(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 December 2017 (07.12.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/210288 A1

(51) International Patent Classification:
A61K 39/395 (2006.01) *C07K 19/00* (2006.01)
A61P 35/00 (2006.01)

(21) International Application Number:
PCT/US2017/035206

(22) International Filing Date:
31 May 2017 (31.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/343,825 31 May 2016 (31.05.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2017/210288 A1

(54) Title: ANTIBODY DRUG CONJUGATES HAVING DERIVATIVES OF AMATOXIN AS THE DRUG

(57) Abstract: There is disclosed derivatives of amanitin conjugated to a targeting antibody to form an ADC (antibody drug conjugate).

Antibody Drug Conjugates Having Derivatives of Amatoxin as the Drug

Cross Reference to Related Application

This patent application claims priority to United States provisional patent application 62/343,825, filed May 31, 2016, the contents of which are incorporated herein by reference.

Technical Field

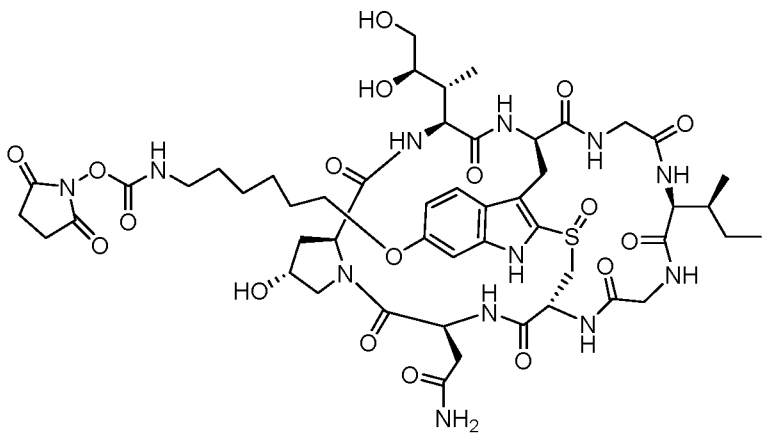
The present disclosure provides derivatives of amanitin conjugated to a targeting antibody to form an ADC (antibody drug conjugate).

Background

The amatoxins are rigid bicyclic peptides having eight amino acid units. These compounds are isolated from a variety of mushroom species *(e.g., Amanita phalloides* (also known as green death cap mushroom), *Galerina marginata, Lepiota brunneo-incamata)* or are prepared synthetically. Different mushroom species contain varying amounts of different Amatoxin family members. A member of this family, alpha-amanitin, is known to be an inhibitor of eukaryotic RNA polymerase II (EC2.7.7.6) and to a lesser degree, RNA polymerase III, thereby inhibiting transcription and protein biosynthesis. Wieland (1983) *Int. J. Pept. Protein Res.* 22(3):257-276. Alpha-amanitin binds non-covalently to RNA polymerase II and dissociates slowly, making enzyme recovery unlikely. Prolonged inhibition of transcription is thought to induce cellular apoptosis.

Exemplary amatoxins include

WO2012/041504



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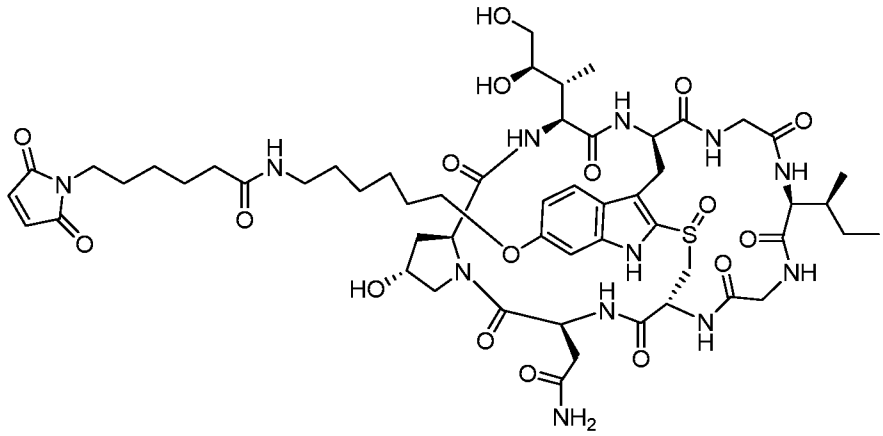

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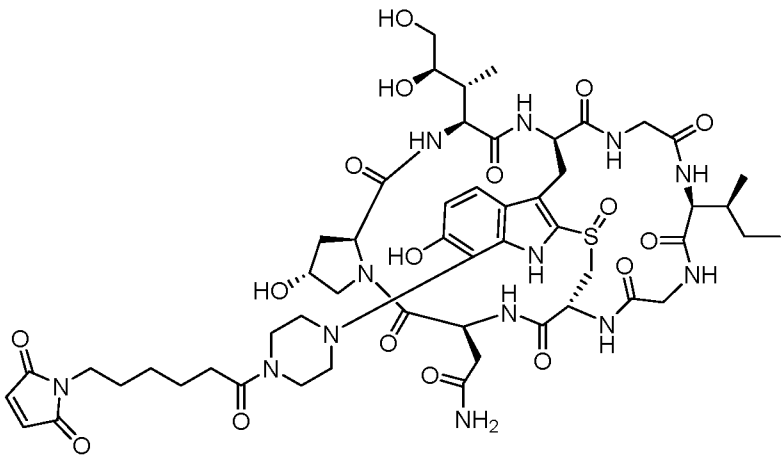
WO2014/043403



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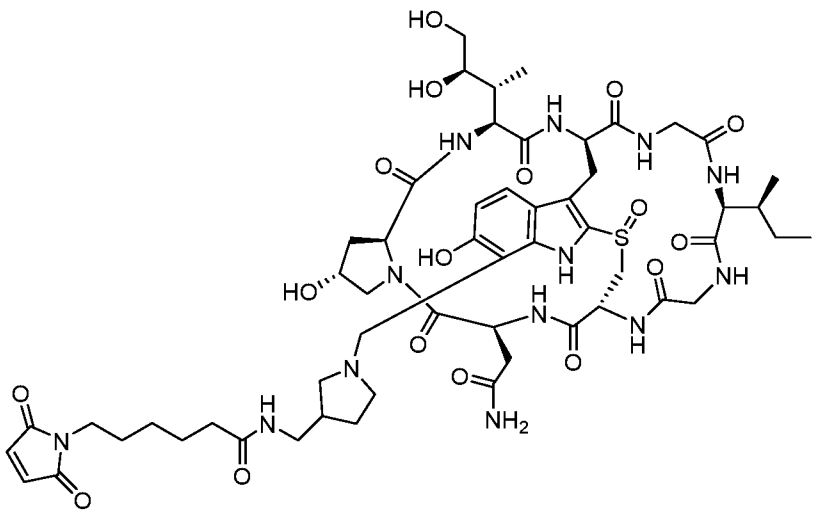

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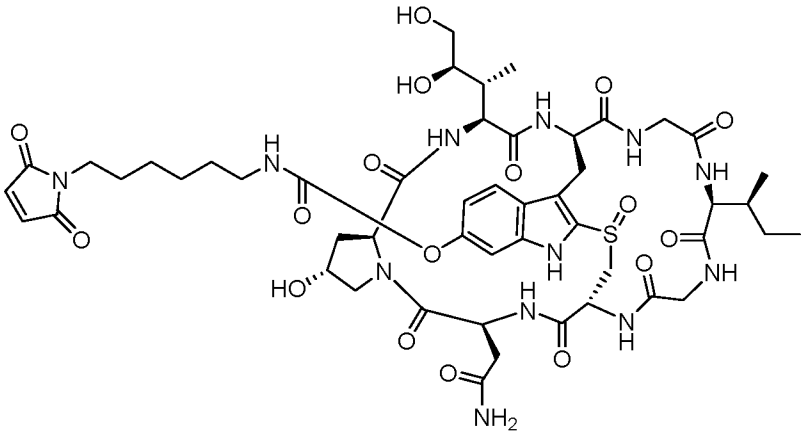
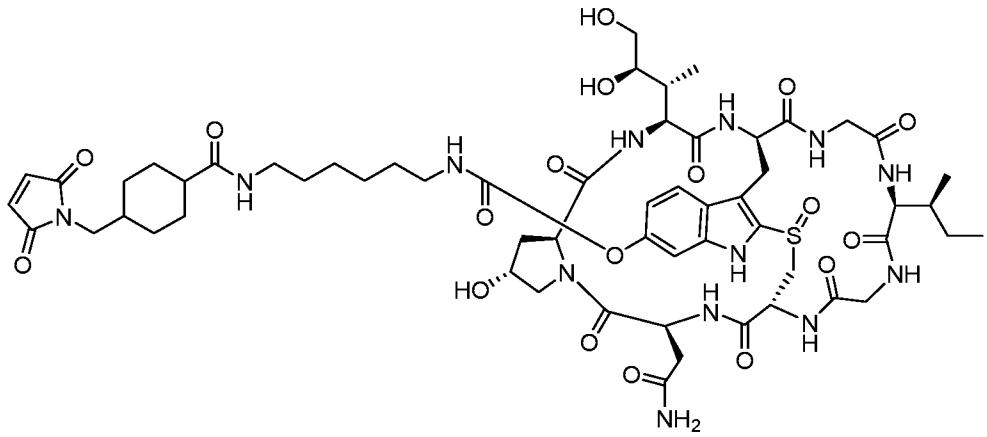


(Example 79) and



(Example 80).

The use of antibody-drug conjugates (ADCs) for the local delivery of cytotoxic or cytostatic agents, including drugs that kill or inhibit tumor cells, allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein. Syrigos and Epenetos (1999) *Anticancer Res.* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drug Delivery Rev.* 26:151-172; U.S. Pat. No. 4,975,278; Baldwin *et al.* (1986) *Lancet* (Mar. 15, 1986):603-05; Thorpe (1985) "Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological and Clinical Applications, A. Pinchera *et al.* (eds.), pp. 475-506. This type of delivery mechanism helps to minimize toxicity to normal cells that may occur from systemic administration of unconjugated drug agents. The toxins may cause their cytotoxic and cytostatic effects through a variety of mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies. Rowland *et al.* (1986) *Cancer Immunol. Immunother.* 21:183-87. Toxins used in antibody-toxin conjugates include radioisotopes, bacterial toxins such as diphtheria toxin, plant toxins such as ricin, fungal toxins such as amatoxins (WO2010/115629, W02012/041504 or WO2012/119787), and small molecule toxins such as geldanamycin (Mandler *et al.* (2000) *J. Natl. Cancer Inst.*

92(19):1573- 1581; Mandler *et al.* (2000) *Bioorg. Med. Chem. Lett.* 10:1025-1028; Mandler *et al.* (2002) *Bioconjugate Chem.* 13:786-791), maytansinoids (EP 1391213; Liu *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:8618-8623), calicheamicin (Lode *et al.* (1998) *Cancer Res.* 58:2928; Hinman*et al.* (1993) *Cancer Res.* 53:3336-3342), daunomycin, doxorubicin, methotrexate, and vindesine (Rowland *et al.* (1986), *supra).*

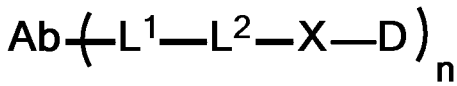
Several antibody-drug conjugates have shown promising results against cancer in clinical trials, including ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec), an antibody-radioisotope conjugate composed of a murine IgGl kappa monoclonal antibody (directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes) connected with an 111In or 90Y radioisotope via a thiourea linker-chelator.

The use of antibody-drug conjugates (ADCs) for the local delivery of cytotoxic or cytostatic agents, including drugs that kill or inhibit tumor cells, allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein. This type of delivery mechanism helps to minimize toxicity to normal cells that may occur from systemic administration of unconjugated drug agents. The toxins may cause their cytotoxic and cytostatic effects through a variety of mechanisms including tubulin binding.

As such, there remains a need for potent RNA polymerase inhibitor antibody conjugates with desirable pharmaceutical properties.

Summary

The present disclosure provides improved amatoxin derivatives used in an ADC (antibody drug conjugate) structure. More specifically, the present disclosure provides an antibody drug conjugate (ADC) having the structure of Formula I



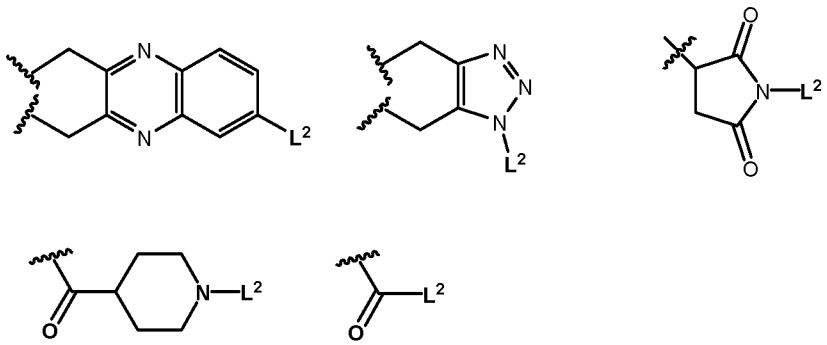
(I)

or a pharmaceutically acceptable salt thereof,

wherein:

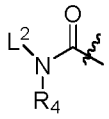
Ab is a monoclonal antibody;

$\mathbf{L^1}$ - $\mathbf{L^2}$ is a linker selected from the group consisting of



whereby the wavy line indicates the point of attachment to Ab;

L^2 - X is a linker having structure of

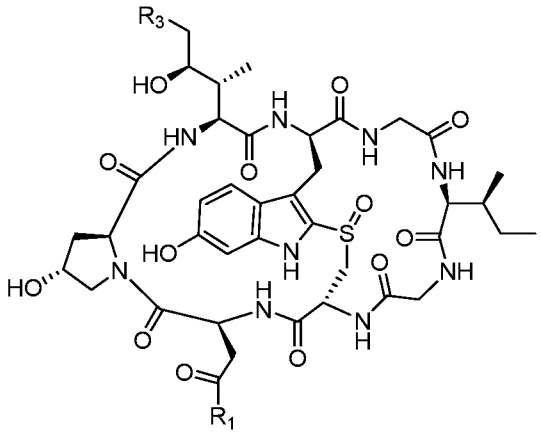


wherein R_4 is hydrogen, C_{1-6} alkyl, $-(CH_2CH_2O)_m-$, or the combination thereof, and

m is an integer from 1-24;

wherein the wavy line indicates the point of attachment to **D**

D is a drug moiety active agent derived from amatoxin and selected from the group consisting of alpha-amanitin, beta-amanitin, gamma-amanitin, and epsilon-amanitin having the structure below:



Name	R1	R3
alpha-amanitin	NH_2	OH
beta-amanitin	OH	OH
gamma-amanitin	NH_2	H
epsilon-amanitin	OH	H

n is an integer from 1-10;

L^2 is a linker selected from the group consisting of an amino acid, peptide consisting of up to 10 amino acids, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, -C(O)NH-, -NHC(O)-, PAB (p-aminobenzyl), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, $-R_6OC(O)NR_5-$, $-R_8-S-S-R_7$, and combinations thereof,

wherein R_5 is selected from the group consisting of hydrogen, C_{1-6} alkyl, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, and combinations thereof;

R_6 is selected from the group consisting of an amino acid, peptide consisting of up to 10 aminoacids, C_{1-6} alkyl, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, -C(O)NH-, -NHC(O)-, PAB, Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, and combinations thereof;

R_7 is C_{2-6} alkylene, or $-(CH_2CH_2O)_m-$;

R_8 is selected from the group consisting of an amino acid, peptide consisting of up to 10 aminoacids, C_{1-6} alkyl, C_{1-6} alkylene, substituted C_{1-6} alkylene, -C(O)NH-, -C(O)-NH-CHR_9-$CR_{10}R_{11}$-, -NHC(O)-CHR_9-$CR_{10}R_{11}$-, $-(CH_2CH_2O)_m-$, PAB, Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, and combinations thereof;

wherein R_9 is selected from the group consisting of hydrogen, C_{1-6} alkyl, C_{1-6} alkylene, $-(CH_2CH_2O)_m-$, -C(O)NH-, -NHC(O)-, -C(O)NH-(CH_2)p-SO_3H, C(O)NH-(CH_2)p-CO_2H, -NHC(O)-(CH_2)p-SO_3H, -NHC(O)-(CH_2)p-CO_2H and combinations thereof;

R_{10} and R_{11} are each independently selected from the group consisting of hydrogen, C_{1-6} alkyl, and combinations thereof;

wherein $-R_6OC(O)NR_5-$ is connected to $\mathbf{L^1}$ through R_5 or R_6;

wherein $-R_8-S-S-R_7-$ is connected to $\mathbf{L^1}$ through R_8;

m is an integer from 1-24**;** and

p is an integer from 1-6.

In another aspect, $\mathbf{L^2}$ in the compounds having the structure of Formula I is a linker selected from the group consisting of an amino acid, peptide consisting of up to 10 amino acids, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, -C(O)NH-, -NHC(O)-, PAB (p-aminobenzyl), -Val-Cit-PAB-, -Val-Ala-PAB-, -Ala-Ala-Asn-PAB-, $-R_6OC(O)NR_5-$, $-R_8-S-S-R_7$, and combinations thereof,

wherein R_5 is selected from the group consisting of hydrogen, C_{1-6} alkyl, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, and combinations thereof;

R_6 is selected from the group consisting of an amino acid, peptide consisting of up to 10 aminoacids, C_{1-6} alkyl, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, -C(O)NH-, -NHC(O)-, PAB, -Val-Cit-PAB-, -Val-Ala-PAB-, -Ala-Ala-Asn-PAB-, and combinations thereof;

R_7 is C_{2-6} alkylene, or $-(CH_2CH_2O)_m-$;

R_8 is selected from the group consisting of an amino acid, peptide consisting of up to 10 aminoacids, C_{1-6} alkyl, C_{1-6} alkylene, substituted C_{1-6} alkylene, -C(O)NH-, -C(O)-NH-

CHR_9-$CR_{10}R_{11}$-, -NHC(O)-CHR_9-$CR_{10}R_{11}$-, -$(CH_2CH_2O)_m$-, PAB, -Val-Cit-PAB-, -Val-Ala-PAB-, -Ala-Ala-Asn-PAB-, and combinations thereof;

wherein R_9 is selected from the group consisting of hydrogen, C_{1-6} alkyl, C_{1-6} alkylene, -$(CH_2CH_2O)_m$-, -C(O)NH-, -NHC(O)-, -C(O)NH-(CH_2)p-SO_3H, C(O)NH-(CH_2)p-CO_2H, -NHC(O)-(CH_2)p-SO_3H, -NHC(O)-(CH_2)p-CO_2H and combinations thereof;

R_{10} and R_{11} are each independently selected from the group consisting of hydrogen, C_{1-6} alkyl, and combinations thereof;

wherein -$R_6OC(O)NR_5$- is connected to $\mathbf{L^1}$ through R_5 or R_6;

wherein -R_8-S-S-R_7- is connected to $\mathbf{L^1}$ through R_8;

m is an integer from 1-24**;** and

p is an integer from 1-6, wherein the remaining values are as described above for Formula I.

In yet another aspect, $\mathbf{L^2}$ in the compounds having the structure of Formula I is a linker selected from the group consisting of an amino acid, peptide consisting of up to 10 amino acids, -$(CH_2)_p$-, -$(CH_2CH_2O)_m$-, -C(O)NH-, -NH(4-phenyl)CH_2O-, -Val-Cit-NH(4-phenyl)CH_2O-, -Val-Ala-NH(4-phenyl)CH_2O-, -Ala-Ala-Asn-NH(4-phenyl)CH_2O-, -$R_6OC(O)NR_5$-, -R_8-S-S-R_7-, and combinations thereof,

wherein R_5 is selected from the group consisting of hydrogen, C_{1-6} alkyl, -$(CH_2)_p$-, -$(CH_2CH_2O)_m$-, and combinations thereof;

R_6 is selected from the group consisting of an amino acid, peptide consisting of up to 10 amino acids, C_{1-6} alkyl, -$(CH_2)_p$-, -$(CH_2CH_2O)_m$-, -C(O)NH-, -NH(4-phenyl)CH_2-, -Val-Cit-NH(4-phenyl)CH_2-, -Val-Ala-NH(4-phenyl)CH_2-, -Ala-Ala-Asn-NH(4-phenyl)CH_2-, and combinations thereof;

R_7 is C_{2-6} alkylene, or -$(CH_2CH_2O)_m$-;

R_8 is selected from the group consisting of an amino acid, peptide consisting of up to 10 amino acids, C_{1-6} alkyl, C_{1-6} alkylene, substituted C_{1-6} alkylene, -C(O)-NH-CHR_9-$CR_{10}R_{11}$-, -NHC(O)-CHR_9-$CR_{10}R_{11}$-, -$(CH_2CH_2O)_m$-, -PAB-, -Val-Cit-NH(4-phenyl)CH_2-, -Val-Ala-NH(4-phenyl)CH_2-, -Ala-Ala-Asn-NH(4-phenyl)CH_2-, and combinations thereof;

wherein R_9 is selected from the group consisting of hydrogen, C_{1-6} alkyl, C_{1-6} alkylene, -$(CH_2CH_2O)_m$-, -C(O)NH-, -NHC(O)-, -C(O)NH-(CH_2)p-SO_3H, -C(O)NH-(CH_2)p-CO_2H, -NHC(O)-(CH_2)p-SO_3H, -NHC(O)-(CH_2)p-CO_2H and combinations thereof;

R_{10} and R_{11} are each independently selected from the group consisting of hydrogen, C_{1-6} alkyl, and combinations thereof;

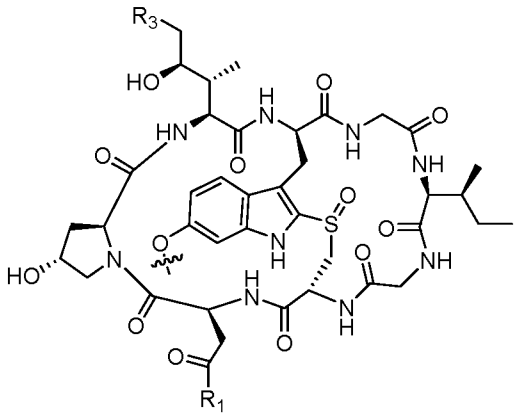
wherein -$R_6OC(O)NR_5$- is connected to $\mathbf{L^1}$ through R_6;

wherein -R_8-S-S-R_7- is connected to $\mathbf{L^1}$ through R_8;

m is an integer from 1-24; and

p is an integer from 1-6, wherein the remaining values are as described above for Formula I.

