 1.8 mL mg⁻¹cm⁻¹) that vary from antibody to antibody were used. In the case of KA0013, the extinction coefficient at 280 nm of 1.768 mL mg⁻¹cm⁻¹ was used as a deduced value based on the amino acid sequence thereof.

Common procedure B: buffer exchange for antibody-drug conjugate

The antibody-drug conjugate was subjected to ultrafiltration concentration and buffer exchange using an ultrafiltration membrane (Millipore P3C030C00, 30 kDa) according to the method specified by the manufacturer, with a 10-fold buffer exchange ratio.

Common procedure C: determination of average number of conjugated drugs per antibody molecule in antibody-drug conjugate

Determination of average number of conjugated drugs per antibody molecule in antibody-drug conjugate using liquid chromatography-mass spectrometry:

The sample was diluted to 2 mg/mL with water. 50 µL of the diluted sample was taken, and 1 µL of 0.5 M TCEP was added. The mixture was reacted at room temperature for 30 min before injection. Chromatographic conditions: column: MabPac RP (2.1 × 50 mm); mobile phase: mobile phase A: 0.1% aqueous formic acid solution; mobile phase B: a 0.1% formic acid in acetonitrile; sample injection volume: 2 µg; column temperature: 80 °C; and detection wavelength: 280 nm.

The liquid phase gradient is shown in Table 2 below.

Table 2

Time (min)	Flow rate (mL/min)	%A	%B
0.00	0.30	90.0	10.0
3.00	0.30	90.0	10.0
7.00	0.30	10.0	90.0

8.00	0.30	10.0	90.0
8.10	0.30	90.0	10.0
10.00	0.30	90.0	10.0

The DAR value was calculated according to the signal values of components.

$$DAR = 2 \times \left(\frac{1 \times LC_1}{LC_0 + LC_1} + \frac{1 \times HC_1 + 2 \times HC_2 + 3 \times HC_3}{HC_1 + HC_2 + HC_3} \right)$$

In the above formula, LC_n represents the signal value of the light chain conjugated with n linker-payloads, and HC_n represents the signal value of the heavy chain conjugated with n linker-payloads.

Specific conjugation examples were as follows.

Reduction of antibody: Patritumab was diluted to about 10 mg/mL with phosphate buffer PB/EDTA (15 mM Na₂HPO₄, 5 mM NaH₂PO₄, 5 mM EDTA-2Na, pH 7.0). The antibody solution (100 mL) was added to a beaker at room temperature, and then a 10 mM aqueous TCEP solution (0.68 mL; 10.0 equivalents relative to one antibody molecule) was added. The mixture was stirred using a magnetic stirrer and incubated at room temperature for 3 h to reduce the disulfide bond in the hinge portion of the antibody.

Conjugation of antibody to drug linker: A solution of 10 mM KA0012 in DMSO (0.748 mL; 11.0 equivalents relative to one antibody molecule) was slowly added to the mixture. The mixture was stirred at room temperature for 1 h to conjugate the drug linker with the antibody.

Purification: Ultrafiltration concentration and buffer exchange were performed using an ultrafiltration membrane. While unconjugated drug linkers and other low-molecular-weight reagents were removed, the buffer was exchanged for a formulation buffer, and the solution was further

concentrated. The resulting purified solution was sterilized by filtration to give a solution containing the antibody-drug conjugate KA0013.

Preparation Example 4: Preparation of Control Drug

The structural formula of control KA0010 is represented by Formula V below:

Formula V

IgG-KA0010: IgG-Mc-GGFG-Dxd, abbreviated as IgG-Dxd.

The preparation methods for KA0010 and KA0011 were performed by referring to the relevant descriptions in Chinese Patent No. CN 106163559 B.

conjugation method commonly used in sulfhydryl conjugation, and the specific preparation method was performed by referring to the above preparation method for KA0013, or by referring to the related descriptions in Chinese Patent No. CN 106163559 B.

