HER2-Binding Molecules

Technical Field

The present disclosure relates to molecular biology, more specifically antibody technology. The present disclosure also relates to methods of medical treatment and prophylaxis.

Background

Cancers remain the leading cause of deaths worldwide. Chemotherapies have good clinical benefits, but due to their low specificity they have very significant side effect and low therapeutic indices. More targeted therapies, such as monoclonal antibody therapies, show good specificity but response rates are smaller. Antibody-drug conjugates (ADCs) are a therapeutic modality that harness an antibody's target specificity to selectively deliver cytotoxic payloads to tumors and are proving increasingly effective in the clinic.

HER2 (also known *e.g.* as ERBB2, neu) is a member of the epidermal growth factor receptor (EGFR) family of transmembrane receptors. Overexpression of HER2 is observed in approximately 20% of human breast cancers, and is implicated in the aggressive growth and poor clinical outcomes in patients having such tumors (Slamon *et al.* Science (1987) 235:177-182).

Anti-HER2 antibody drug conjugates (ADCs) are described in Rassy *et al.*, Breast (2022) 66: 217-226, and include ado-trastuzumab emtansine (DrugBank Acc. No. DB05773; marketed as Kadcyla®)) and fam-trastuzumab deruxtecan-nxki (DrugBank Acc. No. DB14962; marketed as Enhertu®). Ado-trastuzumab emtansine comprises the cytotoxic agent DM1 (a thiol-containing maytansinoid microtubule inhibitor) conjugated to trastuzumab at lysine side chains via an MCC linker. Fam-trastuzumab deruxtecan-nxki comprises the DNA topoisomerase I inhibitor DS-8201a (DXd) conjugated to trastuzumab via a cathepsin-cleavable tetrapeptide linker. Mosele *et al*. Nat Med. (2023) 29(8):2110-2120 reports the results of a phase 2 trial (DAISY trial) relating to the use of fam-trastuzumab deruxtecan-nxki to treat metastatic breast cancer. Disease progression was observed in ~71% of patients (125/177). There remains an unmet clinical need for the effective treatment of HER2-expressing cancers.

Summary

In a first aspect, the present disclosure provides an antigen-binding molecule that binds to HER2, comprising (i) a HER2-binding moiety, and (ii) at least one linker-payload moiety, wherein the antigen-binding molecule comprises (a) DNA damage response (DDR) inhibitor moiety, and (b) a DNA topoisomerase I (TOP1) inhibitor moiety.

In some embodiments, the DDR inhibitor moiety is, or comprises, a DDR inhibitor selected from: an ATR inhibitor, a PARP inhibitor, an ATM inhibitor, a WEE1 inhibitor, a CHK1/2 inhibitor, a DNA-PK inhibitor, or a PLK1 inhibitor.

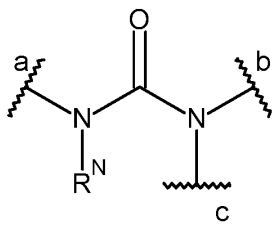
In some embodiments, the DDR inhibitor moiety is, or comprises, a DDR inhibitor which is an ATR inhibitor. In some embodiments, the DDR inhibitor moiety is, or comprises, berzosertib.
In some embodiments, the DDR inhibitor moiety is, or comprises, a DDR inhibitor which is a CHK1/2 inhibitor. In some embodiments, the DDR inhibitor moiety is, or comprises, prexasertib.
In some embodiments, the DDR inhibitor moiety is, or comprises, a DDR inhibitor which is a WEE1 inhibitor. In some embodiments, the DDR inhibitor moiety is, or comprises, adavosertib.
In some embodiments, the DDR inhibitor moiety is, or comprises, a DDR inhibitor which is an ATM inhibitor. In some embodiments, the DDR inhibitor moiety is, or comprises, AZD0156.
In some embodiments, the DDR inhibitor moiety is, or comprises, a DDR inhibitor which is a DNA-PK inhibitor. In some embodiments, the DDR inhibitor moiety is, or comprises, nedisertib.

In some embodiments, the TOP1 inhibitor moiety is, or comprises, a TOP1 inhibitor selected from: camptothecin or a derivative thereof, exatecan, exatecan mesylate (DX-8951f), *N*-glycyl-exatecan, SN-38, DXd(1), DXd(2), irinotecan, etirinotecan, FL118, topotecan, gimatecan, belotecan, deruxtecan, belotecan, rubitecan, lurtotecan, diflomotecan, karenitecan, silatecan, namitecan, elomotecan, DRF-1042, delimotecan, NSC606985, chimmitecan, ZBH-1205, Genz-644282, non-CPT1, indotecan, indimitecan, AZ14170132, SHR9265, Ed-04, KL610023, A1.9, ZD06519, P1003, P1021, VIP126, ZBH-01 and LMP-744.