Preferably, D has a structure of Formula II:

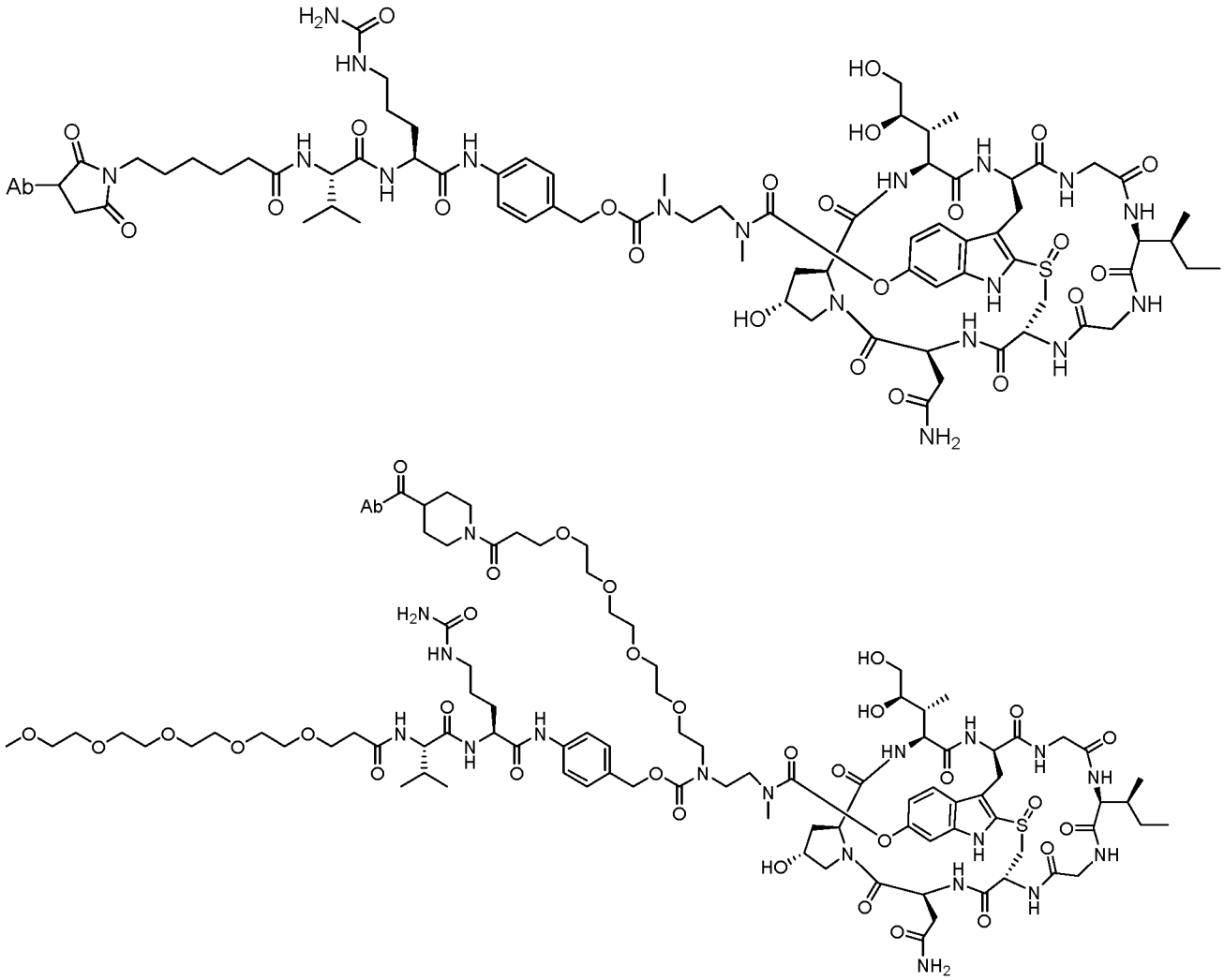


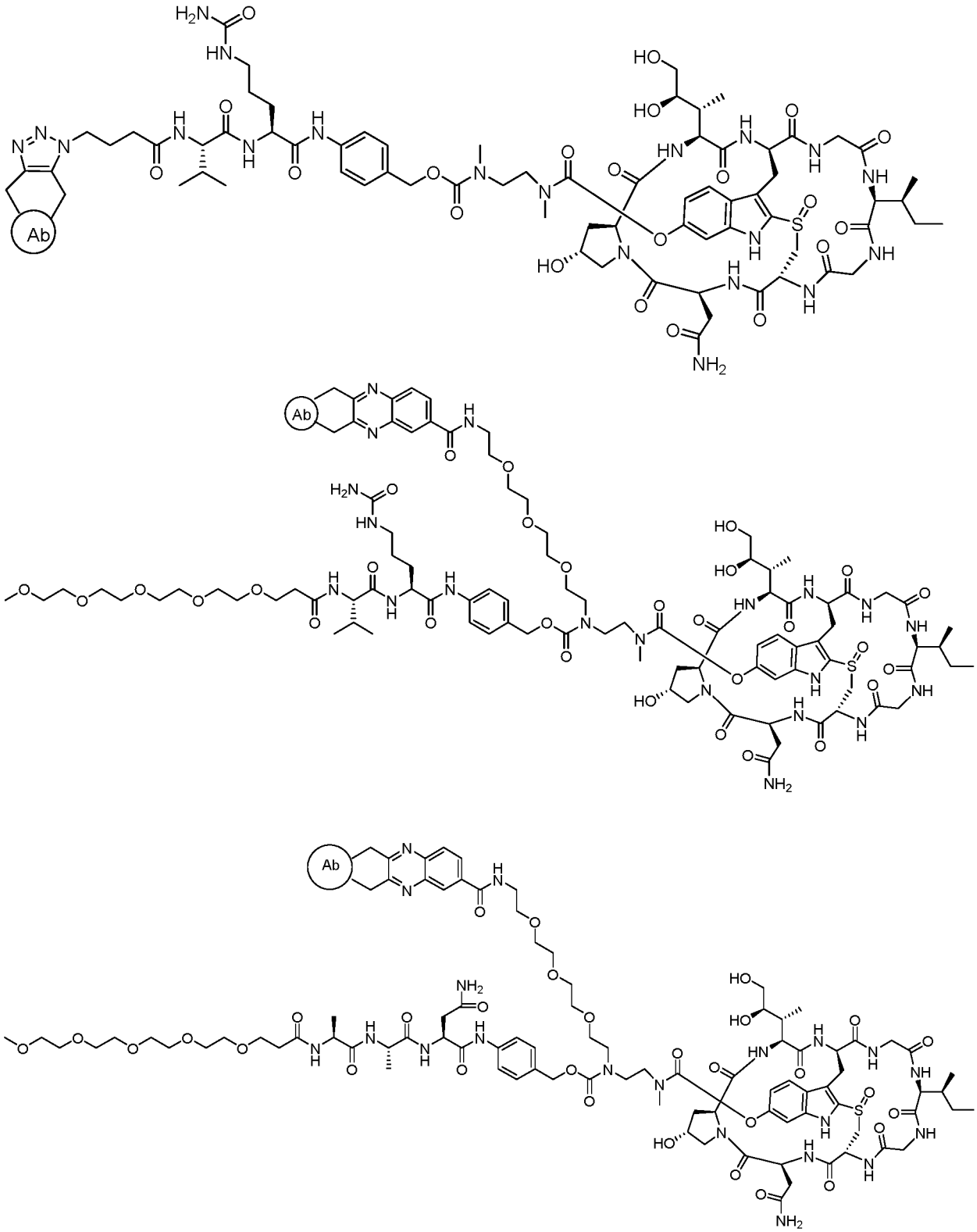
(II)

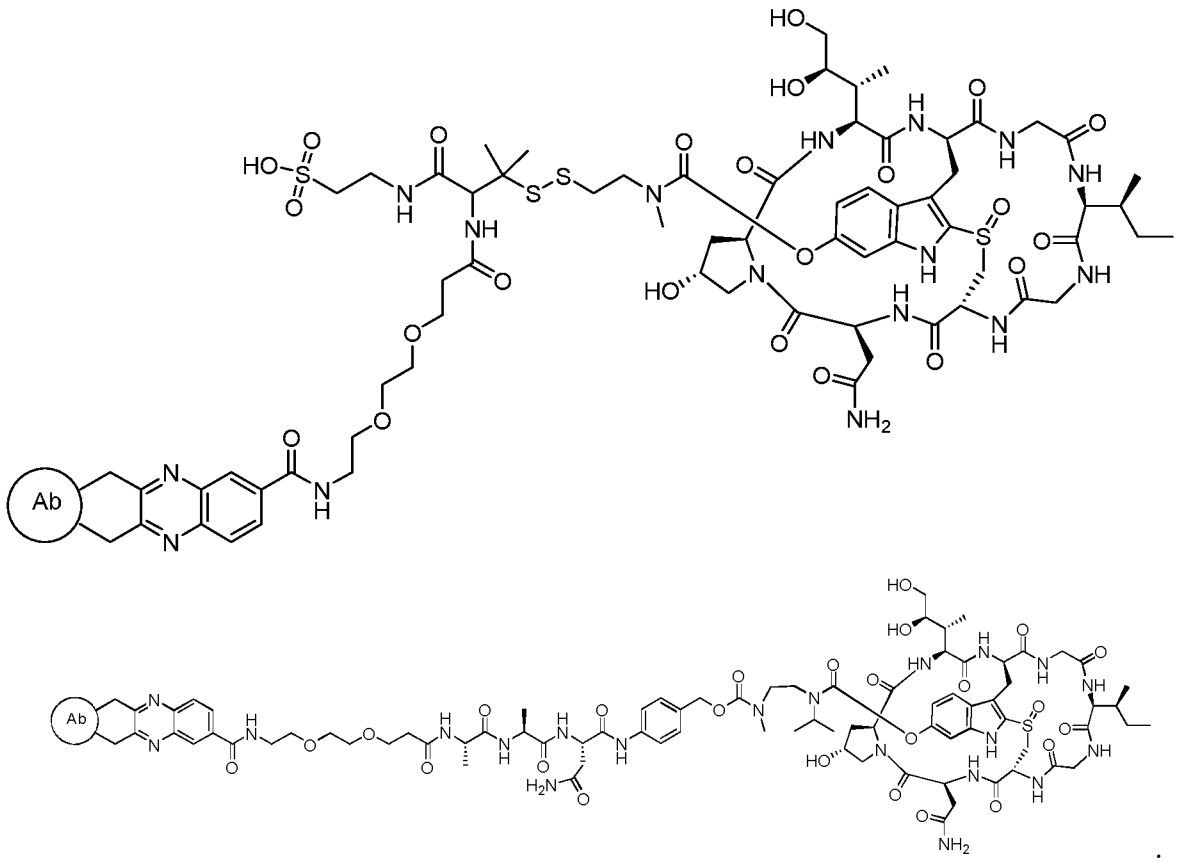
whereby the wavy line indicates the point of attachment to X;

wherein R_1 is NH_2 or OR_2, wherein R_2 is H, or C_1-C_{10} alkyl, and wherein R_3 is H or OH.

Preferably, the disclosed ADC is selected from the group consisting of:







Brief Description of the Figures

Figure 1 shows a comparison of in vitro cytotoxicity of ADC A (22) and ADC B on four cell lines, one cell line in each of the four panels of Figure 1.

Figure 2 shows *in vitro* cytotoxity of ADC24 (see Table 2).

Figure 3 shows *in vitro* cytotoxicity of ADC 22 (see Table 2) on various cell lines.

Figure 4 shows *in vitro* cytotoxicity of ADC 26 on various cell lines.

Figure 5 shows *in vitro* cytotoxicity of ADC 27 on various cell lines.

Figure 6 shows *in vitro* cytotoxicity of ADC 25 on various cell lines.

Figure 7 shows *in vitro* cytotoxicity of ADC 29 on various cell lines.

Figure 8 shows efficacy of cMet/EGFR-22, cMet-22 and Nimo-22 in H292 xenograft: cMet/EGFR-22 and Nimo-22 significantly inhibited H292 tumor growth compared to PBS control group.

Figure 9 shows a tumor size comparison for compound 29. cMet/EFFR-22 and Nimo-22 significantly reduced tumor size/Weight compared to PBS Control group. Nimo-22 had some complete tumor regression (4 out of 7 mice was tumor free).

Figure 10 shows no significant cMet/EGFR-22, cMet-22, Nimo-22 treatment-related body weight loss was observed.

Figure 11 shows cMet/EGFR-23, cMet-23 and Nimo-23 treated groups showed significantly reduced tumor volume compared to PBS Control group.

Figure 12 shows cMet/EGFR-23, cMet-23 and Nimo-23 treated groups showed significantly reduced tumor weight compared to PBS Control group.

Figure 13 shows that no body weight loss was observed in cMet-23, cMet/EGFR-23, and Nimo-23 treated group.

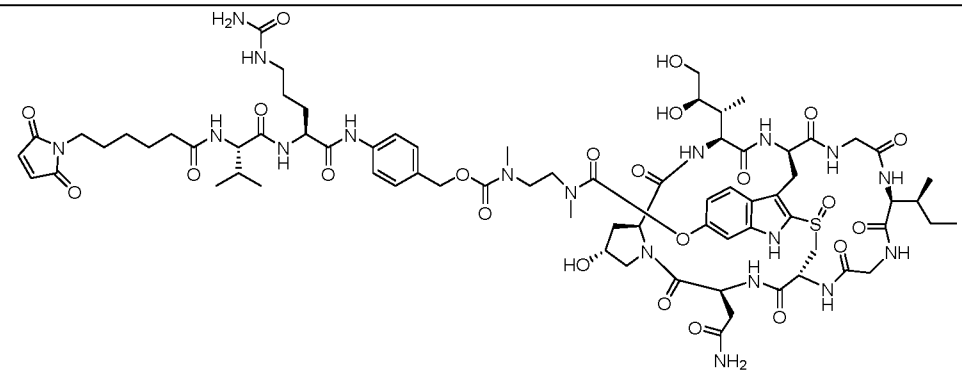
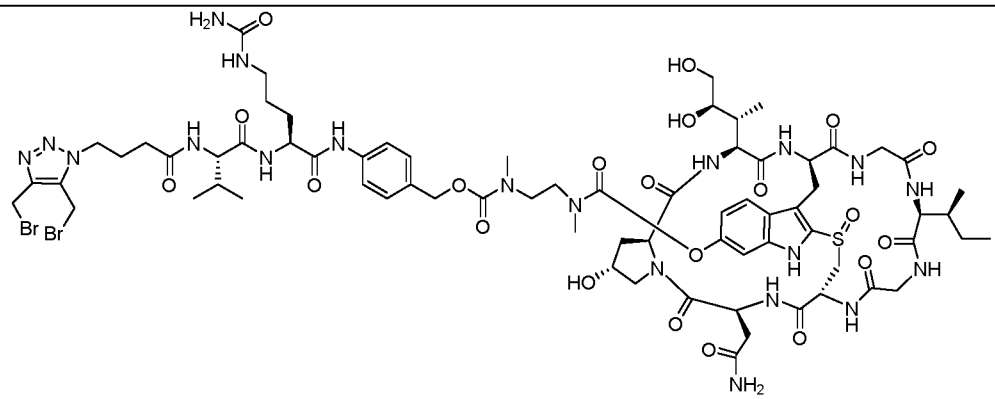
Figure 14 shows that a single dose of cMet/EGFR-25 at 3 mg/kg or 1 mg/kg had no significant tumor growth inhibition in H1975 xenograft.

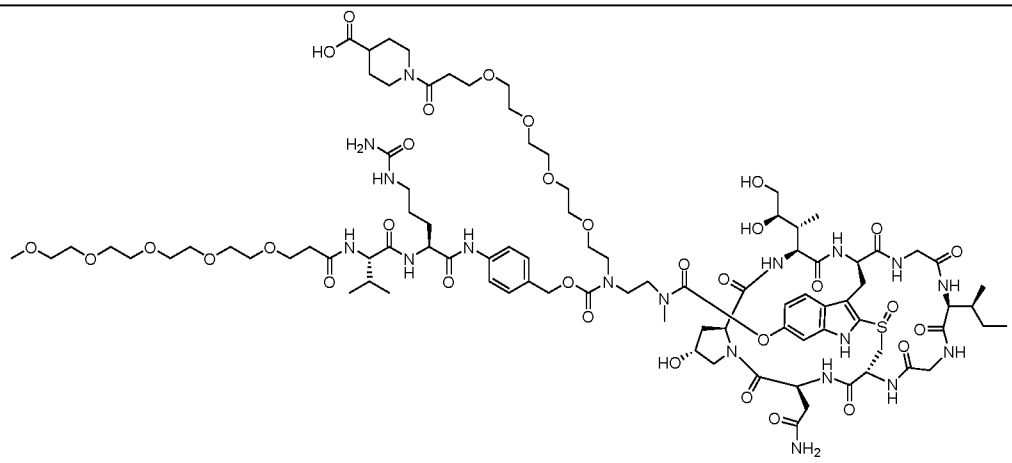
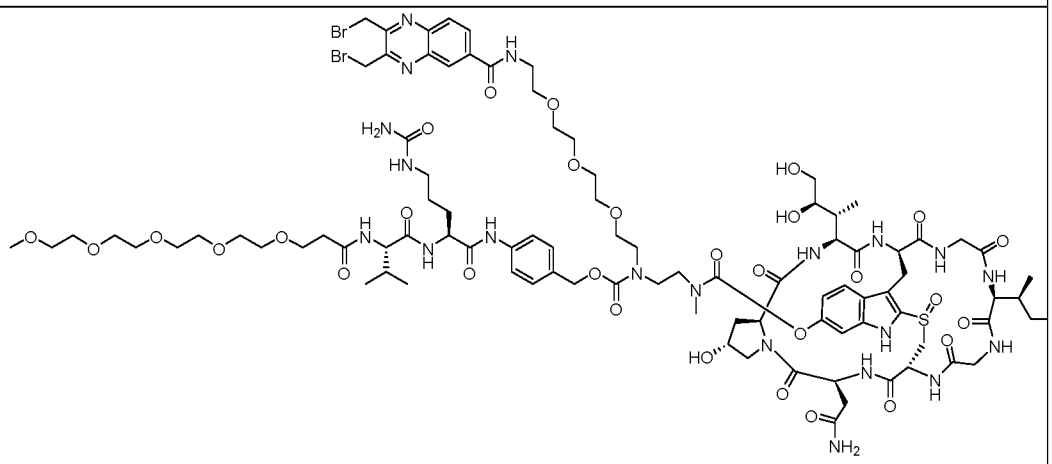
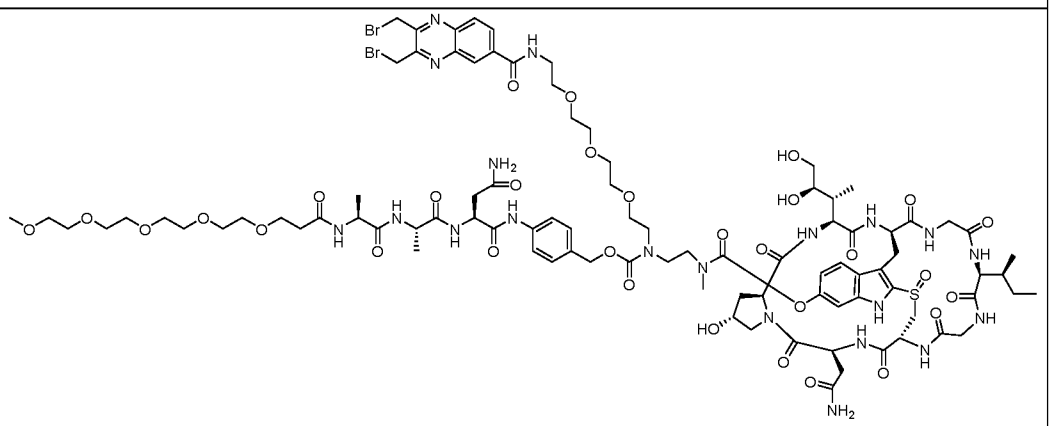
Figure 15 shows that a single dose of cMet/EGFR-27 at 3mg/kg or 1mg/kg, or a sigle dose of cMet-27 had no significant tumor growth inhibition in HCC827 xenograft.