Example 1: FACS Assay for Binding Activity of Anti-HER3 Antibody-Drug Conjugate KA0013 to HER3 on SK-BR-3 Cell Surface

1. Experimental drug

KA0013,

HER3-targeted monoclonal antibody patritumab (prepared according to Preparation Example 1).

KA0011.

2. Experimental method

Following standard procedures, SK-BR-3 cells (purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were collected, washed once with an appropriate amount of PBS, counted, and determined for cell viability. The cell suspension was added to a 96-well plate at 3×10^5 cells/sample and centrifuged at $1000\times g$ for 5 min. The supernatant was discarded. 100 μ L of each of the serially diluted experimental drugs (900 nM, 300 nM, 100 nM, 33.3 nM, 11.1 nM, 3.7 nM, 1.23 nM, 0.41 nM, 0.041 nM, and 0.0041 nM based on antibody working concentrations, respectively) was added, and the cell pellet was resuspended and incubated on ice for 40 min. 1% PBSA (PBS + 1% BSA) was added, and the mixture was centrifuged at $1000\times g$ for 5 min. The supernatant was discarded, and the cells were further washed twice. 100 μ L of 300-fold diluted fluorescent antibody Mouse Anti-Human IgG Fc-Alexa Fluor 647 (Southern Biotech, Cat. No. 9040-31) was added, and the cell pellet was resuspended and incubated on ice for 30 min in the dark. 1% PBSA was added, and the mixture was centrifuged at $1000\times g$ for 5 min.

The supernatant was discarded, and the cells were further washed twice. 200 μ L of 1% PBSA was added, and the cell pellet was resuspended and then loaded into the flow cytometer for assay.

3. Experimental results

The results are shown in FIG. 1.

The results showed that under the same experimental conditions, the EC₅₀ values of KA0013, KA0011, and the HER3-targeted monoclonal antibody patritumab for binding to SK-BR-3 cells were 15.36 nM, 12.89 nM, and 8.481 nM, respectively.

The results indicate that under the same experimental conditions, KA0013, KA0011, and patritumab all have the activity of effectively binding to SK-BR-3 cells expressing HER3.

Example 2: FACS Assay for Internalization Activity of Anti-HER3 Antibody-Drug Conjugate KA0013 in MDA-MB-453 Cells

1. Experimental drug

KA0013,
IgG-Dxd,
KA0012,
KA0011.

2. Experimental method

MDA-MB-453 cells (purchased from Cobioer Biosciences Co., Ltd., Cat. No. CBP60386) were collected, counted, and determined for cell viability. The cell suspension was added to a 96-well low-adsorption plate at 1×10^5 cells/sample and centrifuged at $750 \times g$ for 5 min. The supernatant was discarded. 100 μ L of the experimental drug (at a working concentration of 100 nM) was added, with an isotype control (IgG-Dxd, at a working concentration of 100 nM) designed in parallel. The mixture was mixed uniformly and incubated in an incubator at 37 °C for 1 h, 3 h, and 5 h.

After the incubation was completed, 1% PBSA (PBS + 1% BSA) was added, and the mixture was centrifuged at 750× g for 5 min. The supernatant was discarded, and the cells were washed twice with 1% PBSA. 100 µL of patritumab (at a working concentration of 100 nM) was added, and the mixture was incubated on ice for 30 min. 1% PBSA (PBS + 1% BSA) was added, and the mixture was centrifuged at 750× g for 5 min. The supernatant was discarded, and the cells were further washed twice with 1% PBSA. 100 µL of 300-fold diluted fluorescent antibody Mouse Anti-Human IgG Fc-Alexa Fluor 647 (Southern Biotech, Cat. No. 9040-31) was added, and the cell pellet was resuspended and incubated on ice for 30 min in the dark. 1% PBSA (PBS + 1% BSA) was added, and the mixture was centrifuged at 1000× g for 5 min. The supernatant was discarded, and the cells were further washed twice. 200 µL of 1% PBSA was added, and the cell pellet was resuspended and then loaded into the flow cytometer for assay.

3. Experimental results

The results are shown in FIG. 2.