In some embodiments, the TOP1 inhibitor moiety is, or comprises, a TOP1 inhibitor selected from: camptothecin or a derivative thereof, exatecan, exatecan mesylate (DX-8951f), *N*-glycyl-exatecan, SN-38, DXd(1), DXd(2). In some embodiments, the TOP1 inhibitor moiety is, or comprises exatecan.

In some embodiments, the antigen-binding molecule comprises a linker-payload moiety comprising (a) a DDR inhibitor moiety, and (b) a TOP1 inhibitor moiety.

In some of these embodiments, the linker-payload moiety comprises:
(a) an amino group for conjugation to an antigen-binding moiety;
(b) at least one first payload comprising moiety clicked to a first click group, where the first payload comprising moiety comprises a DDR inhibitor moiety;
(c) at least one second payload comprising moiety clicked to a second click group, where the second payload comprising moiety comprises a TOP1 inhibitor moiety;
(d) the branching group:



wherein R^N is selected from H and -(C_{1-5} alkylene)-C(O)OH, where one CH_2 unit may be replaced by -O-, a indicates where the amino group is linked to the branching group;

b indicates where the at least one first click group is linked to the branching group;
c indicates where the at least one second click group is linked to the branching group.

In some embodiments, the HER2-binding moiety comprises:

(i) a heavy chain variable (VH) region incorporating the following CDRs:

HC-CDR1 having the amino acid sequence of SEQ ID NO:15

HC-CDR2 having the amino acid sequence of SEQ ID NO:16

HC-CDR3 having the amino acid sequence of SEQ ID NO:17; and

(ii) a light chain variable (VL) region incorporating the following CDRs:

LC-CDR1 having the amino acid sequence of SEQ ID NO:23

LC-CDR2 having the amino acid sequence of SEQ ID NO:24

LC-CDR3 having the amino acid sequence of SEQ ID NO:25.

In some embodiments, the antigen-binding moiety that binds to HER2 comprises:

a VH region comprising an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:14; and

a VL region comprising an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:22.

In some embodiments, the antigen-binding moiety that binds to HER2 comprises:

a polypeptide comprising, or consisting of, an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:12; and

a polypeptide comprising, or consisting of, an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:13.

The present disclosure also provides a composition comprising an antigen-binding molecule according to the present disclosure, and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant.

The present disclosure also provides an antigen-binding molecule according to the present disclosure, or a composition according to the present disclosure, for use in a method of medical treatment or prophylaxis, or in a method of diagnosis or prognosis.

The present disclosure also provides an antigen-binding molecule according to the present disclosure, or a composition according to the present disclosure, for use in treating or preventing a cancer.

The present disclosure also provides an antigen-binding molecule according to the present disclosure, or a composition according to the present disclosure, in the manufacture of a medicament for treating or preventing a cancer.

The present disclosure also provides a method of treating or preventing a cancer, comprising administering to a subject a therapeutically- or prophylactically-effective amount of an antigen-binding molecule according to the present disclosure, or a composition according to the present disclosure.

In some embodiments, the cancer is selected from: a cancer comprising cells expressing/overexpressing an EGFR family member, a cancer comprising cells expressing/overexpressing HER2, a cancer comprising cells that do not overexpress an EGFR family member, a cancer comprising cells that do not overexpress HER2, a HER2-low cancer, a HR-positive cancer, a solid tumor, bladder cancer, breast cancer, HER2-positive breast cancer, metastatic HER2-positive breast cancer, HER2-low breast cancer, unresectable or metastatic HER2-low breast cancer, HR-positive breast cancer, triple-negative breast cancer, cervical cancer, gastric cancer, HER2-positive gastric cancer, locally-advanced or metastatic HER2-positive gastric cancer, cholangiocarcinoma, colorectal cancer, esophageal esophagogastric junction cancer, gallbladder cancer, gastric adenocarcinoma, gastroesophageal junction adenocarcinoma, HER2-positive gastric gastroesophageal junction adenocarcinoma, locally-advanced or metastatic HER2-positive gastric gastroesophageal junction adenocarcinoma, gastrointestinal stromal tumor, glioblastoma multiforme, glioma, head and neck carcinoma, hepatocellular carcinoma, intestinal cancer, kidney cancer, lung cancer, non-small cell lung cancer, unresectable or metastatic non-small cell lung cancer comprising activating mutation to *ERBB2*, small cell lung cancer, melanoma, neuroendocrine tumor, oligodendroglioma, ovarian cancer, pancreatic adenocarcinoma, penile cancer, pituitary cancer, prostate cancer, sarcoma, solitary fibrous tumor, testicular cancer, thymic cancer, thyroid cancer and uterine cancer.