Figure 16 shows that no significant body weight loss was observed with a single dose of cMet/EGFR-ADC27 at 3 mg/kg or 1 mg/kg, or a single dose of cMet-ADC27 at 0.3 mg/kg during the study.

Detailed Description

Table 1: Examples of compounds synthesized ("Ab" stands for antibody).

Compound #	Structure
6	
8	

10	
14	
17	

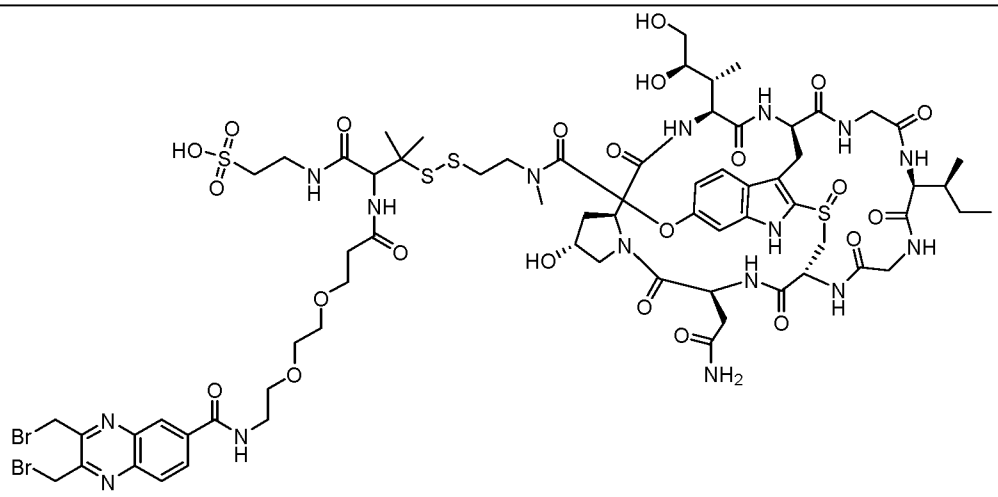
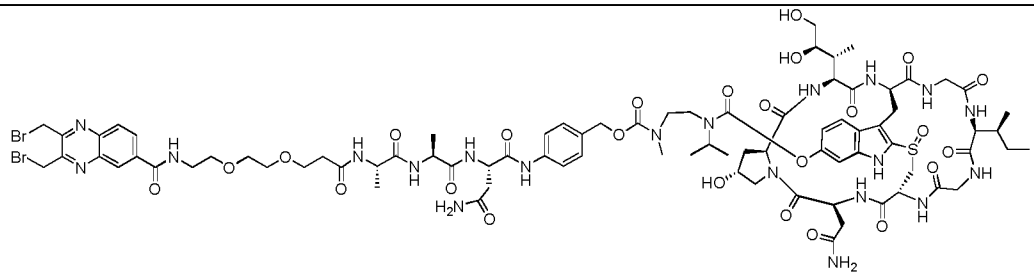
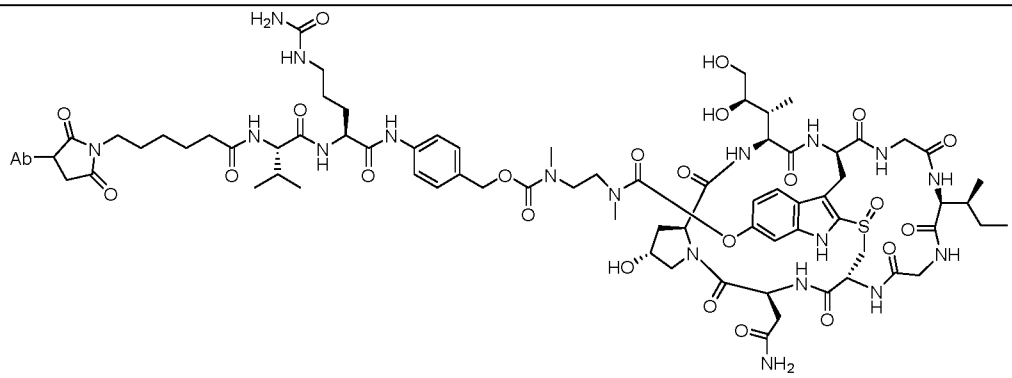
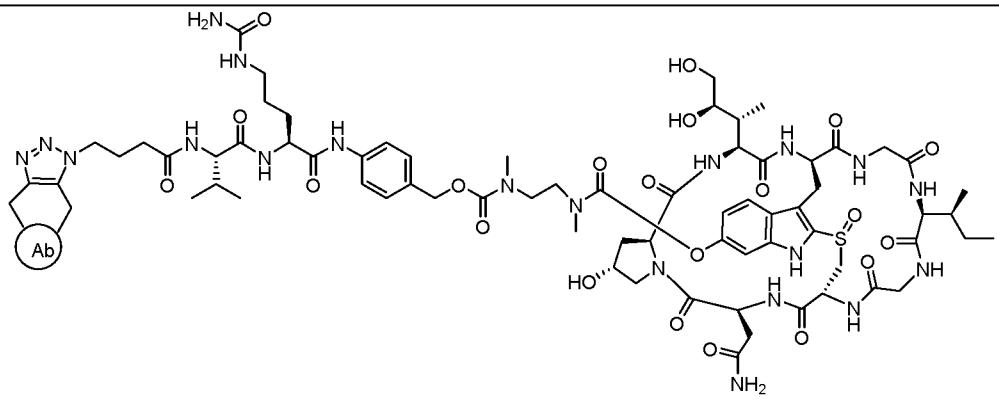
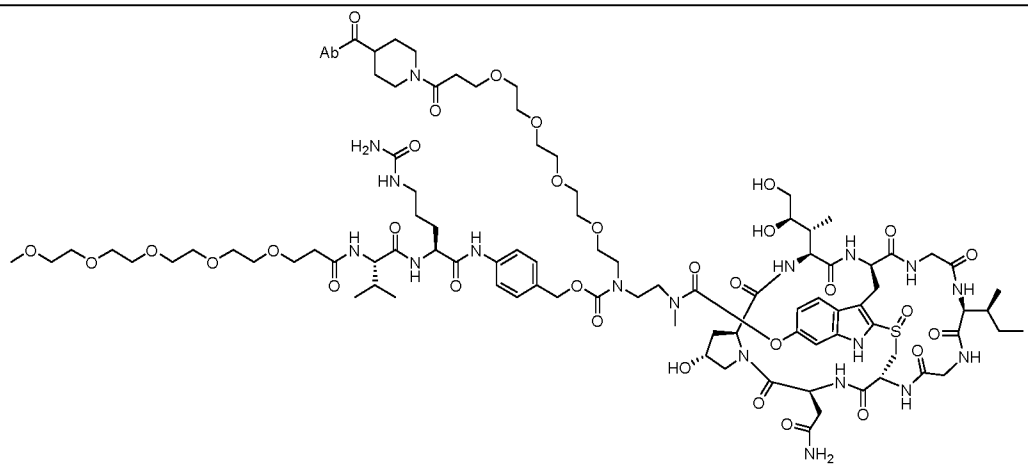
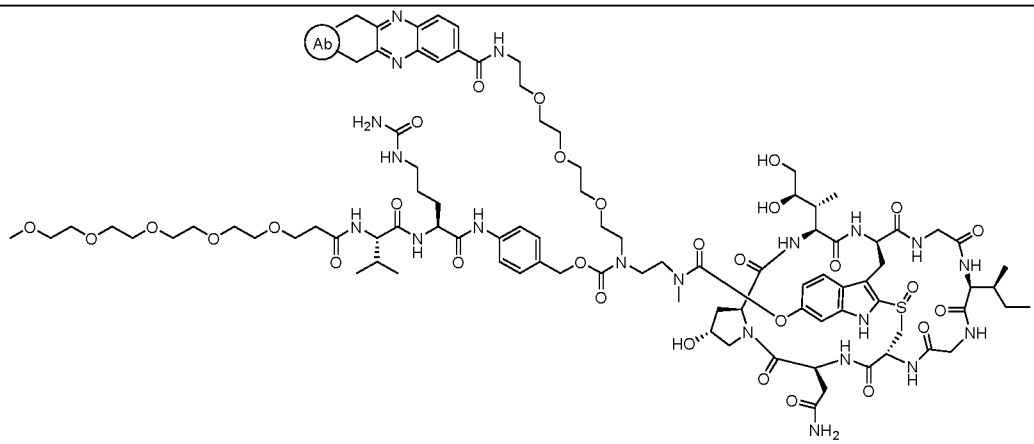
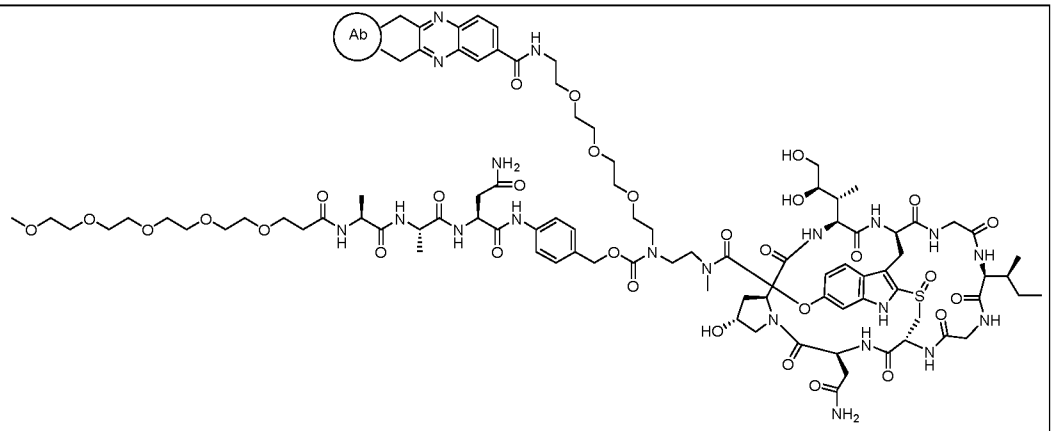
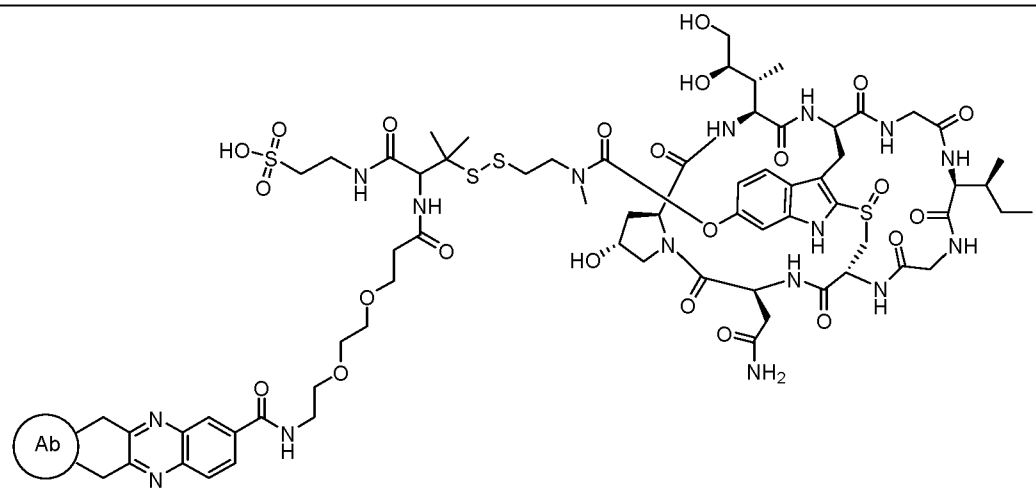
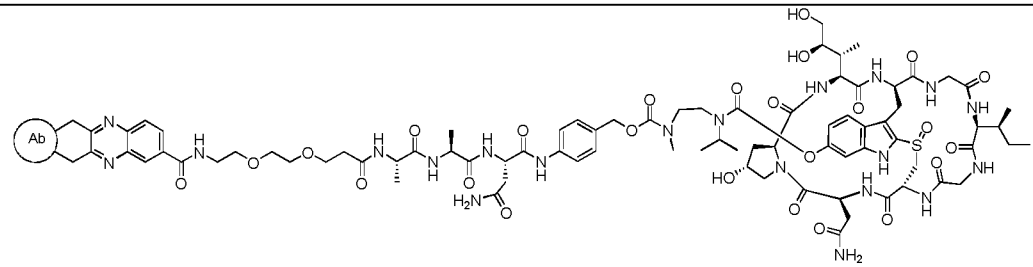
21	
28	

Table 2: Examples of antibody drug conjugates of Formula I

Compound #	**Structure**
22	

23	
24	
25	

26	
27	
29	

Definitions

As used herein, common organic abbreviations are defined as follows:

Ac	Acetyl
aq.	Aqueous
BOC or Boc	tert-Butoxycarbonyl
Bu	n-Butyl
°C	Temperature in degrees Centigrade
Cit	Citrulline
DCM	methylene chloride
DEPC	Diethylcyanophosphonate

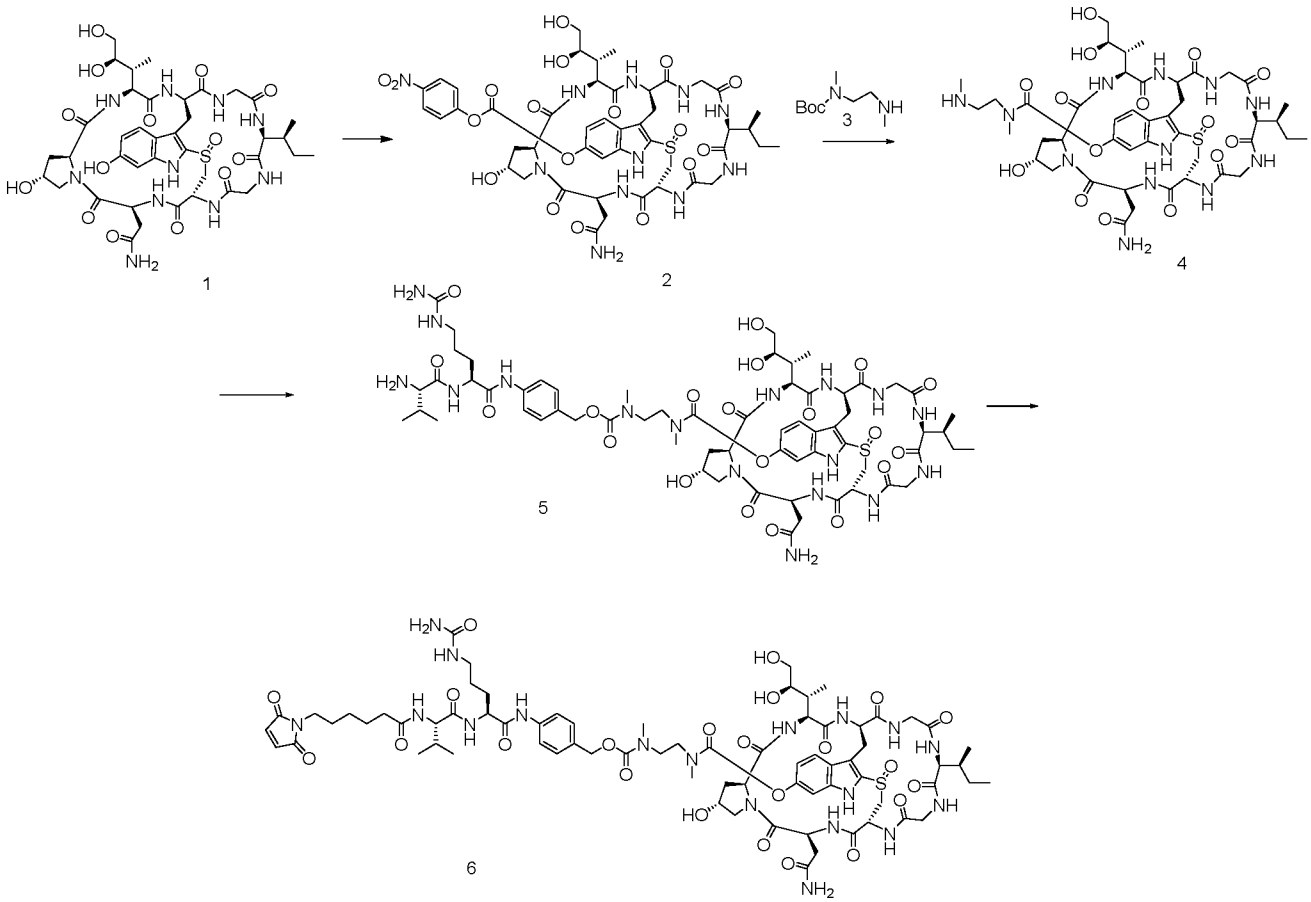
DIC	diisopropylcarbodiimide
DIEA	Diisopropylethylamine
DMA	*N*,*N*'-Dimethylacetamide
DMF	*N*,*N*'-Dimethylformamide
DMSO	Dimethylsulfoxide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et	Ethyl
EtOAc	Ethyl acetate
Eq	Equivalents
Fmoc	9-Fluorenylmethoxycarbonyl
g	Gram(s)
h	Hour (hours)
HATU	2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate
HOBT	N-Hydroxybenzotriazole
HOSu	N-Hydroxysuccinimide
HPLC	High-performance liquid chromatography
LC/MS	Liquid chromatography-mass spectrometry
Me	Methyl
MeOH	Methanol
MeCN	Acetonitrile
mL	Milliliter(s)
MS	mass spectrometry
PAB	p-aminobenzyl
RP-HPLC	reverse phase HPLC
rt	room temperature
t-Bu	tert-Butyl
TEA	Triethylamine
Tert, t	tertiary
TFA	Trifluoracetic acid
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
µL	Microliter(s)

Where used, a hyphen (-) designates the point to which a group is attached to the defined variable. A hyphen on the left side indicates connectivity to the left side structural component of formula (I) and hyphen on the right side indicates connectivity to the right side structural component of formula (I). For example, unless other specified when L_2 is defined as $-(CH_2CH_2O)_m-$, it means that the attachment to L^1 is at the $-CH_2$ carbon and the attachment to X is at the oxygen atom.

General synthesis procedure. – Formation of an activated ester (e.g. NHS) from an acid An acid was dissolved in DCM (methylene chloride) and DMF (N,N' dimethyl formamide) was added to aid dissolution if necessary. N-hydroxysuccinimide (1.5 eq) was added, followed by EDC.HCl (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) (1.5 eq). The reaction mixture was stirred at room temperature for 1 h until most of the acid was consumed. The progress of the reaction was monitored by RP-HPLC. The mixture was then diluted with DCM and washed successively with citric acid (aq. 10%) and brine. The organic layer was dried and concentrated to dryness. The crude product was optionally purified by RP-HPLC or silica gel column chromatography.

Example 1

Preparation of compound **6**



To a solution of alpha-amainitin **1** (46 mg, 50 μmol) in anhydrous dimethylsulfoxide (DMSO) (1 mL) was added bis (4-nitrophenol) carbonate (17 mg, 55 μmol), followed by diisopropylethylamine (DIEA, 10 μL). The mixture was stirred at room temperature for 30 minutes. Compound **3** (12 mg) was added, followed by DIEA (10 μL). LC/MS indicated all the compound **2** was consumed after 1 h. All the solvents were removed under reduced the pressure and the residue was treated with trifluoroacetic acid (TFA) in dichloromethane

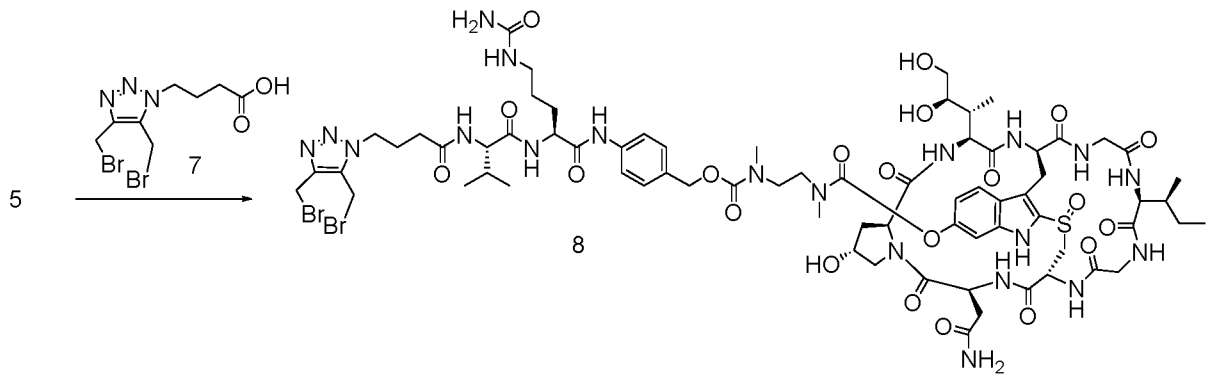
(DCM) (20%, v/v, 2 mL). The reaction mixture was concentrated after 30 min and the residue was purified by reverse phase HPLC to give compound **4** as a white solid in TFA salt form after lyophilization (45 mg, 78%). MS: m/z 1033.4 ($M+H^+$).