The results showed that under the same experimental conditions, KA0013 and KA0011 could be endocytosed in MDA-MB-453 as compared to isotype control IgG-Dxd (i.e., IgG-Mc-GGFG-Dxd, produced by Akeso Biopharma Inc.), and the endocytic activity of the two was significantly different from that of IgG-Dxd.

Example 3: Determination of Non-Specific Killing Activity of Linker-Payload Moiety of Anti-HER3 Antibody-Drug Conjugates

1. Experimental drug

IgG-KA0010,

IgG-KA0012.

2. Experimental method

The non-specific killing activity assay was performed on the linker-payload moiety Mc-AAA-Dxd (named KA0012) of the anti-HER3 antibody-drug conjugate KA0013 and the linker-payload moiety Mc-GGFG-Dxd (named KA0010) of the control KA0011.

After the human venous blood was collected, the heparin sodium anticoagulant tube was gently inverted for uniform mixing, and centrifuged at room temperature at 3500 rpm for 10 min. The plasma layer was collected for the experiment. MDA-MB-453 cells were routinely collected, counted, and determined for cell viability. The cell density was adjusted to 2.5×10^4 cells/mL, and the cell suspension was added to a 96-well black-bottom plate at 100 μ L/well. The plate was incubated in an incubator at 37 °C with 5% CO₂ overnight. The antibody was diluted with a DMEM medium (5% human plasma), and 100 μ L of serially diluted experimental drug (100 nM, 50 nM, 10 nM, 5 nM, 1 nM, 0.5 nM, 0.1 nM, 0.05 nM, and 0.01 nM based on the working concentration of the antibody) was added. A blank control group was designed, with three replicate wells set for each group. The plate was incubated in an incubator at 37 °C with 5% CO₂ for 6 days. After the incubation was completed, the 96-well plate was taken out and centrifuged at 350 $\times$ g for 5 min, and then 135 μ L of the supernatant was discarded by pipetting. 65 μ L of CellTiter-Glo (Promega, Cat. No. G8461) was added. The plate was incubated at room temperature for 20 min. Then the fluorescence value was detected by a multi-label microplate tester.

3. Experimental results

The results are shown in FIG. 3.

The results showed that under the same experimental conditions, IgG-KA0012 had weaker non-specific killing as compared to the isotype control (IgG-KA0010, produced by Akeso Biopharma Inc.). This indicates that Mc-AAA-Dxd is more stable than Mc-GGFG-Dxd.

Example 4: Assay of Plasma Stability of Anti-HER3 Antibody-Drug Conjugate KA0013

1. Experimental drug

KA0013,

KA0011.

2. Experimental method

After the human venous blood was collected, the heparin sodium anticoagulant tube was gently inverted for uniform mixing, and centrifuged at 3500 rpm for 10 min at room temperature. The supernatant was plasma. The experimental drugs (at a working concentration: 100 µg/mL) were diluted with human plasma, and the diluted samples were incubated in an incubator at 37 °C with 5% CO₂ for 0 h, 24 h, 48 h, 72 h, 96 h, and 139 h. After the incubation was completed, 150 µL of the samples were taken out and quickly frozen in liquid nitrogen. After all the samples were collected, changes in free Dxd in plasma were detected by mass spectrometry.

Sample preparation for mass spectrometry: An appropriate amount of the above sample was taken and mixed with acetonitrile in a ratio of 1:1 to precipitate the protein. The mixture was centrifuged at 12,000 rpm for 3 min, and 10 µL of the supernatant was taken for mass spectrometry detection.

Chromatographic conditions: column: MabPac RP (2.1 × 50 mm, 4 µm); mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile; and column temperature: 30 °C.

The gradient is shown in Table 3 below.

Table 3

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0	0.4	95	5
1	0.4	95	5

5	0.4	10	90
6	0.4	10	90
6.5	0.4	95	5
7	0.4	10	90
8	0.4	10	90
8.1	0.4	95	5
10	0.4	95	5

3. Experimental results

The results are shown in FIG. 4.