In some embodiments, the cancer is refractory or relapsed to treatment with a DNA damage repair inhibitor, and/or wherein the cancer is refractory or relapsed to treatment with a DNA topoisomerase I inhibitor.

The present disclosure also the use of an antigen-binding molecule according to the present disclosure, or a composition according to the present disclosure, to deplete or increase killing of cells expressing HER2.

The present disclosure also provides an *in vitro* complex, optionally isolated, comprising an antigen-binding molecule according to the present disclosure bound to HER2.

Description

The present disclosure relates to antigen-binding molecules comprising a HER2-binding moiety and a linker-payload moiety comprising at least two different payload moieties.

The antigen-binding molecules of the present disclosure are provided with unexpected and advantageous properties relative to known anti-HER2 antibody-drug conjugates.

The inventors hypothesize that the resistance to fam-trastuzumab deruxtecan-nxki therapy observed in the DAISY trial might be due to insensitivity to DXd, noting that 65% of patients that progressed on treatment with fam-trastuzumab deruxtecan-nxki retained HER2 expression (Mosele et al. Nat Med. (2023) 29(8):2110-2120).

DNA damage response (DDR) is a key pathway for repair, and might be an important mode of resistance to DNA damaging payloads such as TOP1 inhibitors. Synergistic anticancer effects have previously been observed through combined treatment using a DDR inhibitor and a TOP1 inhibitor (Thomas *et al.*, Cancer Cell. (2021) 39(4):566-79 e7). In embodiments wherein the antigen-binding molecule of the present disclosure comprises a linker-payload moiety comprising (i) a TOP1 inhibitor moiety and (ii) a DNA damage response (DDR) inhibitor moiety, the DDR inhibitor is thought to increase the sensitivity of the cells of the cancer to the TOP1 inhibitor. Moreover, many TOP1 inhibitors and DDR inhibitors are not substrates for P-glycoprotein, and so patients treated with antigen-binding molecules comprising such payload moieties are less likely to develop P-glycoprotein-mediated resistance to such therapy.

HER2

HER2 (also known *e.g.* as ERBB2, neu) is the protein identified by UniProtKB: P04626. The canonical isoform of human HER2 has the amino acid sequence of P04626-1 (v1, 1987-08-13; SEQ ID NO:1). Alternative splicing mRNA encoded by the human *ERBB2* gene yields six main isoforms: isoform 1 (SEQ ID NO:1), isoform 2 (SEQ ID NO:2), isoform 3 (SEQ ID NO:3), isoform 4 (SEQ ID NO:4), isoform 5 (SEQ ID NO:5) and isoform 6 (SEQ ID NO:6). Isoform 2 differs from isoform 1 in that positions 1 to 610 are absent. Positions 1 to 686 of SEQ ID NO:1 are absent from isoform 3. In isoform 4, positions 1 to 23 of SEQ ID NO:1 are replaced with a shorter, 8 amino acid sequence. Positions 1 to 686 of SEQ ID NO:1 are absent from isoform 5. Isoform 6 differs in that positions 633 to 648 and 844 to 1255 of SEQ ID NO:1 are absent, and positions 771 to 883 are replaced with a different sequence of amino acids.

The canonical isoform of human HER2 comprises a 22 amino acid N-terminal signal peptide (SEQ ID NO:8), followed by an extracellular domain (SEQ ID NO:9), a single-pass transmembrane domain (SEQ ID NO:10) and a cytoplasmic domain (SEQ ID NO:11) at the C-terminus. The mature form of human HER2 isoform 1 is shown in SEQ ID NO:7.

The structure and function of HER2 is described *e.g.* in Iqbal *et al.*, Mol Biol Int. (2014) 2014: 852748, which is hereby incorporated by reference in its entirety. Like the other EGFR family members, HER2 comprises a cysteine-rich extracellular region, a lipophilic transmembrane domain and an intracellular domain with tyrosine kinase activity. HER2 lacks any recognized direct activating ligand, and may either exist in a constitutively active state, or become activated upon forming heterodimers with other EGFR family members, such as EGFR and HER3. HER2 homodimerization or heterodimerization with EGFR/HER3 induces autophosphorylation of tyrosine residues of the cytoplasmic domain, initiating a signaling through various different intracellular pathways, most notably the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways. HER2-mediated signaling leads to cell proliferation, survival, differentiation, angiogenesis, and tissue invasion. The HER2-

HER3 heterodimer is a particularly potent stimulator of downstream pathways, particularly the PI3K/Akt pathway.