Compound **4** (45 mg) was dissolved in anhydrous dimethylformamide (DMF, 1 mL) and 9-Fluorenylmethyloxycarbonyl-valyl-citrullyl-(4-aminobenzyl)-(4-nitrophenyl)carbonate (Fmoc-Val-Cit-PAB-PNP, 38 mg) was added, followed by DIEA (20 μL). The mixture was stirred at room temperature for 2 h. LC/MS analysis indicated the completion of reaction. Piperidine (50 μL) was added and after 2 h, the reaction mixture was neutralized by addition of acetic acid (200 μL). The crude mixture was purified directly by reverse phase HPLC to give compound **5** as a white solid in TFA salt form after lyophilization (48 mg, 80%). MS: m/z 1438.7 ($M+H^+$).

To a stirred solution of compound **5** (16 mg, 10 μmol) in DMF (1 mL) was added N-ε-Maleimidocaproyl oxysuccinimide ester (4 mg), followed by DIEA (4 μL). The mixture was stirred at room temperature for 2 h. The crude reaction mixture was injected to a Prep HPLC column for purification. Compound **6** was obtained a white solid after lyophilization. (12 mg). MS: m/z 1631.8 ($M+H^+$).

Example 2

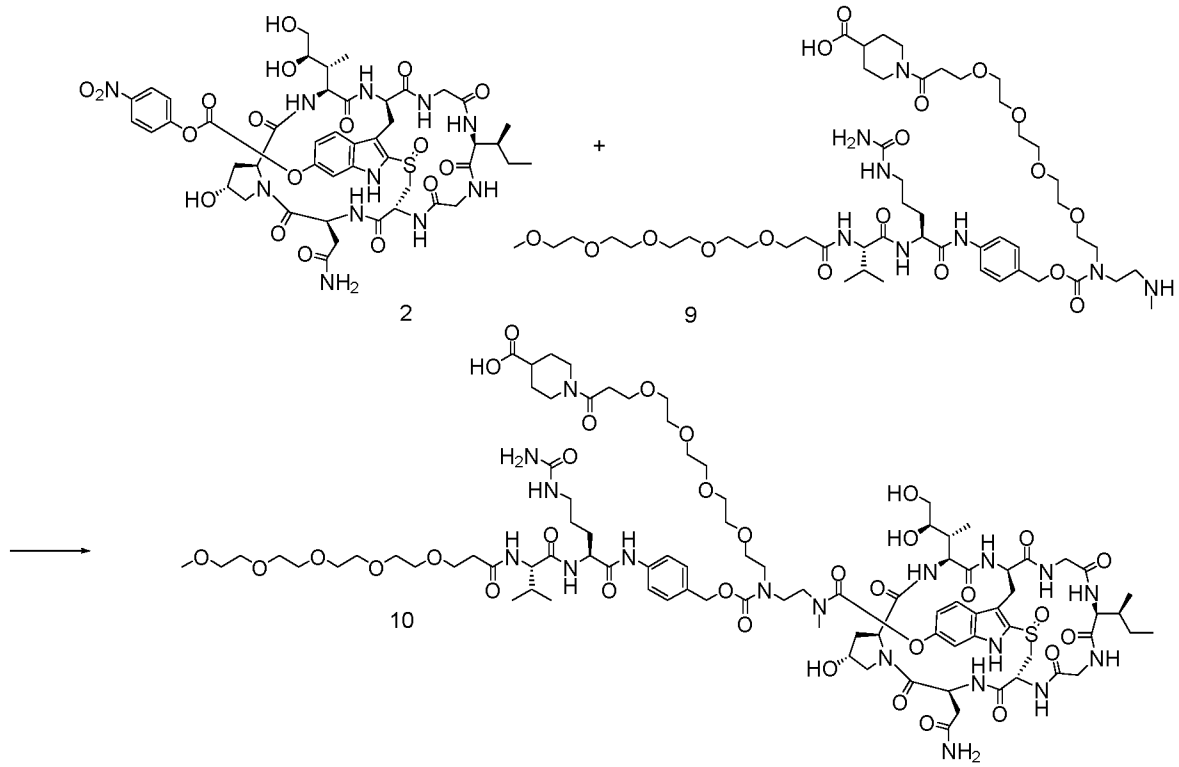
Preparation of compound **8:**



To a stirred solution of compound **5** (16 mg, 10 μmol) in DMF (1 mL) was added acid **7** (6 mg), followed by diisopropylcarbodiimide (5 μL). The mixture was stirred at room temperature for 2 h. The crude reaction mixture was injected to a Prep HPLC column for purification. Compound **8** was obtained a white solid after lyophilization. (8 mg). MS: m/z 1761.8 ($M+H^+$).

Example 3

Preparation of compound **10:**

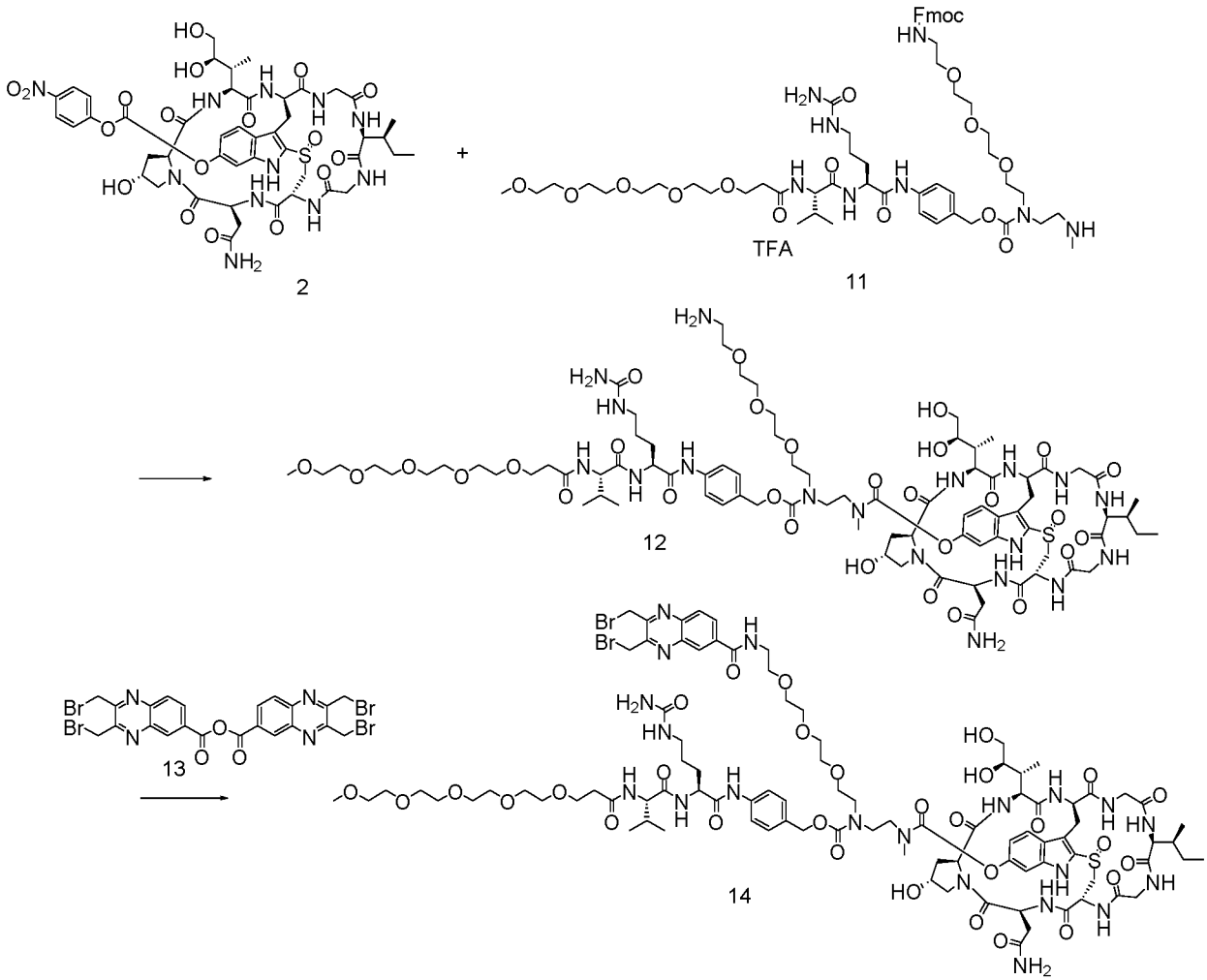


To a stirred solution of compound **2** (30 μmol) in DMSO (1 mL) was added amine **9** (40 mg), followed by DIEA (15 μL). The mixture was stirred at room temperature for 16 h. The crude reaction mixture was injected to a Prep HPLC column for purification. Compound **10** was obtained a white solid after lyophilization. (32 mg). MS: m/z 2046.2 ($M+H^+$).

Compound **10** was converted to the corresponding activated ester following a general procedure prior to conjugating to an antibody.

Example 4

Preparation of compound **14:**



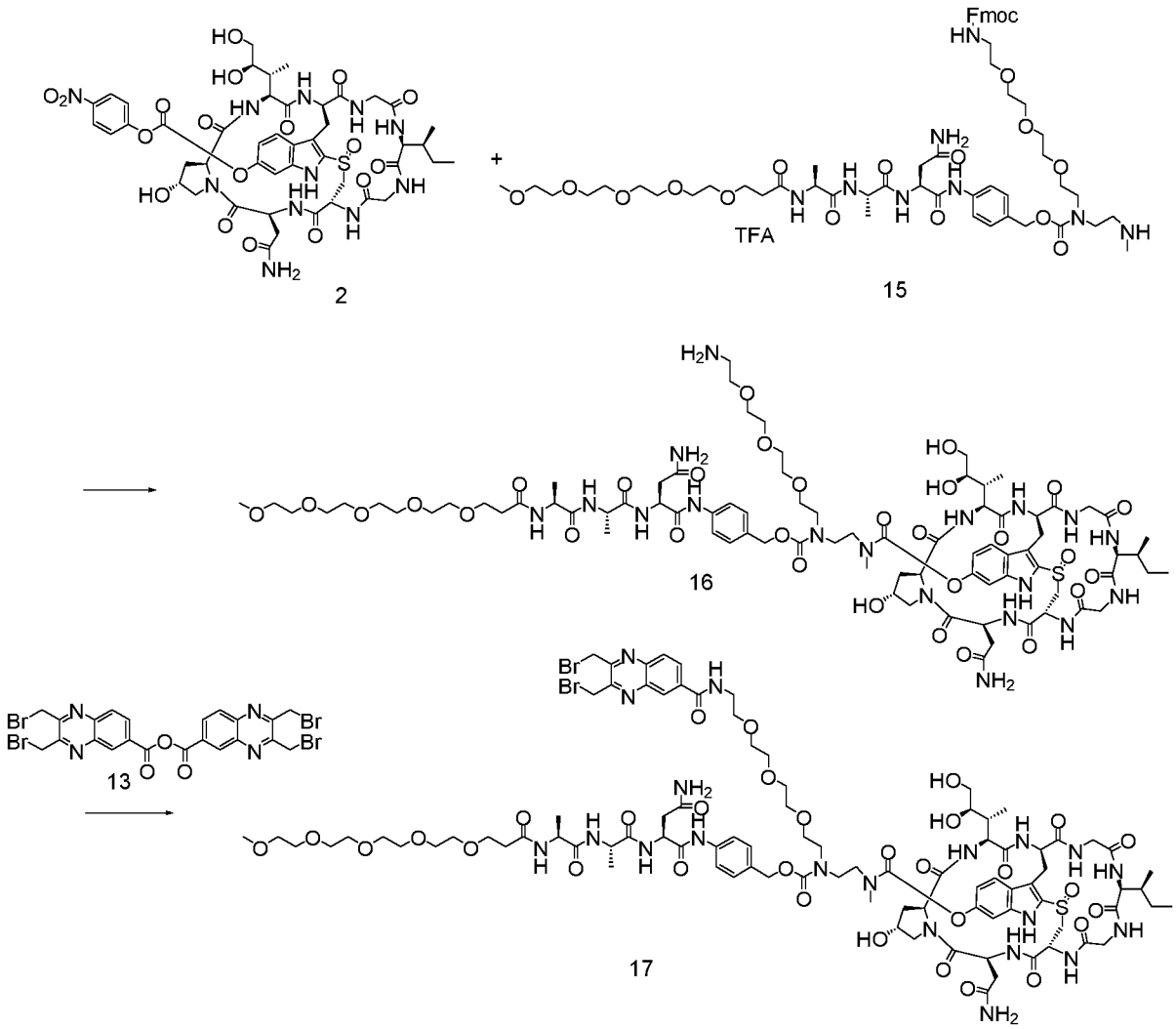
.

To a stirred solution of compound **2** (50 μmol) in DMSO (1 mL) was added amine **11** (65 mg) in DMF (1 mL), followed by DIEA (20 μL). The mixture was stirred at room temperature for 16 h. Piperidine (100 μL) was added. After 30 mins, the mixture was purified directly by reverse phase HPLC to give compound **12** in TFA salt form as a white solid (54 mg). MS: m/z 1862.1 ($M+H^+$).

Compound **12** (20 mg) was dissolved in DMF (1 mL). Anhydride **13** (11 mg) was added, followed by DIEA (5 μL). The reaction mixture was stirred at room temperature for 5 minutes and purified by reverse phase HPLC to give compound **14** as a white solid after lyophilization (19 mg). MS: m/z 2203.9 ($M+H^+$).

Example 5

Preparation of compound **17:**

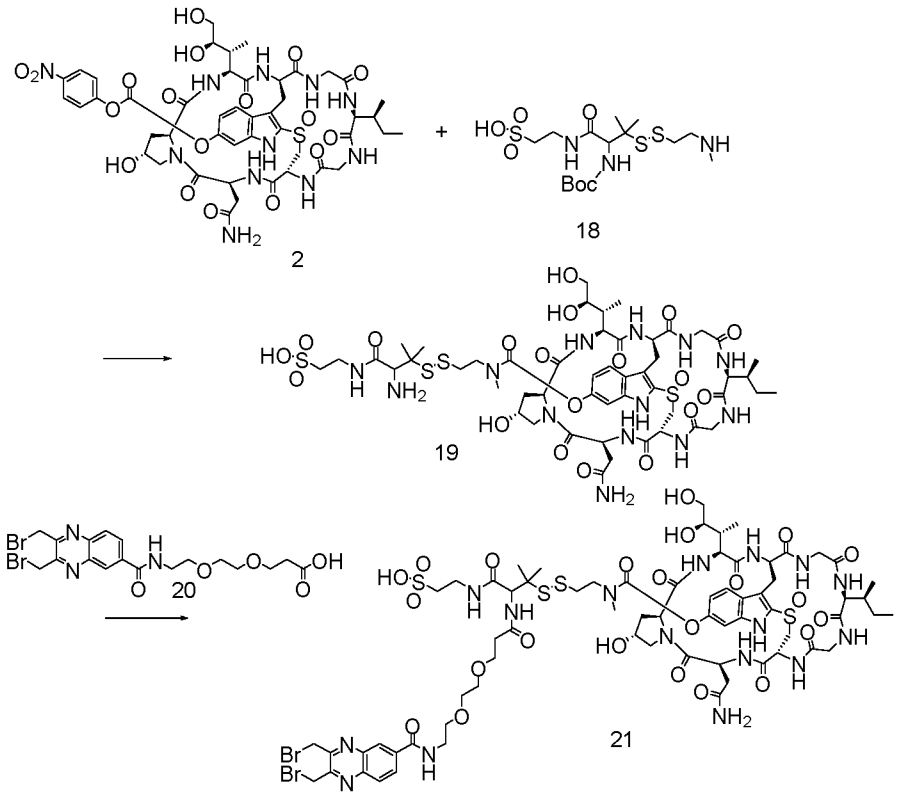


To a stirred solution of compound **2** (50 μmol) in DMSO (1 mL) was added amine **15** (65 mg) in DMF (1 mL), followed by DIEA (20 μL). The mixture was stirred at room temperature for 16 h. Piperidine (100 μL) was added. After 30 mins, the mixture was purified directly by reverse phase HPLC to give compound **16** in TFA salt form as a white solid (49 mg). MS: m/z 1862.3 ($M+H^+$).

Compound **16** (20 mg) was dissolved in DMF (1 mL). Anhydride **13** (11 mg) was added, followed by DIEA (5 μL). The reaction mixture was stirred at room temperature for 5 minutes and purified by reverse phase HPLC to give compound **17** as a white solid after lyophilization (20 mg). MS: m/z 2204.1 ($M+H^+$).

Example 6

Preparation of compound **21:**

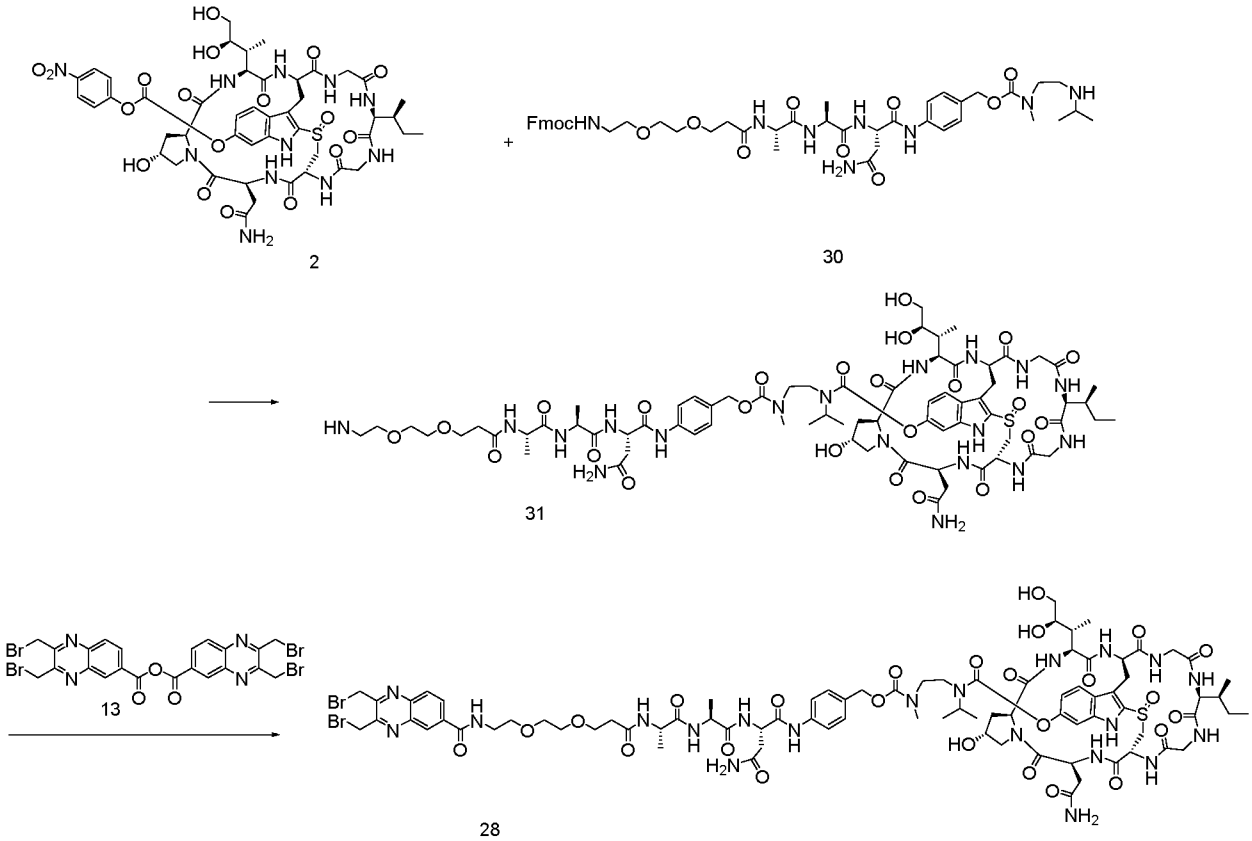


To a stirred solution of compound **2** (50 μmol) in DMSO (1 mL) was added amine **15** (25 mg) in DMF (1 mL), followed by DIEA (20 μL). The mixture was stirred at room temperature for 5 h. The solvents were removed under reduced pressure and the residue was dissolved in 20% TFA/DCM (2 mL). After 30 mins, the mixture was purified directly by reverse phase HPLC to give compound **19** as a white solid (31 mg). MS: m/z 1309.5 ($M+NH_4^+$).