The results showed that under the same experimental conditions, the amount of free Dxd in plasma in the KA0013 group was lower than that in the KA0011 group. This indicates that the stability of KA0013 in plasma is superior to that of KA0011.

Example 5: *In Vitro* Assay of Cleavage Efficiency of Different Linker-Payloads by Cathepsin B

1. Experimental sample:

KA0010,
KA0012.

2. Experimental method

PBS (20× solution) (Sangon, Cat. No. B548117) was subjected to 20-fold dilution to obtain a PBS working solution. Cathepsin B (CTSB) (Sinobio, Cat. No. 10483-H08H) and linker-payload were diluted with the PBS working solution. 150 µL of CTSB (at a working concentration of 3 nM) was mixed uniformly with an equal volume of linker-payload (at a working concentration of 10 µM), and the mixture was incubated in an incubator at 37 °C with 5% CO₂ for 0 h, 0.5 h, 1 h, 2 h, and 4 h. After the incubation

was completed, Halt™ Protease Inhibitor Cocktail, EDTA-Free (30X) (Thermofisher, Cat. No. 87785) was added to terminate the reaction.

The digested samples were directly loaded for mass spectrometry detection.

Chromatographic conditions: column: MabPac RP (2.1 × 50 mm, 4 μm); mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile; loading amount: 1 μL of 10 μM sample; and column temperature: 30 °C.

The gradient is shown in Table 4 below.

Table 4

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0	0.4	95	5
1	0.4	95	5
5	0.4	10	90
6	0.4	10	90
6.5	0.4	95	5
7	0.4	10	90
8	0.4	10	90
8.1	0.4	95	5
10	0.4	95	5

3. Experimental results

The results are shown in FIG. 5.

The results showed that under the same experimental conditions, KA0012 (Mc-AAA-Dxd) and KA0010 (Mc-GGFG-Dxd) could both be efficiently cleaved by CSTB and effectively release the payload, with identical efficiency.

Example 6: Assay for Binding Activity of Anti-HER3 Antibody-Drug

Conjugate KA0013 to Antigen by ELISA

The Elisa plates were coated with human HER3-his and incubated, and the test antibodies were added after blocking. After incubation and washing, goat anti-human IgG Fc, HRP (purchased from Jackson ImmunoResearch Inc., Cat. No. 109-035-098) was added. After incubation and washing, TMB (Neogen, 308177) was added for chromogenic reaction. After the reaction was terminated, the absorbance at 450 nm was detected in a microplate reader. The data were analyzed and processed using SoftMax Pro 6.2.1 software.

The results are shown in FIG. 6 and Table 5.

Table 5: Binding activity of KA0013 and KA0011 for antigen human HER3-his

Coating: huHER3-his(0.5µg/mL, 50µL/well)						
Antibody dilution (nM)	KA0013		KA0011		Patritumab	
1.000	2.848	2.861	2.804	2.819	2.869	2.844
0.333	2.883	2.900	2.814	2.829	2.891	2.856
0.111	2.820	2.841	2.756	2.748	2.831	2.795
0.037	2.538	2.587	2.390	2.438	2.569	2.570
0.012	1.702	1.755	1.508	1.531	1.780	1.775
0.004	0.819	0.847	0.718	0.700	0.870	0.872
0.001	0.362	0.370	0.306	0.315	0.382	0.394
0	0.082	0.092	0.075	0.078	0.091	0.087
Secondary antibody: Goat Anti Human IgG Fc, HRP(1: 5000)						
EC ₅₀ (nM)	0.009		0.011		0.009	

The results showed that the anti-HER3 antibody-drug conjugates KA0013 and KA0011, and patritumab could all effectively bind to the antigen human HER3-his in a dose-dependent manner.