Reference herein to 'HER2' generally refers to the canonical isoform of the human HER2 (*i.e.* isoform 1), but also contemplates isoforms, fragments, variants (including mutants) and homologues thereof (*i.e.* from other species, *e.g.* non-human mammalian species (*e.g.* a non-human primate, *e.g.* rhesus, cynomolgous; *e.g.* a rodent, *e.g.* rat or mouse).

As used herein, a 'fragment', 'variant' or 'homologue' of a protein may optionally be characterised as having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98% or ≥99% amino acid sequence identity to the amino acid sequence of the reference protein (*e.g.* the canonical isoform of the human protein). In some embodiments fragments/variants/ isoforms/homologues may be characterised by ability to perform a function performed by the reference protein.

A 'fragment' generally refers to a fraction of the reference protein. A 'variant' generally refers to a protein having an amino acid sequence comprising one or more amino acid substitutions, insertions, deletions or other modifications relative to the amino acid sequence of the reference protein, but retaining a considerable degree of sequence identity (*e.g.* at least 60%) to the amino acid sequence of the reference protein. An 'isoform' generally refers to a variant of the reference protein expressed by the same species as the species of the reference protein (*e.g.* human HER2 isoforms 1 to 6 are all isoforms of one another). A 'homologue' generally refers to a variant of the reference protein produced by a different species as compared to the species of the reference protein. For example, human HER2 isoform 1 (P04626-1 v1; SEQ ID NO:1) and mouse HER2 (UniProt: P70424-1 v3, 2005-09-27) are homologues of one another. Homologues include orthologues.

A 'fragment' may be of any length (by number of amino acids), although may optionally be at least 20% of the length of the reference protein (that is, the protein from which the fragment is derived) and may have a maximum length of one of 50%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the length of the reference protein. A fragment of HER2 may have a minimum length of one of 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1100, 1200 or 1250 amino acids, and may have a maximum length of one of 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1100, 1200, or 1250 amino acids.

Isoforms, fragments, variants or homologues may optionally be functional isoforms, fragments, variants or homologues, *e.g.* having a functional property/activity of the reference HER2 (*e.g.* human HER2 isoform 1), as determined by analysis by a suitable assay for the functional property/activity. For example, an isoform, fragment, variant or homologue of HER2 may display association with HER3 or EGFR.

In some embodiments, the HER2 is HER2 from a mammal (*e.g.* a primate (rhesus, cynomolgous, non-human primate or human) and/or a rodent (*e.g.* rat or murine) HER2). Isoforms, fragments, variants or

homologues of HER2 may optionally be characterized as having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of an immature or mature HER2 isoform from a given species, *e.g.* human.

In some embodiments, the HER2 comprises, or consists of, an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to one of SEQ ID NOs:1 to 7.

In some embodiments, a fragment of HER2 comprises, or consists of, an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to SEQ ID NO:7 or 9.

HER2-binding antigen-binding moieties

An 'antigen-binding molecule' refers to a molecule that binds to a given target antigen. Antigen-binding molecules comprise an antigen-binding moiety through which the antigen-binding molecule binds to its target antigen. The antigen-binding molecules of the present disclosure comprise an antigen-binding moiety that binds to HER2 (*i.e.* a HER2-binding moiety).

Antigen-binding moieties may comprise, or may be derived from, antibodies (*i.e.* immunoglobulins (Igs)) and antigen-binding fragments of antibodies. As used herein, 'antibodies' include monoclonal antibodies, polyclonal antibodies, monospecific and multispecific (*e.g.*, bispecific, trispecific, *etc.*) antibodies, and antibody-derived antigen-binding molecules such as scFv, scFab, diabodies, triabodies, scFv-Fc, minibodies, single domain antibodies (*e.g.* VhH, *etc.*). Antigen-binding fragments of antibodies include *e.g.* Fv, Fab, $F(ab')_2$ and F(ab') fragments.