To a stirred solution of compound **19** (25 mg, 20 μmol) in DMF (1 mL) was added acid **20** (16 mg), followed by diisopropylcarbodiimide (8 μL). The mixture was stirred at room temperature for 2 h. The crude reaction mixture was injected to a Prep HPLC column for purification. Compound **21** was obtained a white solid after lyophilization. (12 mg). MS: m/z 1791.4 ($M+H^+$).

Example 7

Preparation of compound **28:**

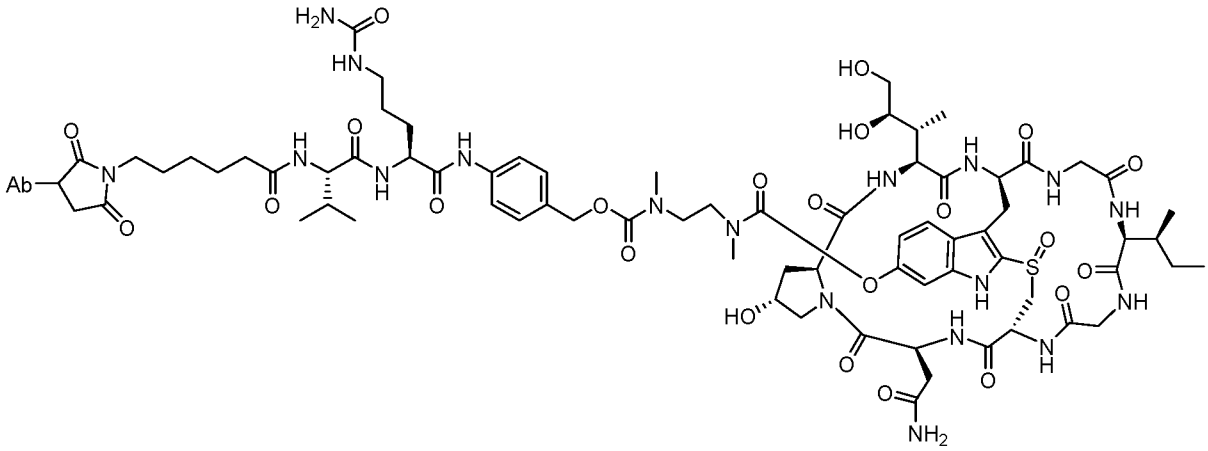


To a stirred solution of compound **2** (50 μmol) in DMSO (1 mL) was added amine **30** (46 mg, 50 μmol) in DMF (1 mL), followed by DIEA (20 μL). The mixture was stirred at room temperature for 16 h. Piperidine (100 μL) was added. After 30 mins, the mixture was purified directly by reverse phase HPLC to give compound **31** in TFA salt form as a white solid (25 mg). MS: m/z 1640.5 ($M+H^+$).

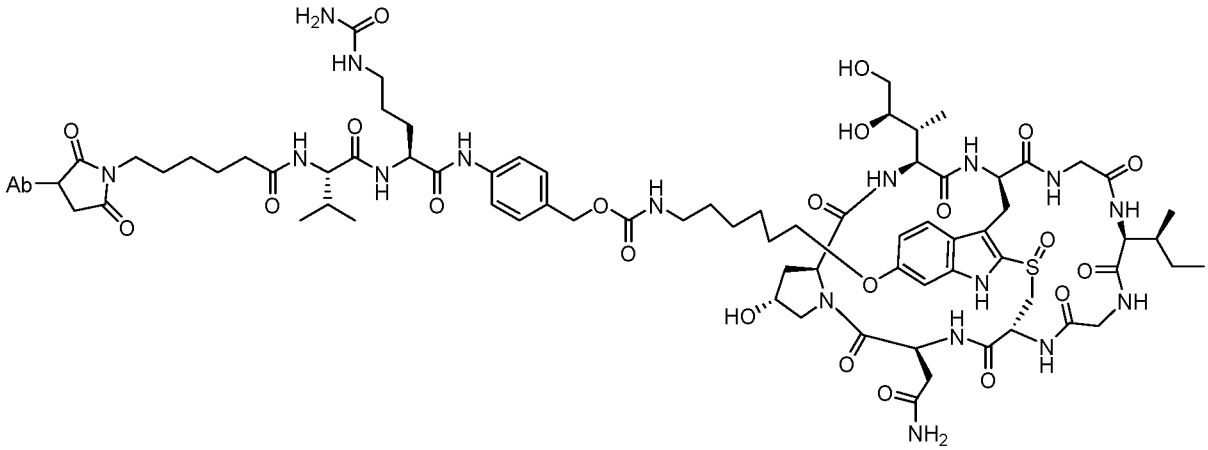
Compound **31** (20 mg, 11.4 μmol) was dissolved in DMF (1 mL). Anhydride **13** (8 mg) was added, followed by DIEA (5 μL). The reaction mixture was stirred at room temperature for 5 minutes and purified by reverse phase HPLC to give compound **28** as a white solid after lyophilization (16 mg). MS: m/z 1981.9 ($M+H^+$).

Example 8

This example provides a comparative study, comparing two different amatinin conjugates shown as "A" and "B" below.



Amanitin antibody conjugate Structure A (ADC **22**)



Amanitin antibody conjugate Structure B

A comparative study was carried out to evaluate the efficacy of amanitin antibody conjugate structure A wherein alpha-amaintin was attached to the linker via a cleavable carbamate bond (reported in this disclosure) and amanitin antibody conjugate structure B wherein alpha amanitin was attached through a non-cleavable ether bond (reported in WO2012/041504) in various *in vitro* cell killing assays (Figure 1 four panels for four different cell lines. ADC A completely outperformed ADC B in all 4 Her-2 positive cell lines tested.

Example 9

This example provides the results of EC50 assays (nM) of the designated drug conjugated antibodies measured *in vitro* in specified cells. The antibody used was an anti-

HER2 IgG class of antibody. Seven breast cancer cell lines with various level of Her2 expression as indicated with plus or minus signs in the table below were plated in 96 well plate. The ADCs were serial diluted and added onto cells for treatment for 5 days. At the end of the study, cell proliferation was measured by Promega's CellTitreGlo. EC50 (in nM) was determined as the concentration of 50% cell growth inhibition. The selection criteria for a successful compound included high efficacy, such as killing cell lines with high expression of the target receptor, with EC50 less than 3 nM. Also, the successful candidate should have low toxicity and good therapeutic window, as determined by relatively low killing of the control cell line (MDA468) with low expression of the target receptor. Both ADCs **22** (Figure 3) and **24** (Figure 2) were selected as successful candidates with high efficacy and good therapeutic window.

Example 10

This example provides the results of EC50 assays (nM) of designated ADCs described herein measured *in vitro* in specified cells. The antibody used targets a receptor tyrosine kinase on cell surface. Eight cancer cell lines with various level of receptor expression, as indicated with plus or minus signs in the table below, were plated in 96 well plate. The ADCs were serial diluted and added onto cells for treatment for 5 days. At the end of the study, cell proliferation was measured by Promega's CellTitreGlo. EC50 (in nM) was shown below and determined as the concentration of 50% cell growth inhibition. The selection criteria for a successful compound includes high efficacy, such as killing cell lines with high expression of the target receptor, with EC50 less than 3 nM. Also, the successful candidate should have low toxicity and good therapeutic window, as determined by relatively low killing of the control cell lines (T-47D) with low expression of the target receptor. ADC 25 (Figure 6) shows good cell killing efficacy in cell lines H1993, HCC827, SNU-5, and H292, but did not show efficacy in Hs746T, EBC-1 and U 87. It showed good therapeutic window since it did not kill the negative control cell line T-47 D. ADC 26 (Figure 4) shows good cell killing activity in H1993 and SNu-5. However, it is not active in the other 6 cell lines. ADC 27 (Figure 5) shows excellent cell killing activity in H1993 (EC50 = 11 pM) and SNu-5 (EC50 = 75 pM). It also shows good efficacy in Hs746T (EC 50 = 0.4 nM). ADC 29 (Figure 7) shows good cell killing efficacy in cell lines Hs746T, but did not show efficacy in EBC-1, U87, HCC827, H1993 and T-47.

Example 11

This example provides the results for the efficacy of ADCs conjugated with small molecule 22, 23, 25, or 27 in a model of H292, HCC827, and H1975 Human Xenograft

Tumor Growth in Nude Mice. HCC827, H292, H1975 cell lines were obtained from ATCC. The cells were cultured in RPMI 1640 1X (Corning 10-041-CV) medium with 10% FBS (Seradigm 1500-500) and penicillin streptomycin (Corning 30-002-CI) at 37 °C in a 5% carbon dioxide humidified environment. Cells were cultured for a period of 2 weeks and passaged 4 times before harvest. The cells were harvested with 0.25% trypsin (Corning 25-050-CI). Prior to injection, HCC827 cells were mixed in a 1:1 ratio of HBSS (Hank's balanced salt solution; Ward's 470180-784) and matrigel (Corning 354234) mixture, and 7 million cells per 0.2 ml were injected subcutaneously into the upper right flank of each mouse. H292 cells were resuspended in HBSS, and 5 million cells per 0.2 ml were injected subcutaneously into the upper right flank of each mouse. H1975 cells were resuspended in HBSS, and 3 million cells per 0.2 ml were injected subcutaneously into the upper right flank of each mouse.

Female Nu/Nu mice aged 5-7 weeks (Charles River) were used throughout these studies.

Upon receipt, mice were housed 5 mice per cage in a room with a controlled environment. Rodent chow and water was provided *ad libitum*. Mice were acclimated to laboratory conditions for 72 hours before the start of dosing. The animals' health status was monitored during the acclimation period. Each cage was identified by group number and study number, and mice were identified individually by ear tags.

The study design and dosing regimens are shown in Table 3.

Table 3

Tumor model	**Groups**	**Animals per Group**		**Treatment volume/route**	**Dose / Frequency**
H292	1	7	PBS	200 µl/i.v.	0 mg/kg, single dose
	2	7	*cMet/EGFR-22*	200 µl/i.v.	3 mg/kg, single dose
	3	7	*cMet-22*	200 µl/i.v.	3 mg/kg, single dose
	4	7	*Nimo-22*	200 µl/i.v.	3 mg/kg, single dose
HCC827	1	7	PBS	200 µl/i.v.	0 mg/kg, single dose
	2	7	*cMet/EGFR-23*	200 µl/i.v.	3 mg/kg, single dose
	3	7	*cMet-23*	200 µl/i.v.	3 mg/kg, single dose

	4	7	*Nimo-23*	200 µl/i.v.	3 mg/kg, single dose
H1975	1	8	*PBS*	200 µl/i.v.	0 mg/kg, single dose
	2	8	*cMet/EGFR-25*	200 µl/i.v.	1 mg/kg, single dose
	3	8	*cMet/EGFR-25*	200 µl/i.v.	3 mg/kg, single dose
HCC827	1	8	*PBS*	200 µl/i.v.	0 mg/kg, single dose
	2	8	*cMet-27*	200 µl/i.v.	0.3 mg/kg, single dose
	3	8	cMet/EGFR-27	200 µl/i.v.	1 mg/kg, single dose
	4	8	cMet/EGFR-27	200 µl/i.v.	3 mg/kg, single dose

Tumor growth was monitored by measurement of tumor width and length using a digital caliper starting day 5-7 after inoculation, and followed twice per week until tumor volume reached ~100-250 mm^3. Tumor volume was calculated using the formula: Volume (mm^3) = [Length (mm) x Width $(mm)^2$]/ 2. Once tumors were staged to the desired volume, animals were randomized, and mice with very large or small tumors were culled. Mice were divided into groups with animal numbers per group as indicated in study design. Mice were then treated intravenously (0.2 ml/animal) with either PBS or antibody conjugated with 22, 23, 25, or 27 as dose indicated in study design. Tumor growth was monitored, and each group of mice was sacrificed when the average tumor load for the control group exceeded 2000 mm^3.

Tumor volume was measured twice weekly throughout the experimental period to determine TGI (tumor growth inhibition %). The body weight of each mouse was measured twice weekly by electric balance. Group average and standard deviation were calculated, and statistical analyses (one-way ANOVA with Dunnett's multiple comparison test; GraphPad Prism 6.0) was carried out. All treatment groups were compared with the PBS group. $P<0.05$ was considered statistically significant.

A single dose of cMet/EGFR-22 and Nimo-22 treatment at 3mg/kg significantly inhibited H292 tumor growth when compared to PBS treated control group. While cMet-22 inhibited tumor growth in the first 10 days after treatment, tumor regained growth after 10 days (Figures 8 and 9). In this study, a single dose of cMet/EGFR-22 and cMet-22 at 3 mg/kg showed skin rash at 3-6 days after treatment, and dry, flaky skin between day 6 to 14. Those

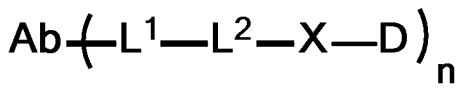
skin issues recovered after day 14. There was no significant treatment-related body weight loss observed during the study. (Figure 10). Although there was body weight loss during the first week in cMet/EGFR-22 treated group, the weight loss was transient and less than 10% of total body weight. Also, the animals regained weight and was healthier overall compared to PBS treated control group

A single dose of cMet/EGFR-23, cMet-23, or Nimo-23 treatment at 3 mg/kg each significantly inhibited H292 tumor growth when compared to PBS treated control group (Figures 11 and 12). No body weight loss was observed in cMet-23, cMet/EGFR-23, and Nimo-23 treated group (3 mg/kg) (Figure 13).

A single dose of cMet/EGFR-25 at 3 mg/kg or 1 mg/kg had no significant tumor growth inhibition in H1975 xenograft (Figure 14). A single dose of cMet/EGFR-27 at 3 mg/kg or 1 mg/kg, or a single dose of cMet-27 at 0.3 mg/kg had no significant tumor growth inhibition in HCC827 xenograft (Figure 15). No significant body weight loss was observed during the study (Figure 16).

We claim:

1. An antibody drug conjugate (ADC) having the structure of Formula I



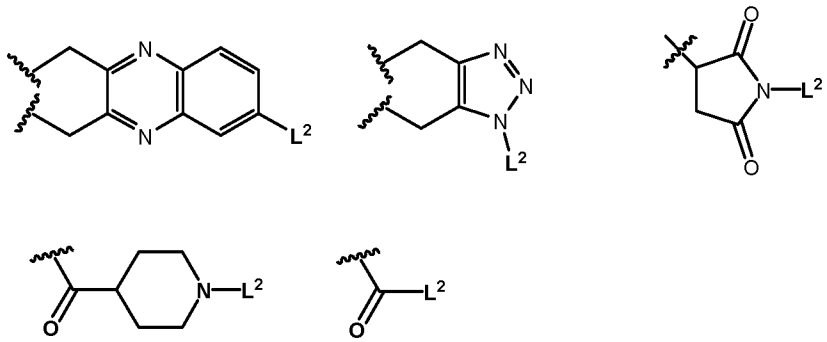
(I)

or a pharmaceutically acceptable salt thereof,

wherein:

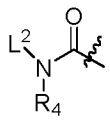
Ab is a monoclonal antibody;

$\mathbf{L^1}$ - $\mathbf{L^2}$ is a linker selected from the group consisting of



whereby the wavy line indicates the point of attachment to Ab;

$\mathbf{L^2}$ - **X** has a structure of



wherein R_4 is hydrogen, $C_{1\text{-}6}$ alkyl, or $-(CH_2CH_2O)_m-$, wherein m is an integer from 1-24, whereby the wavy line indicates the point of attachment to **D**;

$\mathbf{L^2}$ is a linker selected from the group consisting of a single amino acid, a peptide having a length of 2-10 amino acids, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, -C(O)NH-, -NHC(O)-, PAB (p-aminobenzyl), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, $-R_6OC(O)NR_5-$, $-R_8-S-S-R_7$, or combinations thereof, wherein R_5 is selected from the group consisting of hydrogen, $C_{1\text{-}6}$ alkyl, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, or combinations thereof; R_6 is selected from the group consisting of an amino acid, peptide consisting of up to 10 amino acids, $C_{1\text{-}6}$ alkyl, $-(CH_2)_p-$, $-(CH_2CH_2O)_m-$, -C(O)NH-, -NHC(O)-, PAB, Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, or combinations thereof; R_7 is selected from the group consisting of $C_{2\text{-}6}$ alkylene, $-(CH_2CH_2O)_m-$; R_8 is selected from the group consisting of an amino acid, peptide consisting of up to 10 amino acids, $C_{1\text{-}6}$ alkyl, $C_{1\text{-}6}$ alkylene, substituted $C_{1\text{-}6}$ alkylene, -C(O)NH-, -C(O)-

NH-CHR$_9$-CR$_{10}$R$_{11}$-, -NHC(O)-CHR$_9$-CR$_{10}$R$_{11}$-, -(CH$_2$CH$_2$O)$_m$-, PAB, Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, or combinations thereof;

wherein R$_9$ is selected from the group consisting of hydrogen, C$_{1-6}$ alkyl, C$_{1-6}$ alkylene, -(CH$_2$CH$_2$O)$_m$-, -C(O)NH-, -NHC(O)-, -C(O)NH-(CH$_2$)p-SO$_3$H, C(O)NH-(CH$_2$)p-CO$_2$H, -NHC(O)-(CH$_2$)p-SO$_3$H, -NHC(O)-(CH$_2$)p-CO$_2$H or combinations thereof;

R$_{10}$ and R$_{11}$ each independently selected from the group consisting of hydrogen, C$_{1-6}$ alkyl, or combinations thereof;

wherein -R$_6$OC(O)NR$_5$- is connected to **L^1** through R$_5$ or R$_6$;

wherein -R$_8$-S-S-R$_7$- is connected to **L^1** through R$_8$;

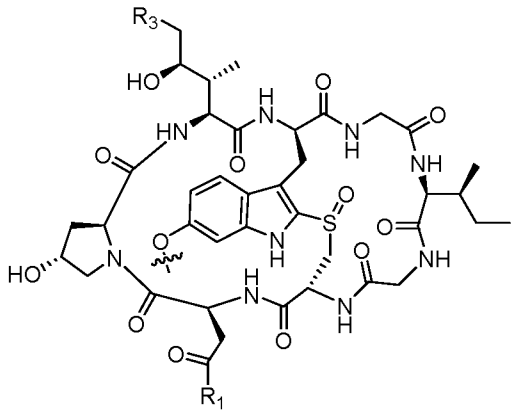
D is a drug moiety active agent derived from amatoxin and selected from the group consisting of alpha-amanitin, beta-amanitin, gamma-amanitin, and epsilon-amanitin; and

n is an integer from 1-10;

m is an integer from 1-24**;**

and **p** is an integer from 1-6.