Example 7: Determination of Kinetic Parameters of Anti-HER3 Antibody-Drug Conjugates

1. Experimental sample

KA0013

KA0011

2. Experimental method

The dilution buffer for experimental samples was PBS containing 0.02% Tween-20 and 0.1% BSA (pH 7.4). 1 µg/mL huHER3-his-Biotin was immobilized on the SA sensor at an immobilization height of 1 nm. The sensor was equilibrated in a buffer for 60 s, and then the binding of the immobilized huHER3-his-Biotin on the sensor to the antibodies at concentrations of 0.2469-20 nM (three-fold dilution) was determined for 120 s. The antibodies were then dissociated in the buffer for 300 s. The sample plate shaking speed was 1000 rpm, the determination temperature was 30 °C, and the frequency was 5.0 Hz. The data were analyzed by 1:1 model fitting to obtain affinity constants. The data acquisition software was Fortebio Data Acquisition 12.0, and the data analysis software was Fortebio Data Analysis 12.0.

3. Experimental results

The results are shown in Table 6 and FIGs. 7 to 8.

Table 6: Kinetic parameters for binding of anti-HER3 antibody-drug conjugates to human HER3-his-biotin

Sample	KD (M)	kon (1/Ms)	S E (kon)	kdis (1/s)	S E (kdis)	Rmax (nm)
KA0013	8.08E-11	1.49E+06	4.63E+03	1.21E-04	2.77E-06	0.116-0.243
KA0011	5.52E-11	1.40E+06	6.33E+03	7.70E-05	3.99E-06	0.128-0.197

KD is affinity constant; kon is binding rate of antigen and antibody; kdis is dissociation rate of antigen and antibody; $KD = kdis/kon$.

The results showed that the anti-HER3 antibody-drug conjugates KA0013 and KA0011 both had good affinity for the antigen human HER3-his-biotin.

Example 8: Assay for Specific Cell Killing Activity of Anti-HER3 Antibody-Drug Conjugates

1. Experimental drug

KA0013

KA0011

IgG-KA0012

2. Experimental method

NCI-H1781 cells (Cobioer Biosciences Co., Ltd., Cat. No. CBP60116) were collected, and centrifuged at $1000\times g$ for 5 min, and then the supernatant was removed. The cells were resuspended and counted, and the viability was determined. The cells were diluted, and NCI-H1781 (2500 cells/100 μL) was added to each well of a black-bottom 96-well plate. The plate was incubated in an incubator at $37\text{ }^{\circ}\text{C}$ with 5% CO_2 overnight. The experimental drug was diluted with a medium (10% human ab serum), and 100 μL of the experimental drug was added to each well according to the experimental design. A blank control was designed, with three replicate wells set for each group. The plate was incubated in an incubator at $37\text{ }^{\circ}\text{C}$ with 5% CO_2 for 6 days. After incubation, the 96-well plate was centrifuged at $350\times g$ for 5 min, and then 135 μL of the supernatant was discarded. 60 μL of CellTiter-Glo (Promega, Cat. No. G8461) was added to each well. The plate was incubated at room temperature for 15 min. Then the fluorescence value was detected by a multi-label microplate tester.

3. Experimental results

The results are shown in FIG. 9. The results showed that the anti-HER3 antibody-drug conjugates KA0013 and KA0011 could all specifically kill

NCI-H1781 cells.