Antigen-binding moieties also include target antigen-binding aptamers, *e.g.* a nucleic acid aptamers (reviewed, for example, in Zhou and Rossi, Nat Rev Drug Discov. (2017) 16(3):181-202). In some embodiments, an antigen-binding moiety comprises or consists of an antigen-binding peptide/polypeptide, *e.g.* a peptide aptamer, thioredoxin, monobody, anticalin, Kunitz domain, avimer, knottin, fynomer, atrimer, DARPin, affibody, nanobody (*i.e.* a single-domain antibody (sdAb)), affilin, armadillo repeat protein (ArmRP), OBody or fibronectin – reviewed *e.g.* in Reverdatto *et al.*, Curr Top Med Chem. 2015; 15(12): 1082–1101, which is hereby incorporated by reference in its entirety (see also *e.g.* Boersma *et al.*, J Biol Chem (2011) 286:41273-85 and Emanuel *et al.*, Mabs (2011) 3:38-48).

Commonly employed techniques for the production of fully human antibodies include (i) phage display, in which human antibody genes are expressed in phage display libraries, and (ii) production of antibodies in transgenic mice engineered to have human antibody genes (described in Park and Smolen, Advances in Protein Chemistry (2001) 56: 369-421). Briefly, in the human antibody gene-phage display technique,

genes encoding the VH and VL chains are generated by PCR amplification and cloning from 'naive' human lymphocytes, and assembled into a library from which they can be expressed either as disulfide-linked Fab fragments or as single-chain Fv (scFv) fragments. The Fab- or scFv-encoding genes are fused to a surface coat protein of filamentous bacteriophage and Fab or scFv capable of binding to the target of interest can then be identified by screening the library with antigen. Molecular evolution or affinity maturation procedures can be employed to enhance the affinity of the Fab/scFv fragment. In the transgenic mouse technique, mice in which the endogenous murine Ig gene loci have been replaced by homologous recombination with their human homologues are immunised with antigen, and monoclonal antibody is prepared by conventional hybridoma technology, to yield a fully human monoclonal antibody.

In some embodiments, an antigen-binding moiety according to the present disclosure comprises, or consists of, the antigen-binding region of an antibody (*e.g.* an antigen-binding fragment of an antibody). Antigen-binding moieties may be derived from antibodies. Antibody-derived antigen-binding moieties may comprise, or consist of, the antigen-binding region of an antibody (*e.g.* an antigen-binding fragment of an antibody). In some embodiments, an antigen-binding moiety may be or comprise the Fv (*e.g.* provided as an scFv) or the Fab region of an antibody that binds to a given target antigen, or the whole antibody.

The antigen-binding moieties of the present disclosure may be designed and prepared using the sequences of monoclonal antibodies (mAbs) capable of binding to a given target antigen (*e.g.* HER2). Antigen-binding regions of antibodies, such as variable fragment (Fv), Fab and $F(ab')_2$ fragments may also be used/provided. An 'antigen-binding region' is any fragment of an antibody that binds to the target for which the given antibody is specific.

In some embodiments, an antigen-binding moiety comprises the antibody heavy chain variable region (VH) and the antibody light chain variable region (VL) of an antibody capable of specific binding to the target antigen. In some embodiments, the antigen-binding moiety is or comprises the Fv (*e.g.* provided as an scFv) of an antibody. In some embodiments, the antigen-binding moiety is or comprises the Fab region of an antibody. In some embodiments, the antigen-binding moiety is or comprises the whole antibody (*i.e.* comprising variable and constant regions).

An antigen-binding moiety may be, or may comprise, an antigen-binding polypeptide, or an antigen-binding polypeptide complex. An antigen-binding moiety may comprise more than one polypeptide which together form an antigen-binding moiety. The polypeptides may associate covalently or non-covalently. In some embodiments, the polypeptides form part of a larger polypeptide comprising the polypeptides (*e.g.* in the case of scFv comprising VH and VL, or in the case of scFab comprising VH-CH1 and VL-CL).

In some embodiments, an antigen-binding moiety according to the present disclosure comprises, or consists of, a polypeptide complex formed by protein:protein interaction between constituent peptides/polypeptides of the antigen-binding moiety. An antigen-binding moiety may refer to a non-covalent or covalent complex of more than one polypeptide (*e.g.* 2, 3, 4, 6, or 8 polypeptides), *e.g.* an

IgG-like antigen-binding moiety comprising two heavy chain polypeptides and two light chain polypeptides.

Antibodies generally comprise six complementarity-determining regions CDRs; three in the heavy chain variable (VH) region: HC-CDR1, HC-CDR2 and HC-CDR3, and three in the light chain variable (VL) region: LC-CDR1, LC-CDR2, and LC-CDR3. The six CDRs together define the paratope of the antibody, which is the part of the antibody that binds to the target antigen.