2. The ADC of claim 1, wherein D has a structure of Formula II:

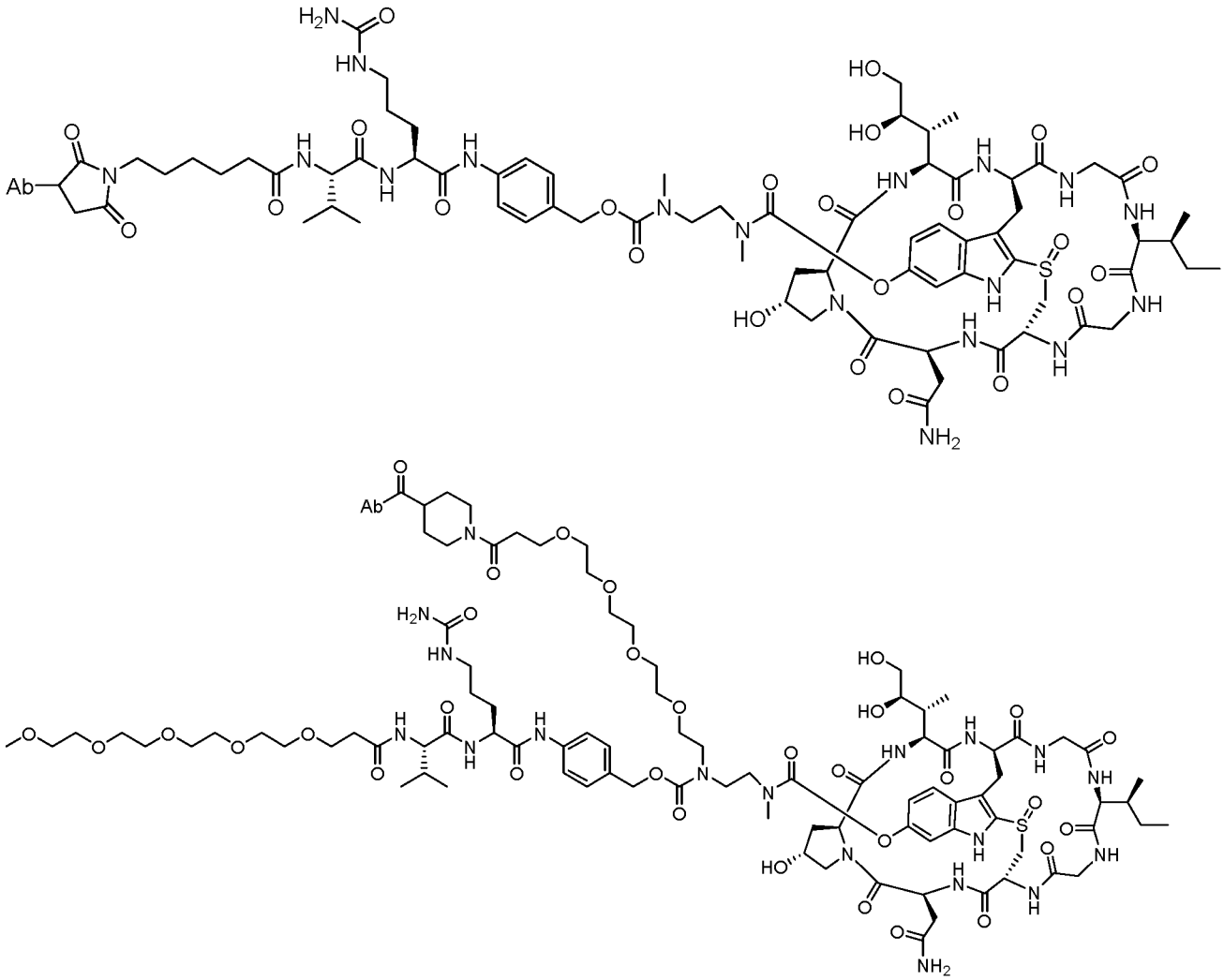


(II)

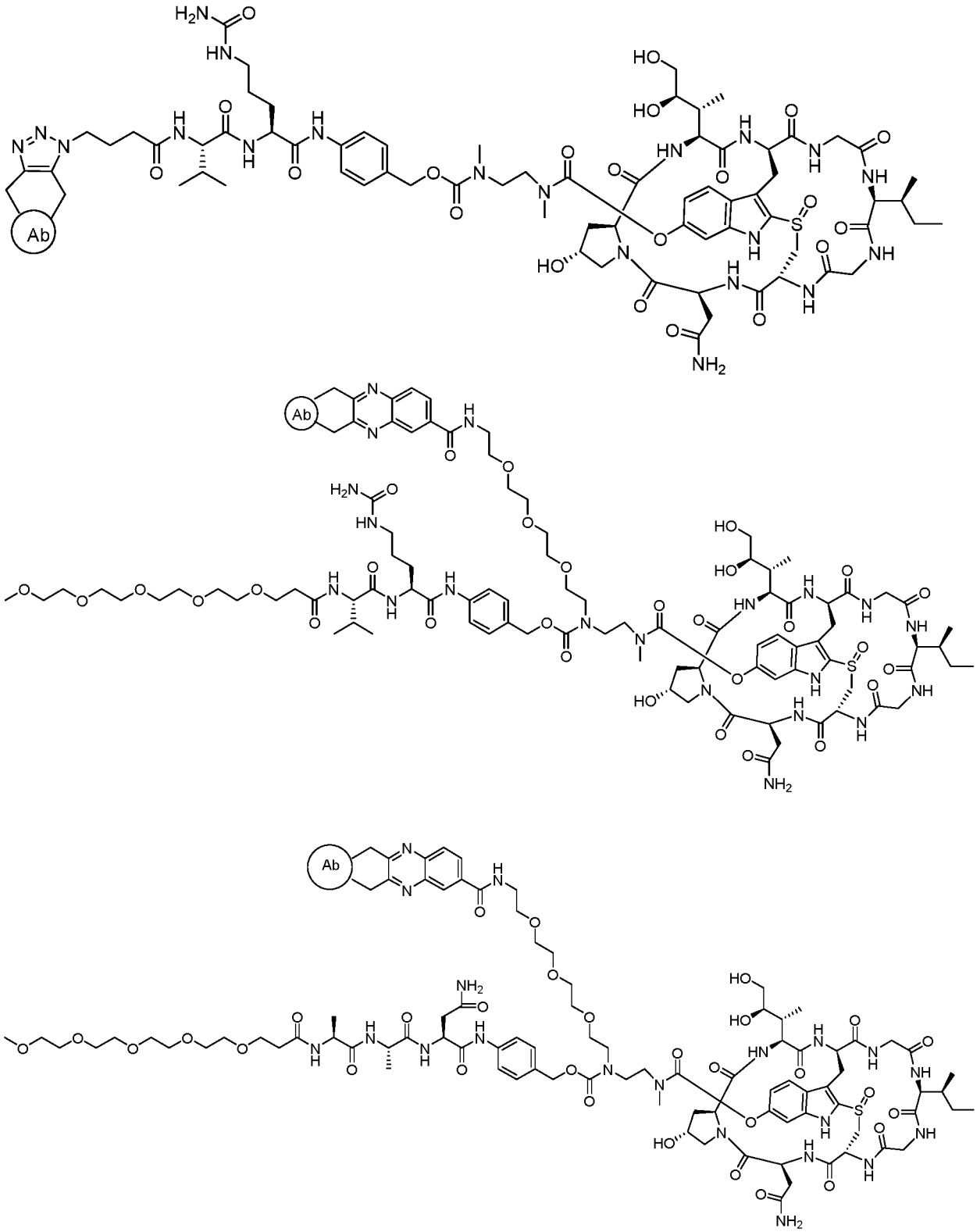
whereby the wavy line indicates the point of attachment to X;

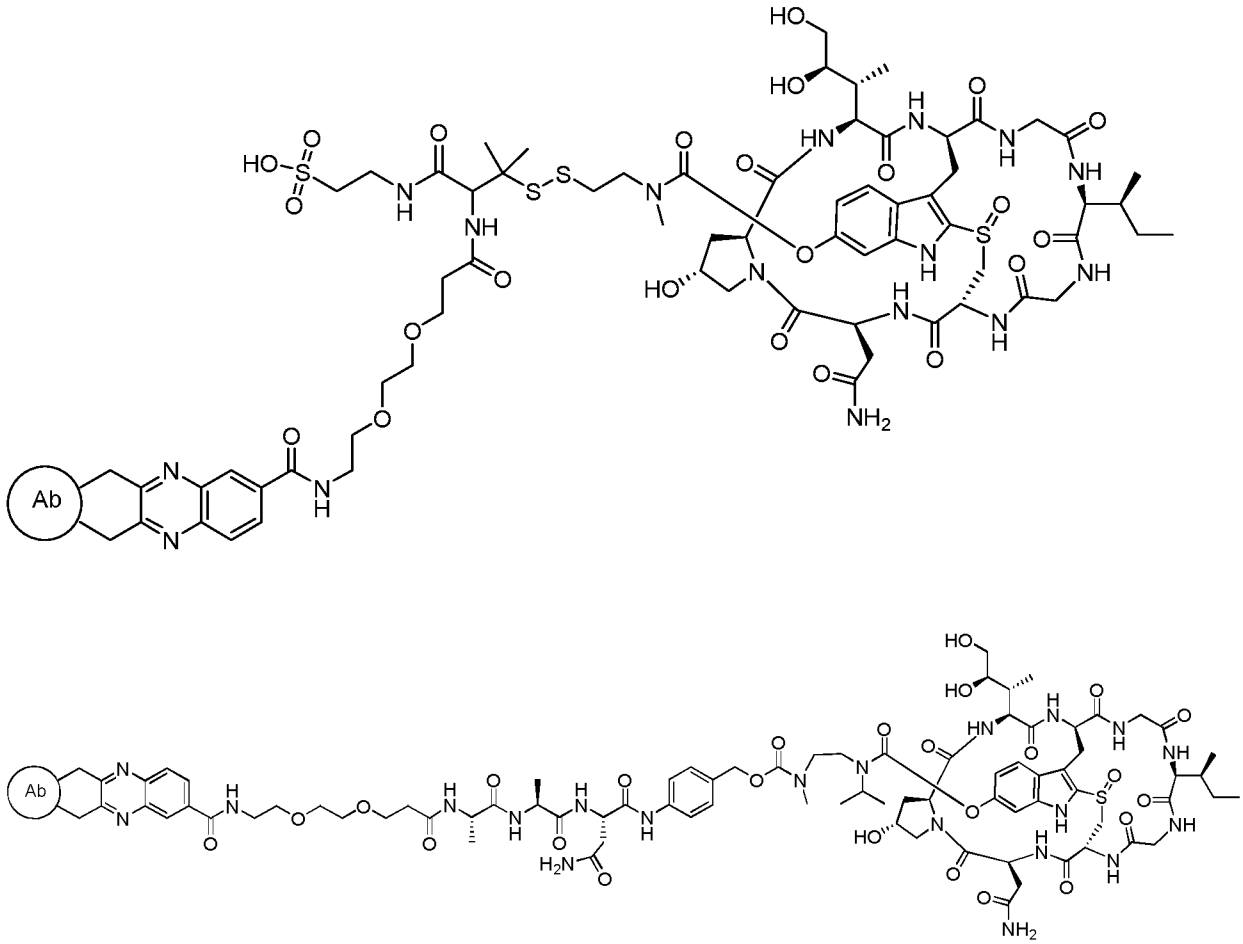
wherein R$_1$ is NH$_2$ or OR$_2$, wherein R2 is H, or C1-C10 alkyl, and wherein R3 is H or OH.

3. The ADC of claim 1, wherein the ADC is selected from the group consisting of:

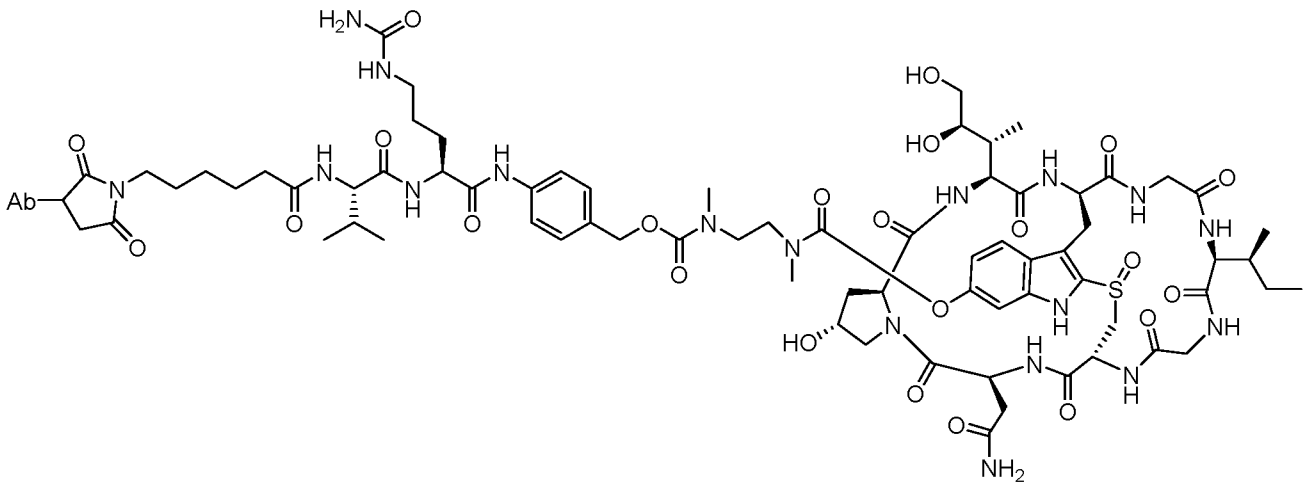


5

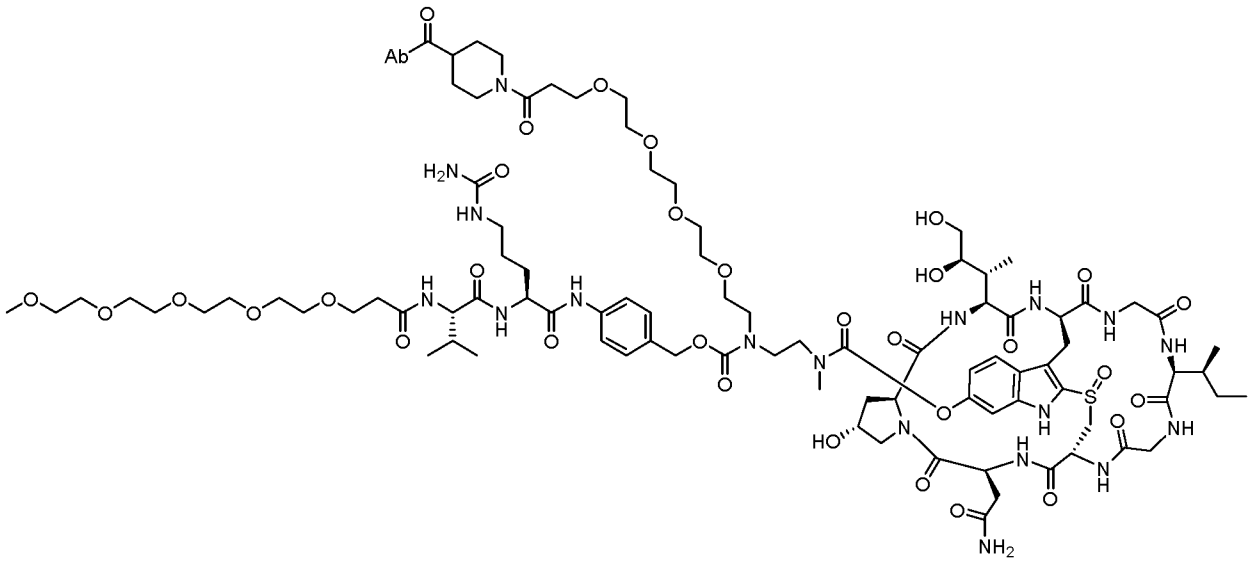




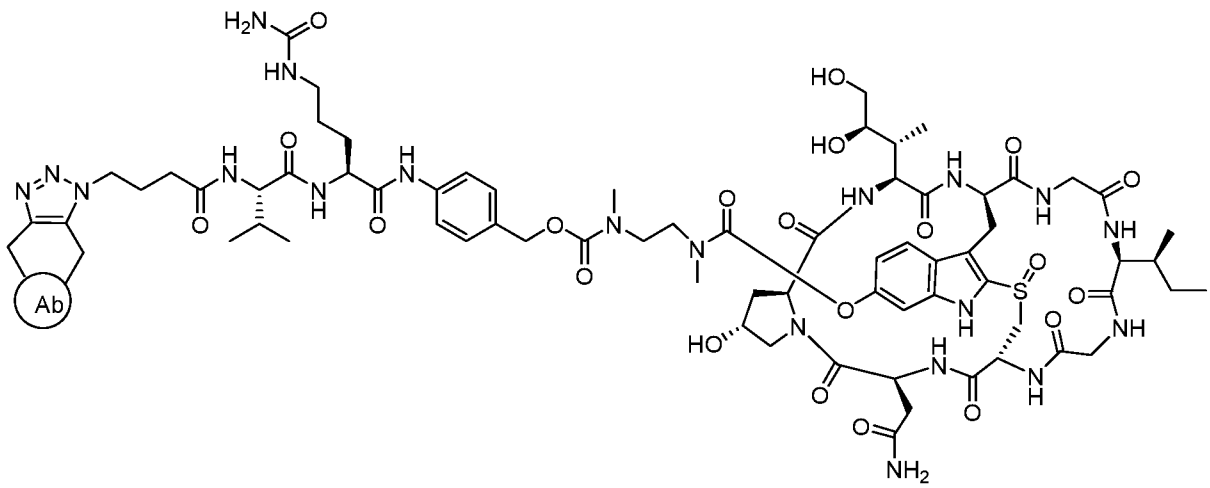
4. An ADC having the formula:



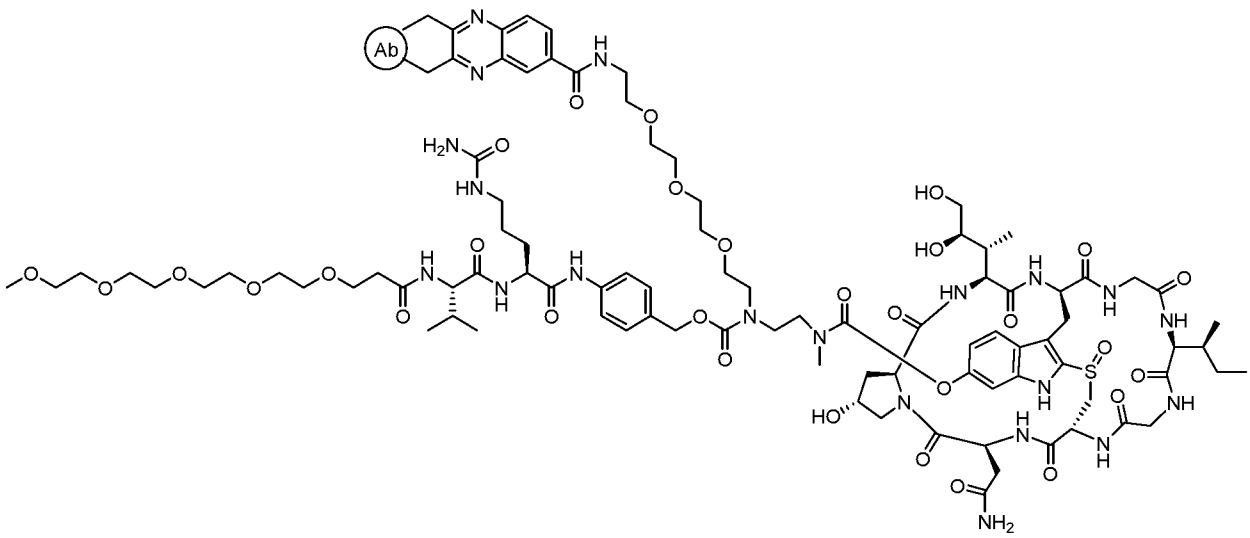
5. An ADC having the formula:



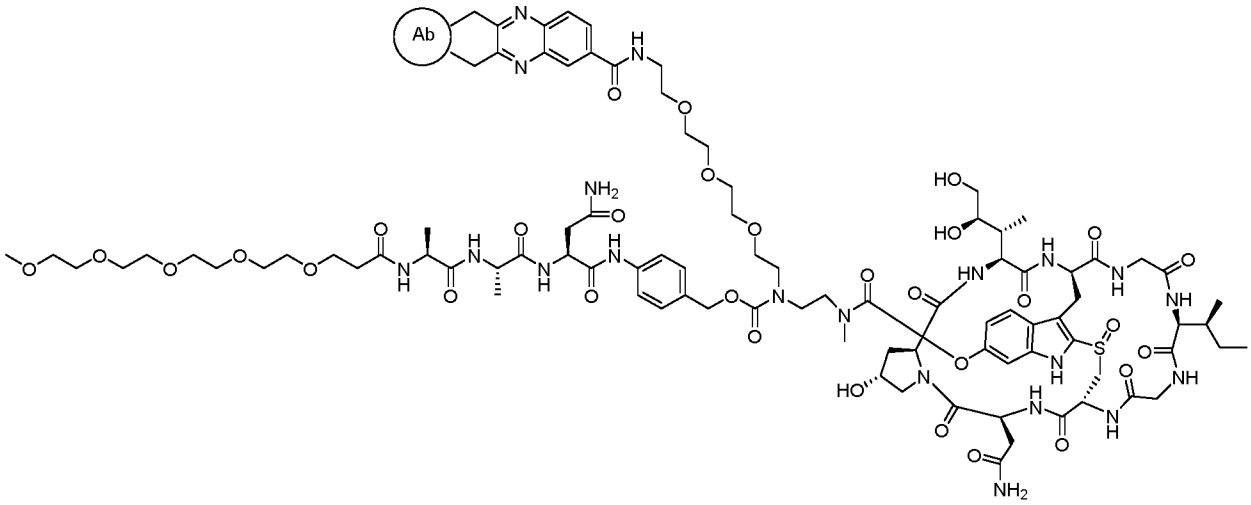
6. An ADC having the formula:



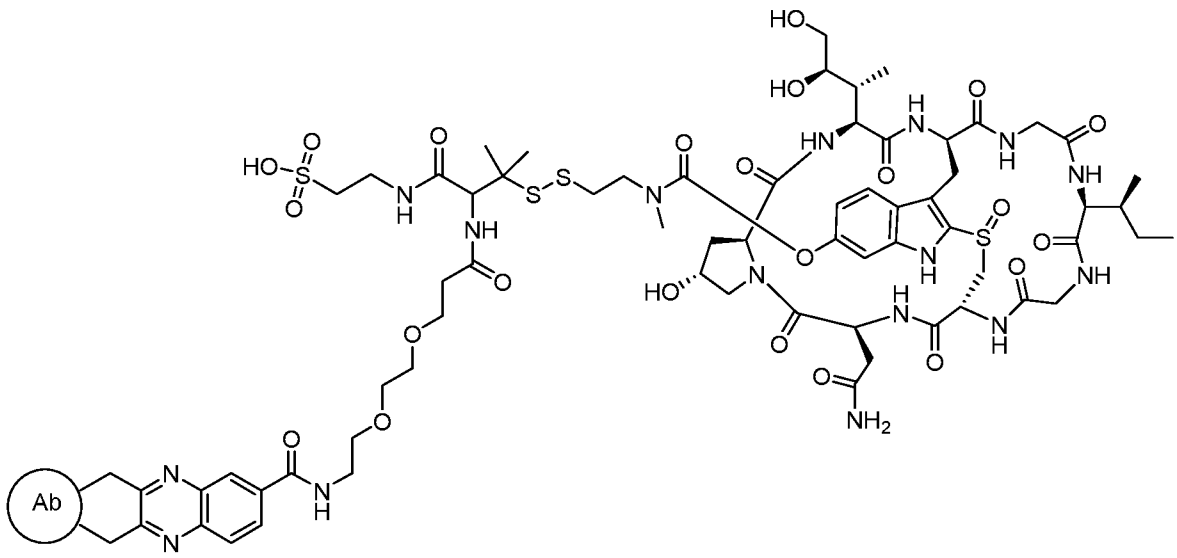
7. An ADC having the formula:



8. An ADC having the formula:



9. An ADC having the formula:



10. An ADC having the formula:

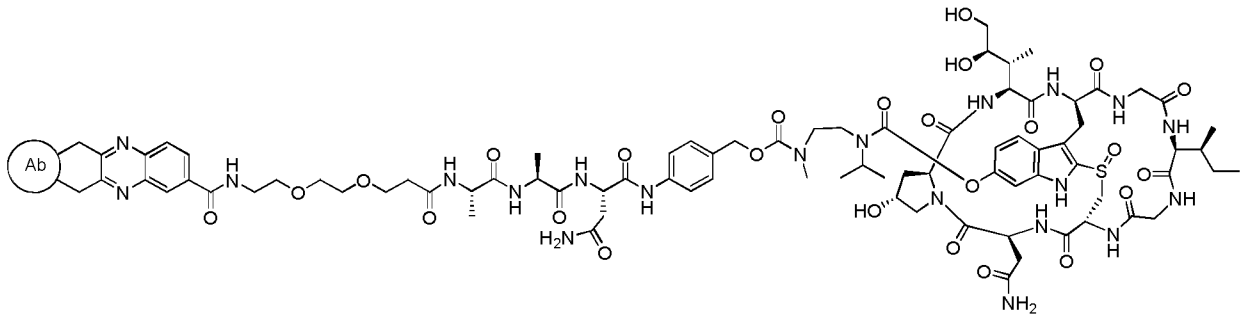


Figure 1

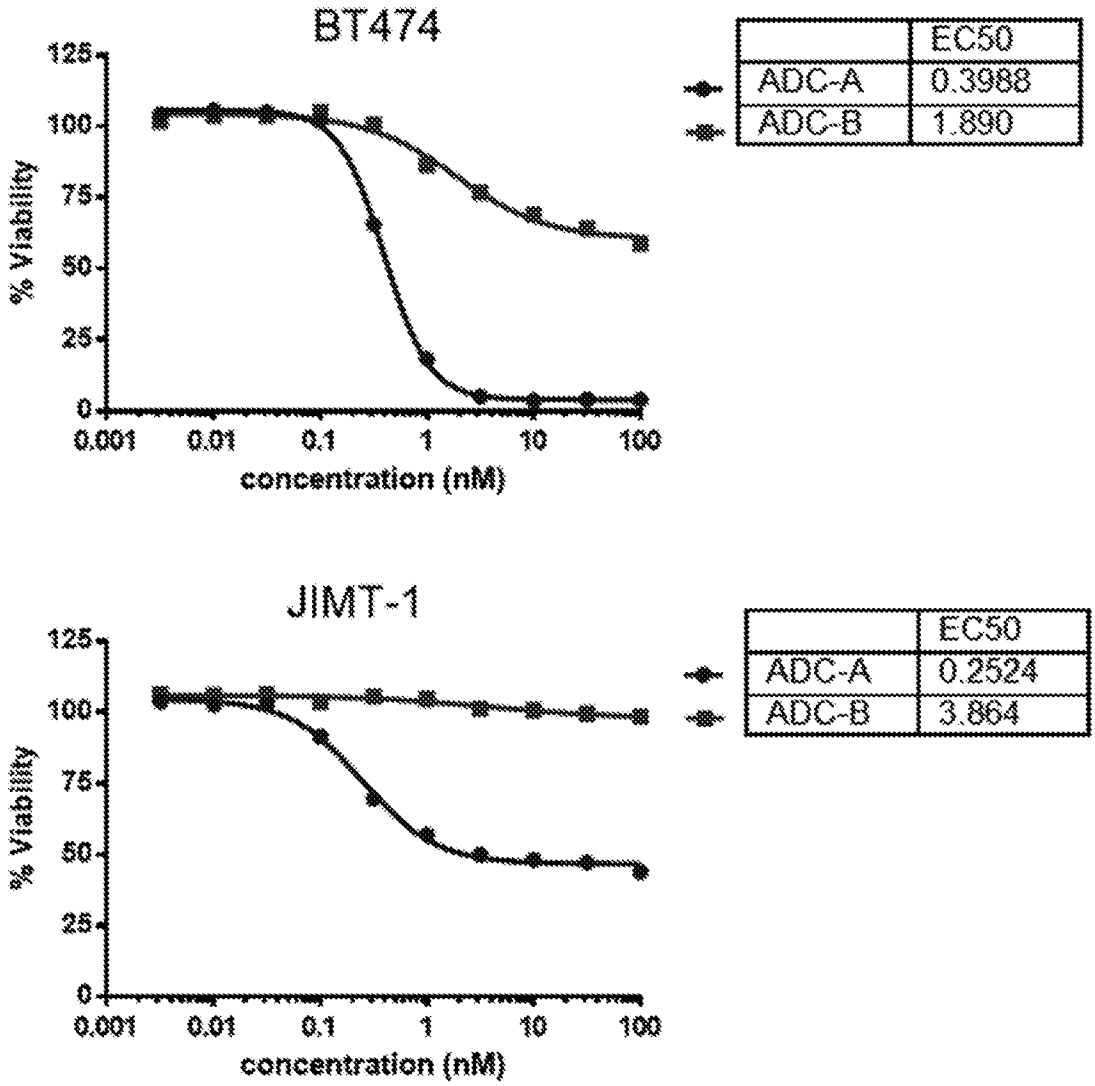
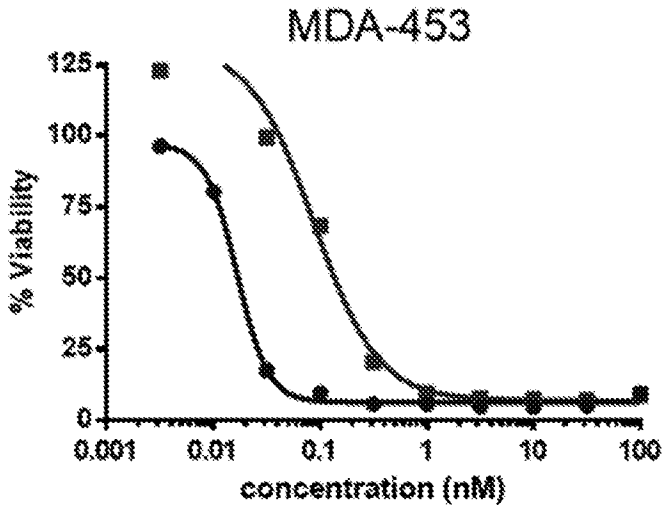
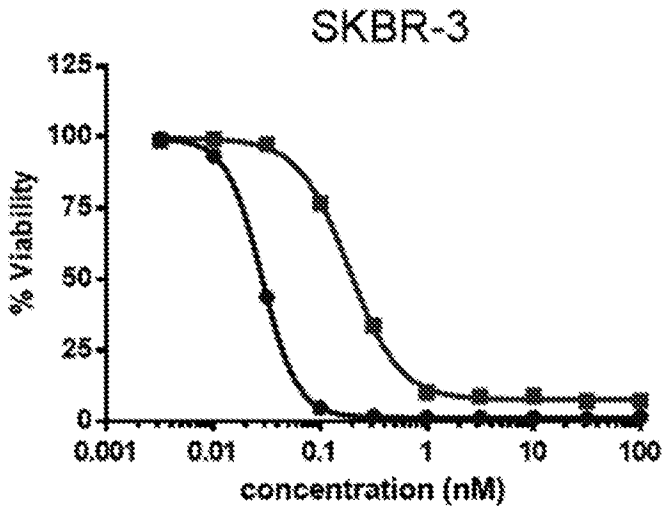

BT474
% Viability
125
100
75
50
25
0
0.001
0.01
0.1
1
10
100
concentration (nM)
EC50
ADC-A 0.3988
ADC-B 1.890
JIMT-1
% Viability
125
100
75
50
25
0
0.001
0.01
0.1
1
10
100
concentration (nM)
EC50
ADC-A 0.2524
ADC-B 3.864

Figure 1 - continued



	EC50
ADC-A	0.01663
ADC-B	0.08750



	EC50
ADC-A	0.02861
ADC-B	0.1873

Figure 2

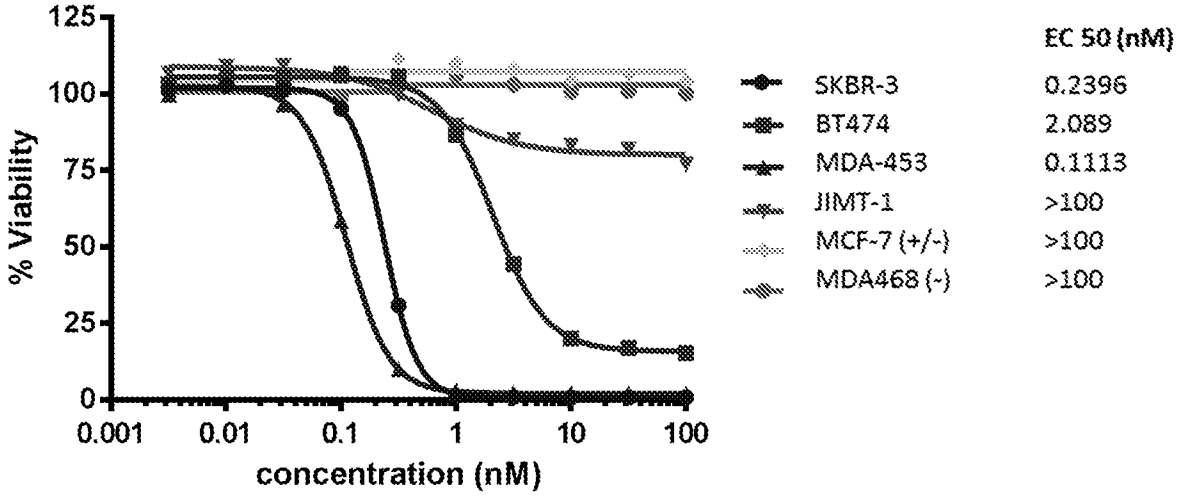


Figure 3

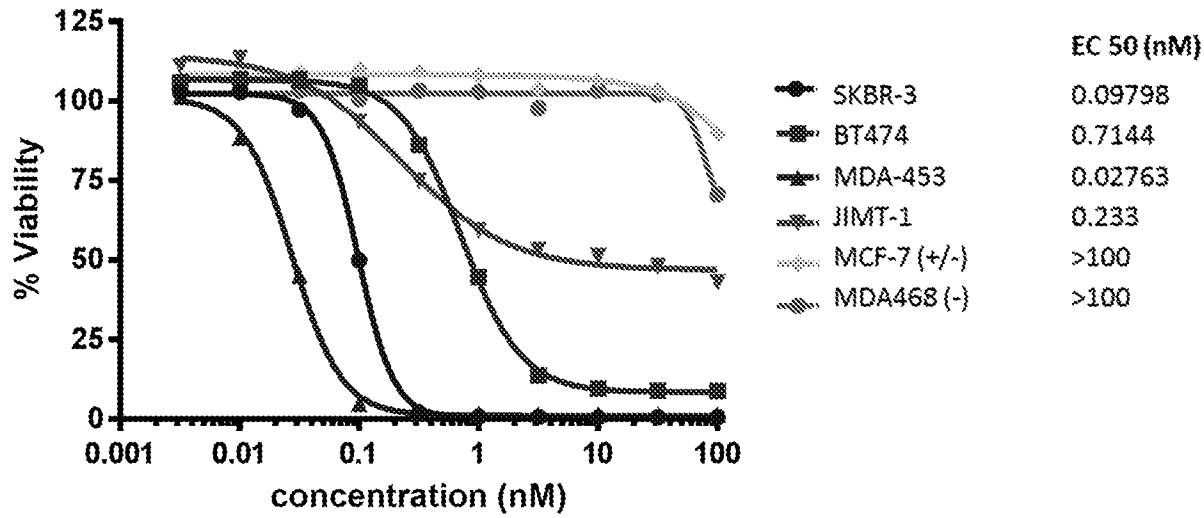


Figure 4

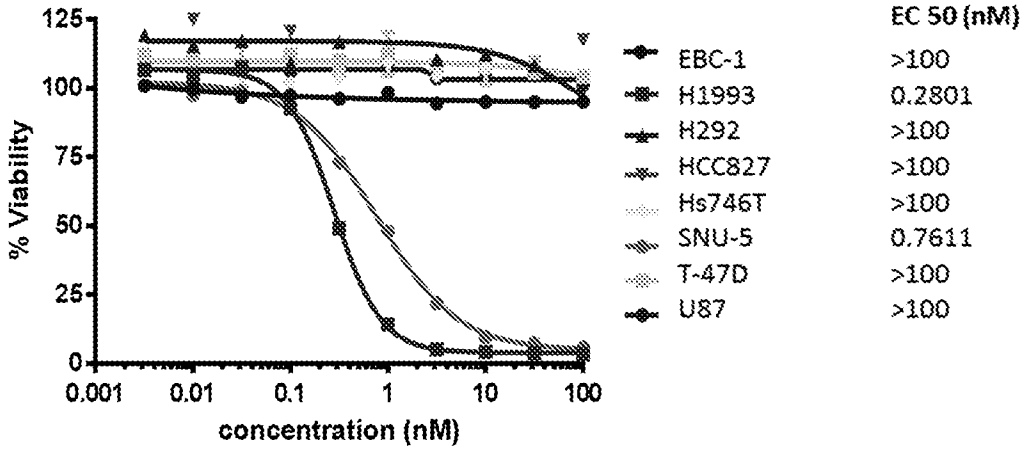


Figure 5

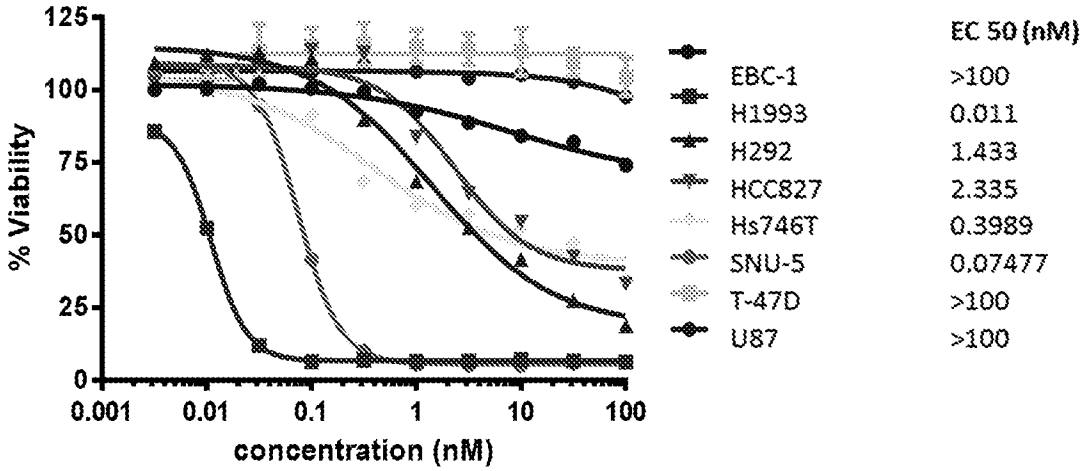
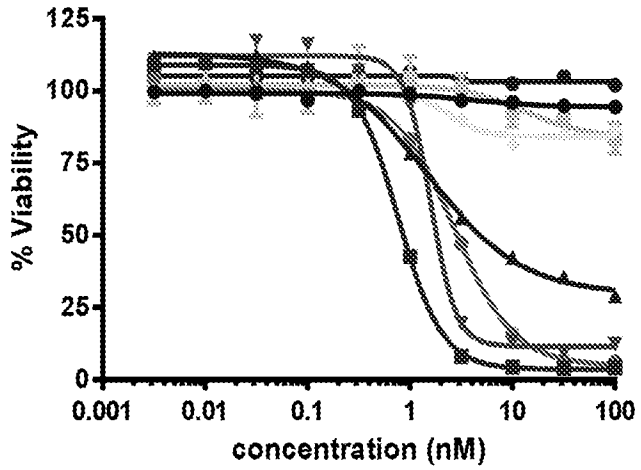
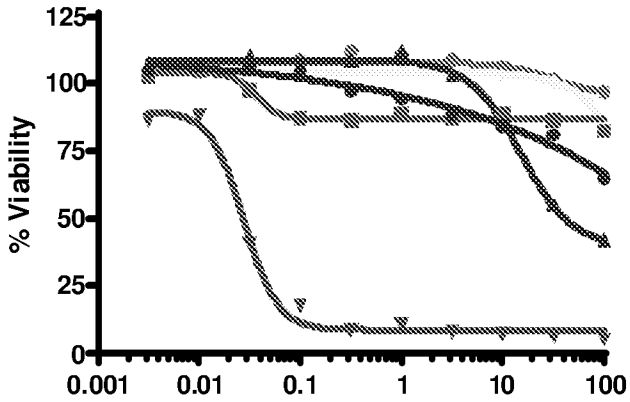


Figure 6



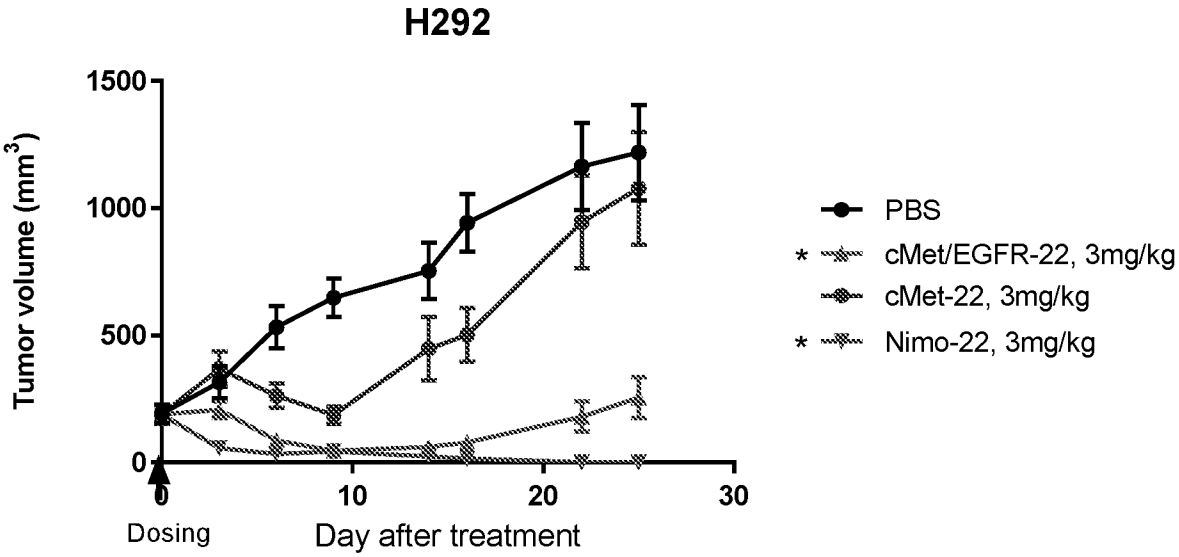
	EC 50 (nM)
EBC-1	>100
H1993	07644
H292	1.474
HCC827	1.583
Hs746T	>100
SNU-5	2.694
T-47D	>100
U87	>100

Figure 7



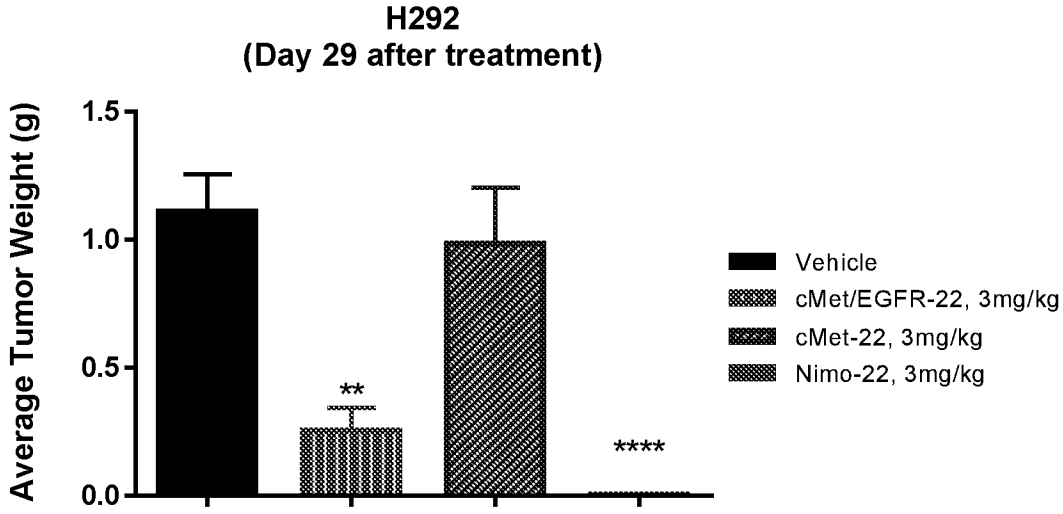
	EC 50 (nM)
EBC-1	>100
H1993	>100
HCC827	15.26
Hs746T	0.028
T-47	>100
U87	>100

Figure 8



* p<0.05. One way ANOVA, multiple comparisons compared to Control group.