Example 9: Study on ADCC Activity of Anti-HER3 Antibody-Drug Conjugates

MDA-MB-453 cells (purchased from Cobioer Biosciences Co., Ltd., Cat. No. CBP60386) and PBMCs (provided by Zhongshan Blood Center) in the logarithmic growth phase were collected and centrifuged at $170\times g$ for 5 min, and the supernatant was discarded. The cell pellet was resuspended in an assay medium (RPMI-1640 containing 1% FBS), centrifuged and washed twice, and the cells were counted. The cell density was adjusted with the assay medium, and the target cell suspension was seeded into a 96-well plate (containing about 3×10^4 target cells/well) at 50 μL /well. Then, serially diluted drugs (5 $\mu\text{g/mL}$, 15 $\mu\text{g/mL}$, and 45 $\mu\text{g/mL}$ based on the working concentration of the antibody, respectively) were added, and the plate was pre-incubated at room temperature for 1 h. After the pre-incubation was completed, the PBMC suspension was added to the sample wells at 100 μL /well (each well contained about 9×10^5 PBMCs with an effector-to-target ratio of 30:1), mixed uniformly, and incubated in an incubator at 37 °C with 5% CO_2 for 4 h. After the incubation was completed, the 96-well plate was taken out and centrifuged at $250\times g$ for 5 min. 100 μL of culture supernatant was carefully pipetted and transferred to another 96-well plate. The freshly prepared reaction solution was added at 100 μL /well according to the instructions of the Cytotoxicity Detection Kit (Roche, Cat. No. 11644793001), and the plate was incubated at room temperature for 30 min in the dark. OD values at 490 nm and 650 nm were measured. The OD value of each group = $\text{OD}_{490 \text{ nm}} - \text{OD}_{650 \text{ nm}}$. The ADCC activity (expressed as ADCC%) was calculated based on the measured OD value of each group.

$\text{ADCC\%} = (\text{OD of experimental group} - \text{average OD of target cell})$

spontaneous group)/(average OD of positive control – average OD of target cell spontaneous group) × 100%

The results are shown in FIG. 10. The results showed that in the MDA-MB-453 target cell system, KA0011 had no ADCC activity for MDA-MB-453 cells, while KA0013 had ADCC activity for MDA-MB-453 cells.

Example 10: *In Vivo* Efficacy Assay of Anti-HER3 Antibody-Drug Conjugates

To determine the anti-tumor activity of the anti-HER3 antibody-drug conjugates *in vivo*, HCC827 human lung cancer cells (purchased from GuangZhou Jennio Biotech Co., Ltd.) were first inoculated subcutaneously into 5- to 8-week-old female CB-17 SCID mice (purchased from Guangdong Model Organisms Center, Inc.) on the upper posterior region of the right hind thigh. The day of grouping was defined as D0. The route of administration was intravenous injection (iv), once a week for a total of 3 doses. The modeling and specific dosing regimen are shown in Table 7. After the administration, the length and width of the tumors in each group were measured, and the tumor volume was calculated.

Table 7: Dosing regimen of anti-HER3 antibody-drug conjugates for treatment of CB-17 SCID mouse model of HCC827 human lung cancer cell xenograft tumor

Group	Number of animals	Modeling	Dosing regimen
hIgG1 (1.5 mg/kg) group	6	HCC827 cells were inoculated	hIgG1 (1.5mg/kg), IV, weekly×3
IgG-KA0012 (1.5 mg/kg) group	6	subcutaneously at 6×10^6 cells/200	IgG-KA0012, 1.5mg/kg, IV, weekly×3

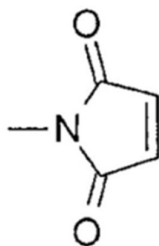
Patritumab (3 mg/kg) group	6	μL/mouse into CB-17 SCID mice	Patritumab, 3mg/kg, IV, weekly×3
KA0013 (3 mg/kg) group	6	on the upper posterior region	KA0013, 3mg/kg, IV, weekly×3
KA0013 (1.5 mg/kg) group	6	of the right hind thigh.	KA0013, 1.5mg/kg, IV, weekly×3

The results are shown in FIG. 11. The results showed that the anti-HER3 antibody-drug conjugate KA0013 could effectively inhibit the growth of mouse tumors compared to the isotype control antibody. In addition, as shown in FIG. 12, the tumor-bearing mice exhibited good tolerance to test drug KA0013, and the body weights of the tumor-bearing mice in each group were not affected.

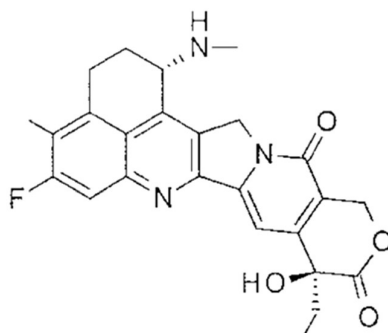
Although the specific embodiments of the present disclosure have been described in detail, those skilled in the art will understand that various modifications and substitutions can be made to those details according to all the teachings that have been disclosed, and these changes shall all fall within the protection scope of the present disclosure. The full scope of the present disclosure is given by the appended claims and any equivalent thereof.