The VH region and VL region comprise framework regions (FRs) either side of each CDR, which provide a scaffold for the CDRs. From N-terminus to C-terminus, VH regions comprise the following structure: N term-[HC-FR1]-[HC-CDR1]-[HC-FR2]-[HC-CDR2]-[HC-FR3]-[HC-CDR3]-[HC-FR4]-C term; and VL regions comprise the following structure: N term-[LC-FR1]-[LC-CDR1]-[LC-FR2]-[LC-CDR2]-[LC-FR3]-[LC-CDR3]-[LC-FR4]-C term.

There are several different conventions for defining antibody CDRs and FRs, such as those described in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), Chothia *et al.*, J. Mol. Biol. 196:901-917 (1987), and VBASE2, as described in Retter *et al.*, Nucl. Acids Res. (2005) 33 (suppl 1): D671-D674. The CDRs and FRs of the VH regions and VL regions of the antibody clones described herein were defined according to the international IMGT (ImMunoGeneTics) information system (LeFranc *et al.*, Nucleic Acids Res. (2015) 43 (Database issue):D413-22), which uses the IMGT V-DOMAIN numbering rules as described in Lefranc *et al.*, Dev. Comp. Immunol. (2003) 27:55-77. In preferred embodiments, the CDRs and FRs of antigen-binding molecules referred to herein are defined according to the IMGT information system.

In some embodiments, an antigen-binding moiety according to the present disclosure comprises, or consists of, an Fv region that binds to HER2. In some embodiments, the VH and VL regions of the Fv are provided as single polypeptide joined by a linker sequence, *i.e.* a single chain Fv (scFv).

The VL and light chain constant (CL) region, and the VH region and heavy chain constant 1 (CH1) region of an antigen-binding region of an antibody together constitute the Fab region. In some embodiments, the antigen-binding moiety comprises a Fab region comprising a VH, a CH1, a VL and a CL (*e.g.* Cκ or Cλ). In some embodiments, the Fab region comprises a polypeptide comprising a VH and a CH1 (*e.g.* a VH-CH1 fusion polypeptide), and a polypeptide comprising a VL and a CL (*e.g.* a VL-CL fusion polypeptide). In some embodiments, the Fab region comprises a polypeptide comprising a VH and a CL (*e.g.* a VH-CL fusion polypeptide) and a polypeptide comprising a VL and a CH1 (*e.g.* a VL-CH1 fusion polypeptide); that is, in some embodiments, the Fab region is a CrossFab region. In some embodiments, the VH, CH1, VL and CL regions of the Fab or CrossFab are provided as single polypeptide joined by linker regions, *i.e.* as a single chain Fab (scFab) or a single chain CrossFab (scCrossFab).

In some embodiments, an antigen-binding moiety described herein comprises, or consists of, a whole antibody which binds to HER2. As used herein, 'whole antibody' refers to an antibody having a structure

which is substantially similar to the structure of an immunoglobulin (Ig). Different kinds of immunoglobulins and their structures are described *e.g.* in Schroeder and Cavacini J Allergy Clin Immunol. (2010) 125(202): S41-S52, which is hereby incorporated by reference in its entirety.

Immunoglobulins of type G (*i.e.* IgG) are ~150 kDa glycoproteins comprising two heavy chains and two light chains. From N- to C-terminus, the heavy chains comprise a VH followed by a heavy chain constant region comprising three constant domains (CH1, CH2, and CH3), and similarly the light chains comprise a VL followed by a CL. Depending on the heavy chain, immunoglobulins may be classed as IgG (*e.g.* IgG1, IgG2, IgG3, IgG4), IgA (*e.g.* IgA1, IgA2), IgD, IgE, or IgM. The light chain may be kappa (κ) or lambda (λ).

In some embodiments, the antigen-binding moiety comprises, or consists of, an IgG (*e.g.* IgG1, IgG2, IgG3, IgG4), IgA (*e.g.* IgA1, IgA2), IgD, IgE, or IgM which binds to HER2.

In some embodiments, an antigen-binding moiety of the present disclosure comprises one or more regions (*e.g.* CH1, hinge, CH2, CH3, *etc.*) of an immunoglobulin heavy chain constant sequence. In some embodiments, the immunoglobulin heavy chain constant sequence is, or is derived from, the heavy chain constant sequence of an IgG (*e.g.* IgG1, IgG2, IgG3, IgG4), IgA (*e.g.* IgA1, IgA2), IgD, IgE or IgM, *e.g.* a human IgG (*e.g.* hIgG1, hIgG2, hIgG3, hIgG4), hIgA (*e.g.* hIgA1, hIgA2), hIgD, hIgE or hIgM. In some embodiments, the immunoglobulin heavy chain constant sequence is, or is derived from, the heavy chain constant sequence of a human IgG1 allotype (*e.g.* G1m1, G1m2, G1m3 or G1m17).