Figure 9



* p<0.05. One way ANOVA, multiple comparisons compared to Control group.

Figure 10

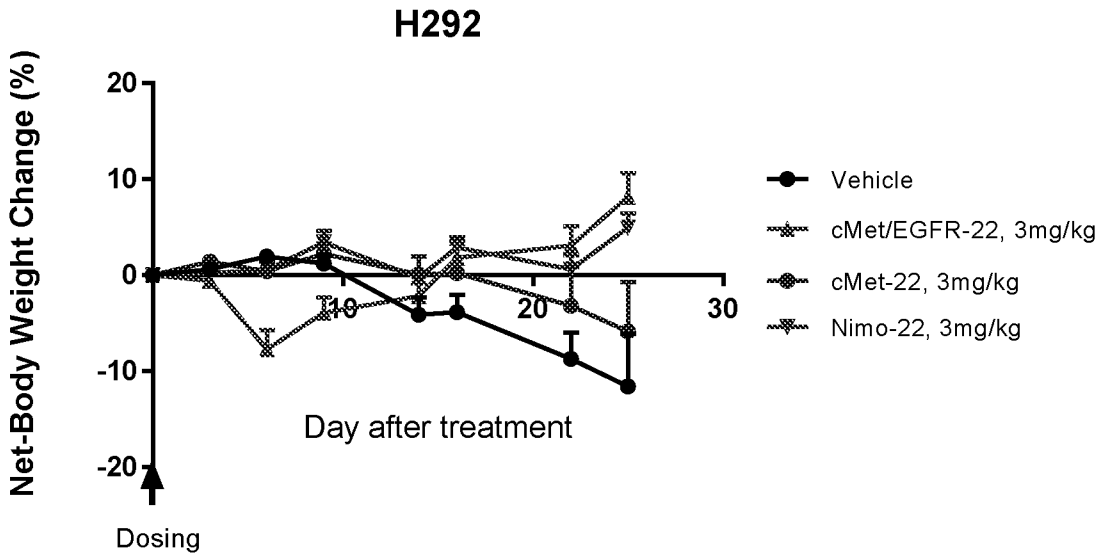


Figure 11

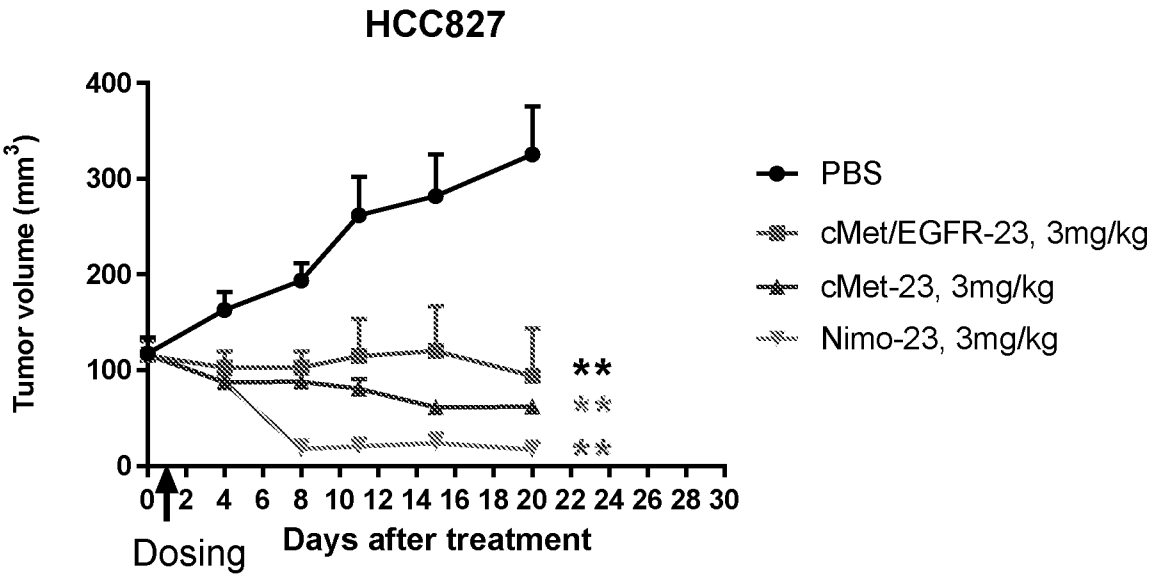
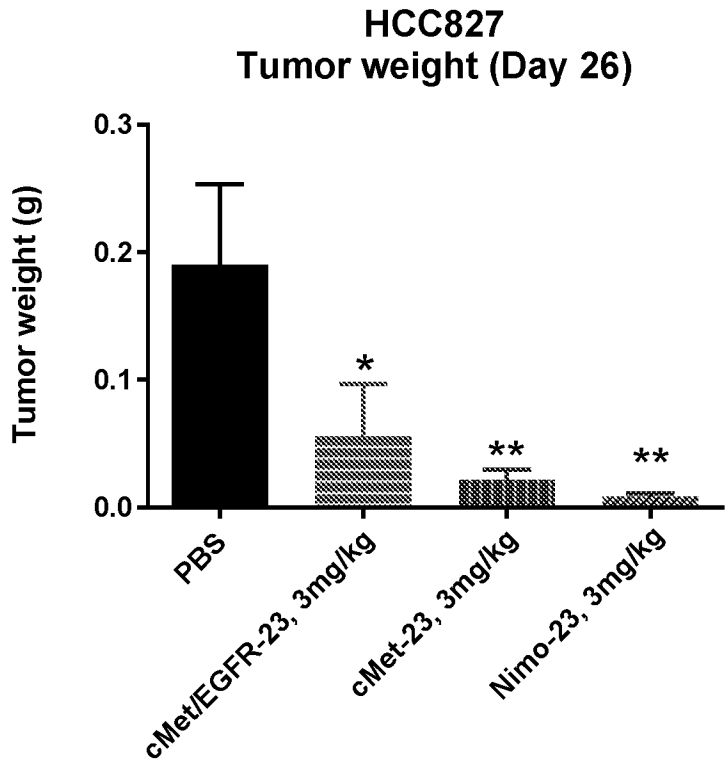


Figure 12



* P < 0.05, ** P < 0.01, One Way Anova with post hoc Dunnett's multiple comparison test to vehicle, N=7

Figure 13

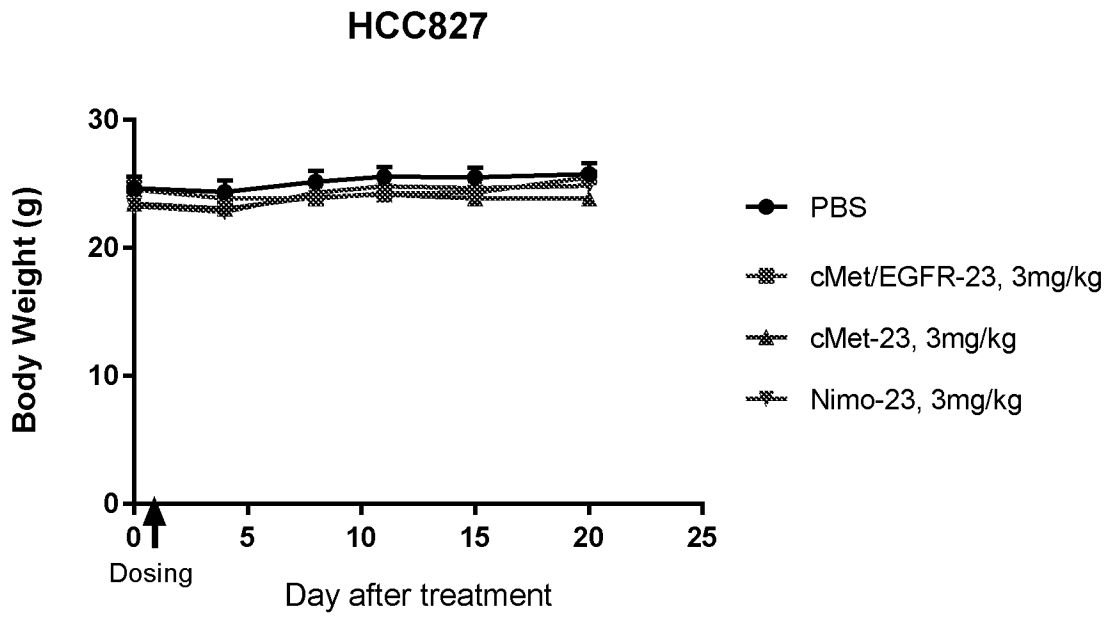


Figure 14

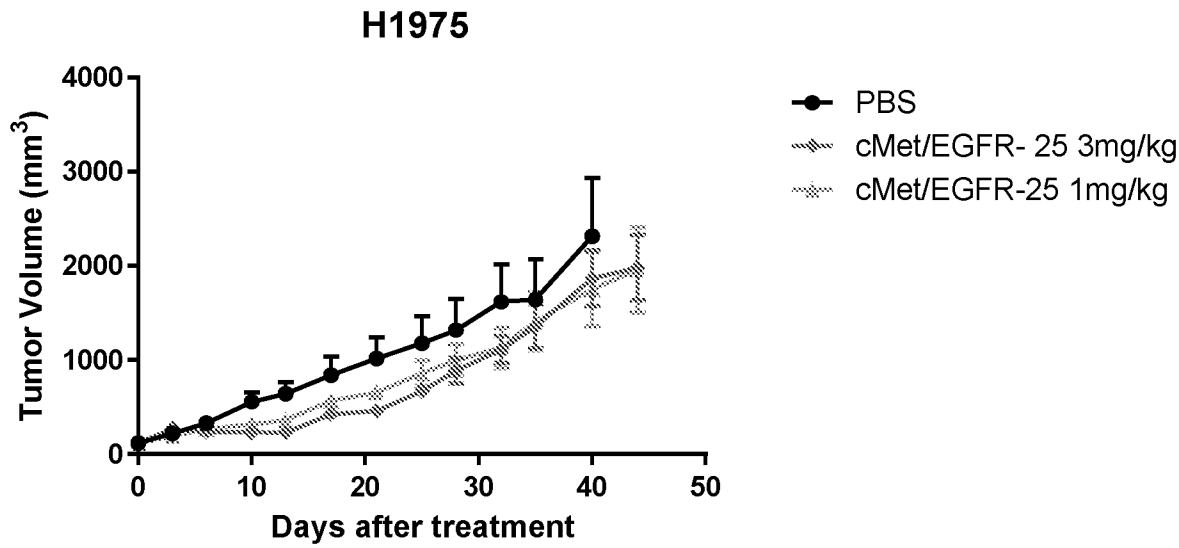


Figure 15

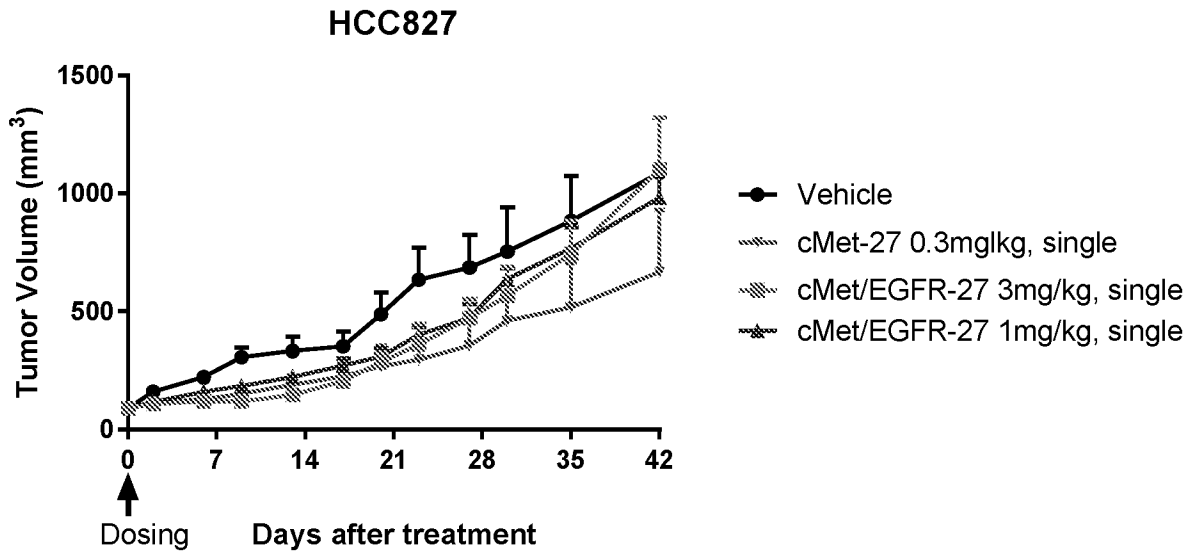
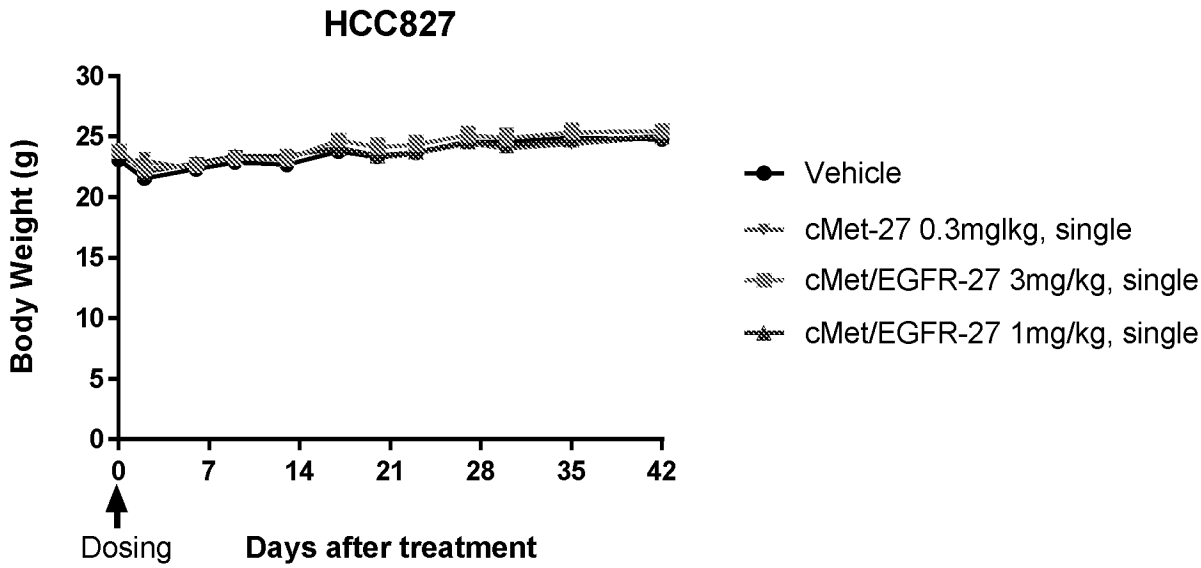


Figure 16



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/35206

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/395; A61P 35/00; C07K 19/00 (2017.01)
CPC - A61K47/48492; A61K47/48561; A61K39/395; C07K19/00; C07K16/30; A61K2039/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0100161 A1 (FAULSTICH et al.) 26 April 2012 (26.04.2012) para [0016], [0134], [0137]	1-4
Y	KUHN, 'The Design and Synthesis of Small-Molecule Anticancer Agents Targeted Through Antibody-Drug Conjugates', A thesis Submitted to the Faculty of Baylor University In Partial Fulfillment of the Requirements for the Honors Program, May 2014, pages 1-86. pg 19, para 2 to pg 20, para 2; pg 22, para 2; Fig 7	1-4
A	WO 2016/001485 A1 (GLYKOS FINLAND OY) 07 January 2016 (07.01.2016) Entire Document	1-4
A	WO 2016/004043 A1 (BLEND THERAPEUTICS INC.) 07 January 2016 (07.01.2016) Entire Document	1-4
A	US 2015/0105540 A1 (MIAO et al.) 16 April 2015 (16.04.2015) para [0161], Example 6	1-4
A	US 2015/0160192 A1 (CHEN et al.) 11 June 2015 (11.06.2015) Entire Document	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 October 2017 (02.10.2017)	18 OCT 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/35206

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

--Please see attached sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/35206

Attachment to Box.No.III:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+: Claims 1-10 directed to an antibody-drug conjugate compound of Formula I, containing an amatoxin drug, selected from the various structures listed in the claims. The antibody-drug conjugate will be searched to the extent that it encompasses the first-listed structure of claim 3. It is believed that claims 1-4 read on this first named invention, and thus these claims will be searched without fee to the extent that they encompass the first-listed structure of claim 3. Applicant is invited to elect additional compounds, wherein each additional compound elected will require one additional invention fee. Applicants must specify the claims that encompass any additionally elected compound. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the '+' group(s) will result in only the first claimed invention to be searched. Additionally, an exemplary election wherein different actual variables are selected is suggested. An exemplary election would be an antibody-drug conjugate represented by the second-listed structure of claim 3 (i.e., claims 1-3 and 5).

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ includes the technical feature of a unique antibody-drug conjugate, which is not required by any other invention of Group I+.

Common technical features:

The inventions of Group I+ share the technical feature of a compound having the structure of Formula I.

This shared technical feature, however, does not provide a contribution over the prior art, as being obvious over US 2012/0100161 A1 to Faulstich et al. (hereinafter 'Faulstich') in view of the thesis entitled, 'The Design and Synthesis of Small-Molecule Anticancer Agents Targeted Through Antibody-Drug Conjugates', A thesis Submitted to the Faculty of Baylor University In Partial Fulfillment of the Requirements for the Honors Program, May 2014 (Kuhn).

Faulstich teaches an amatoxin-antibody conjugate having therapeutic utility in the treatment of cancer (para [0016]) analogous to Formula I, wherein n is 1; D is alpha-amanitin and Ab is a monoclonal antibody, wherein a linker connects Ab and the phenolic OH of D (para [0132], alpha-amanitin-Herceptin conjugate (3)). Faulstich does not specifically teach a linker comprising L1-L2-X, where L1, L2 and X are as described in claim 1. However, Kuhn teaches a linker of structure L1-L2-X suitable for linking a phenolic OH to an antibody, wherein
L1 is the structure listed in row 1, col 3 of claim 1; X has the structure shown in claim 1, where R4 is C1-alkyl;
L2 is a combination of -(CH2)p, where p is an integer from 1-6; and R6OCOR5, where R5 is C1-alkyl and R6 is val-cit-PAB (pg 22, para 2; Fig 7), wherein said linker is cleavable by cathepsin B, which is upregulated in a tumor environment (pg 19, para 2 to pg 20, para 1). It would have been obvious to one of ordinary skill in the art to include the linker disclosed in Kuhn in the amatoxin-antibody conjugate of Faulstich, in order to ensure tumor targeted release of amatoxin from said conjugate, because both Faulstich and Kuhn teach antibody-drug conjugates useful in cancer therapy.

As said compound was obvious at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the inventions of Groups I+.

The inventions of Group I+ thus lack unity under PCT Rule 13.