CLAIMS

1. A compound or a pharmaceutically acceptable salt thereof, wherein the compound comprises a compound of Formula III, n alanine residues, and a compound of Formula IV that are sequentially linked,



Formula III,



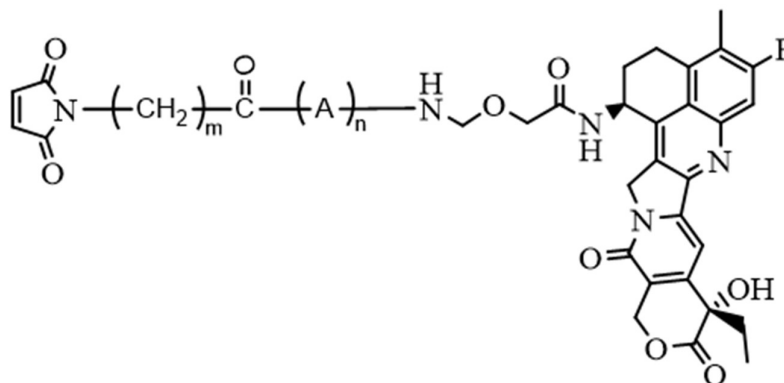
Formula IV,

wherein

n is 2, 3, 4, 5, or 6;

any two of the compound of Formula III, the n alanine residues, and the compound of Formula IV are independently either linked directly or via a chemical group.

2. The compound or the pharmaceutically acceptable salt thereof according to claim 1, having a structural formula represented by Formula I below,



Formula I

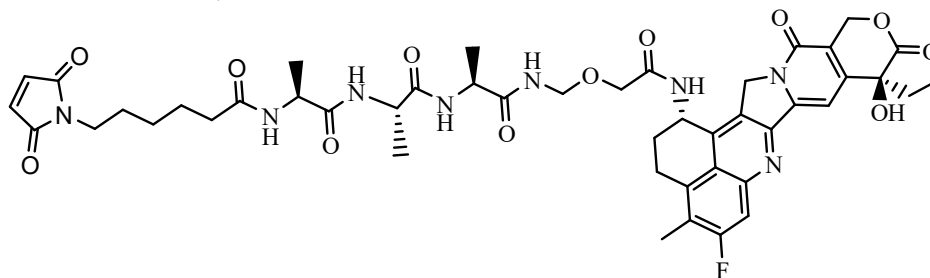
wherein

A represents alanine;

m is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12;

n is 2, 3, 4, 5, or 6.

3. The compound or the pharmaceutically acceptable salt thereof according to any one of claims 1 to 2, wherein the compound is represented by Formula II below,



Formula II

4. An antibody-drug conjugate, comprising an antibody or an antigen-binding fragment thereof, a linker, and a payload, wherein the linker and the payload are the compound or the pharmaceutically acceptable salt thereof according to any one of claims 1 to 3; preferably, the linker is linked to the antibody or the antigen-binding fragment thereof via one or more thioether bonds;

preferably, the linker forms a thioether bond with a sulfur atom at a disulfide bond position of the hinge portion of the antibody.

5. The antibody-drug conjugate according to claim 4, wherein the antibody is an IgG antibody, preferably an IgG1 antibody.

6. The antibody-drug conjugate according to any one of claims 4 to 5, wherein the antibody is an anti-HER3 antibody.

7. The antibody-drug conjugate according to any one of claims 4 to 6, wherein the antibody comprises a heavy chain variable region and a light chain variable region,

wherein

the heavy chain variable region comprises an HCDR1 set forth in SEQ ID NO: 1, an HCDR2 set forth in SEQ ID NO: 2, and an HCDR3 set forth in SEQ ID NO: 3;

the light chain variable region comprises an LCDR1 set forth in SEQ ID NO: 4, an LCDR2 set forth in SEQ ID NO: 5, and an LCDR3 set forth in SEQ ID NO: 6.