In some embodiments, an antigen-binding moiety comprises one or more polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:31 or 36. In some embodiments, an antigen-binding moiety comprises one or more polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:32. In some embodiments, an antigen-binding moiety comprises one or more polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:33 or 48. In some embodiments, an antigen-binding moiety comprises one or more polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:34 or 37. In some embodiments, an antigen-binding moiety comprises one or more polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or

100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:38, 39, 49 or 50. In some embodiments, an antigen-binding moiety comprises one or more polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:30, 35, 46 or 47.

In some embodiments, an antigen-binding moiety of the present disclosure comprises one or more regions of an immunoglobulin light chain constant sequence. In some embodiments, the immunoglobulin light chain constant sequence is human immunoglobulin kappa constant (IGKC; Cκ). In some embodiments, the immunoglobulin light chain constant sequence is a human immunoglobulin lambda constant (IGLC; Cλ), *e.g.* IGLC1, IGLC2, IGLC3, IGLC6 or IGLC7.

In some embodiments, an antigen-binding moiety comprises one or more polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:40, 41, 42, 43, 44 or 45.

In some embodiments described herein, one or more amino acids of an amino acid sequence referred to herein (*e.g.* an amino acid sequence of an antigen-binding moiety, *e.g.* an amino acid sequence of a CDR or VH/VL region) are substituted with another amino acid. A substitution comprises substitution of an amino acid residue with a non-identical 'replacement' amino acid residue. A replacement amino acid residue of a substitution according to the present disclosure may be a naturally-occurring amino acid residue (*i.e.* encoded by the genetic code) which is non-identical to the amino acid residue at the relevant position of the equivalent, unsubstituted amino acid sequence, selected from: alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile): leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val). In some embodiments, a replacement amino acid may be a non-naturally occurring amino acid residue – *i.e.* an amino acid residue other than those recited in the preceding sentence. Examples of non-naturally occurring amino acid residues include norleucine, ornithine, norvaline, homoserine, aib, and other amino acid residue analogues such as those described in Ellman, *et al.*, Meth. Enzym. 202 (1991) 301-336.

In some embodiments, a substitution may be biochemically conservative. In some embodiments, where an amino acid to be substituted is provided in one of rows 1 to 5 of the table below, the replacement amino acid of the substitution is another, non-identical amino acid provided in the same row:

Row	Shared property	Amino acids
1	Hydrophobic	Met, Ala, Val, Leu, Ile, Trp, Tyr, Phe, Norleucine
2	Neutral hydrophilic	Cys, Ser, Thr, Asn, Gln

3	Acidic or negatively-charged	Asp, Glu
4	Basic or positively-charged	His, Lys, Arg
5	Orientation influencing	Gly, Pro

By way of illustration, in some embodiments wherein substitution is of a Met residue, the replacement amino acid may be selected from Ala, Val, Leu, Ile, Trp, Tyr, Phe and Norleucine.

In some embodiments, a replacement amino acid in a substitution may have the same side chain polarity as the amino acid residue it replaces. In some embodiments, a replacement amino acid in a substitution may have the same side chain charge (at pH 7.4) as the amino acid residue it replaces:

Amino Acid	**Side-chain polarity**	**Side-chain charge (pH 7.4)**
Alanine	nonpolar	neutral
Arginine	basic polar	positive
Asparagine	polar	neutral
Aspartic acid	acidic polar	negative
Cysteine	nonpolar	neutral
Glutamic acid	acidic polar	negative
Glutamine	polar	neutral
Glycine	nonpolar	neutral
Histidine	basic polar	positive (10%) neutral (90%)
Isoleucine	nonpolar	neutral
Leucine	nonpolar	neutral
Lysine	basic polar	positive
Methionine	nonpolar	neutral
Phenylalanine	nonpolar	neutral
Proline	nonpolar	neutral
Serine	polar	neutral
Threonine	polar	neutral
Tryptophan	nonpolar	neutral
Tyrosine	polar	neutral
Valine	nonpolar	neutral

That is, in some embodiments, a nonpolar amino acid is substituted with another, non-identical nonpolar amino acid. In some embodiments, a polar amino acid is substituted with another, non-identical polar amino acid. In some embodiments, an acidic polar amino acid is substituted with another, non-identical acidic polar amino acid. In some embodiments, a basic polar amino acid is substituted with another, non-identical basic polar amino acid. In some embodiments, a neutral amino acid is substituted with another, non-identical neutral amino acid. In some embodiments, a positive amino acid is substituted with another,

non-identical positive amino acid. In some embodiments, a negative amino acid is substituted with another, non-identical negative amino acid.