8. The antibody-drug conjugate according to claim 7, wherein the heavy chain variable region comprises an amino acid sequence set forth in SEQ ID NO: 7, and the light chain variable region comprises an amino acid sequence set forth in SEQ ID NO: 9.

9. The antibody-drug conjugate according to any one of claims 4 to 8, wherein a heavy chain constant region of the antibody is an Ig gamma-1 chain C region or an Ig gamma-4 chain C region, and a light chain constant region is an Ig kappa chain C region.

10. The antibody-drug conjugate according to any one of claims 4 to 9, wherein the heavy chain constant region of the antibody comprises an amino acid sequence set forth in SEQ ID NO: 11, and the light chain constant region comprises an amino acid sequence set forth in SEQ ID NO: 13.

11. The antibody-drug conjugate according to any one of claims 4 to 10, wherein an average number of linker-payloads conjugated to each antibody molecule is 1-8.

12. The antibody-drug conjugate according to any one of claims 4 to 11, having ADCC activity.

**13. The antibody-drug conjugate according to any one of claims 4 to 12, wherein
an EC₅₀ for binding of the antibody-drug conjugate to an antigen targeted by the antibody-drug conjugate is identical to or substantially identical to an EC₅₀ for binding of an antibody or an antigen-binding fragment thereof comprised in the antibody-drug conjugate to the same antigen.**

14. A pharmaceutical composition, comprising the antibody-drug conjugate according to any one of claims 4 to 13 and one or more pharmaceutically acceptable auxiliary materials.

**15. Use of the antibody-drug conjugate according to any one of claims 4 to 13 in the preparation of a medicament for treating or preventing a tumor, wherein
preferably, the tumor is an HER3-positive tumor;**

preferably, the tumor is selected from one or more of lung cancer, clear cell sarcoma, skin cancer, renal cancer, urothelial cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, gastrointestinal stromal tumor, cervical cancer, head and neck cancer, esophageal cancer, epidermoid carcinoma, peritoneal cancer, adult glioblastoma multiforme, colon cancer, rectal cancer, uterine cancer, salivary gland cancer, vulvar cancer, thyroid cancer, anal cancer, and penile cancer;
preferably, the lung cancer is non-small cell lung cancer;
preferably, the colon cancer is metastatic colon cancer.

16. The antibody-drug conjugate according to any one of claims 4 to 13 for use in treating or preventing a tumor, wherein
preferably, the tumor is an HER3-positive tumor;
preferably, the tumor is selected from one or more of lung cancer, clear cell sarcoma, skin cancer, renal cancer, urothelial cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, gastrointestinal stromal tumor, cervical cancer, head and neck cancer, esophageal cancer, epidermoid carcinoma, peritoneal cancer, adult glioblastoma multiforme, colon cancer, rectal cancer, uterine cancer, salivary gland cancer, vulvar cancer, thyroid cancer, anal cancer, and penile cancer;
preferably, the lung cancer is non-small cell lung cancer;
preferably, the colon cancer is metastatic colon cancer.

17. A method for treating or preventing a tumor, comprising a step of administering to a subject in need an effective amount of the antibody-drug conjugate according to any one of claims 4 to 13, wherein
preferably, the tumor is an HER3-positive tumor;

preferably, the tumor is selected from one or more of lung cancer, clear cell sarcoma, skin cancer, renal cancer, urothelial cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, gastric cancer, gastrointestinal stromal tumor, cervical cancer, head and neck cancer, esophageal cancer, epidermoid carcinoma, peritoneal cancer, adult glioblastoma multiforme, colon cancer, rectal cancer, uterine cancer, salivary gland cancer, vulvar cancer, thyroid cancer, anal cancer, and penile cancer;
preferably, the lung cancer is non-small cell lung cancer;
preferably, the colon cancer is metastatic colon cancer.