In some embodiments, substitution(s) may be functionally conservative. That is, in some embodiments, the substitution may not affect (or may not substantially affect) one or more functional properties (*e.g.* target binding) of the antigen-binding moiety comprising the substitution as compared to the equivalent unsubstituted molecule.

The antigen-binding molecules of the present disclosure comprise an antigen-binding moiety that binds to HER2.

In some embodiments, the antigen-binding moiety comprises the CDRs of an antigen-binding moiety which is capable of binding to HER2. In some embodiments, the antigen-binding moiety comprises the FRs of an antigen-binding moiety which is capable of binding to HER2. In some embodiments, the antigen-binding moiety comprises the CDRs and the FRs of an antibody that is capable of binding to HER2. That is, in some embodiments the antigen-binding moiety comprises the VH region and the VL region of an antibody that is capable of binding to HER2.

In some embodiments, an antigen-binding moiety which is capable of binding to HER2 according to the present disclosure may be, or may be derived from, a HER2-binding antibody selected from: trastuzumab (DrugBank Acc. No. DB00072; which is formed of the polypeptides having the amino acid sequences of SEQ ID NO:12 and SEQ ID NO:13), pertuzumab (DrugBank Acc. No. DB06366), margetuximab (DrugBank Acc. No. DB14967), timigutuzumab (described *e.g.* in Fiedler *et al.*, ESMO Open. (2018) 3(4): e000381), CT-P6 (described *e.g.* in Jeong *et al.*, Expert Opin Biol Ther (2019) 19(10):1085-1095), PF-05280014 (described *e.g.* in Paik, BioDrugs (2018) 32(5):515-518), SB3 (described *e.g.* in Lamb, BioDrugs (2018) 32(3):293-296), ABP-980 (described *e.g.* in Dhillon, BioDrugs (2018) 32(5):511-514), MYL-1410 (described *e.g.* in Rugo *et al.*, JAMA (2017) 317:37-47) BCD-022 (described *e.g.* in Alexeev *et al.*, BMC Cancer (2020) 20: 783, HD201 (described *e.g.* in Pivot *et al.*, Clin Ther. (2018) 40(3):396-405.e4) and HLX22 (described *e.g.* in Yang *et al.* BioDrugs. 2022; 36(3): 393-409). In some embodiments, the antigen-binding moiety is, or is derived from, trastuzumab.

In some embodiments the antigen-binding moiety is capable of binding the same region of DLL3, or an overlapping region of HER2, to the region of HER2 which is bound by an antigen-binding molecule comprising the VH and VL sequences of a HER2-binding antibody described hereinabove. In some embodiments the antigen-binding moiety is capable of binding the same region of HER2, or an overlapping region of HER2, to the region of HER2 which is bound by an antigen-binding molecule comprising the VH and VL sequences of trastuzumab (*i.e.* an antigen-binding molecule comprising a VH having the amino acid sequence of SEQ ID NO:14, and a VL having the amino acid sequence of SEQ ID NO:22).

In some embodiments, the antigen-binding moiety is capable of binding to a polypeptide comprising, or consisting of, the amino acid sequence of one of SEQ ID NOs:1, 7 or 9.

The ability of an antigen-binding moiety to bind to a given peptide/polypeptide can be analysed by methods well known to the skilled person, including analysis by ELISA, immunoblot (*e.g.* western blot), immunoprecipitation, Surface Plasmon Resonance (SPR; see *e.g.* Hearty *et al.*, Methods Mol Biol (2012) 907:411-442) or Bio-Layer Interferometry (see *e.g.* Lad *et al.*, (2015) J Biomol Screen 20(4): 498-507).

In embodiments where the antigen binding moiety is capable of binding to a peptide/polypeptide comprising a reference amino acid sequence, the peptide/polypeptide may comprise one or more additional amino acids at one or both ends of the reference amino acid sequence. In some embodiments the peptide/polypeptide comprises *e.g.* 1-5, 1-10, 1-20, 1-30, 1-40, 1-50, 5-10, 5-20, 5-30, 5-40, 5-50, 10-20, 10-30, 10-40, 10-50, 20-30, 20-40 or 20-50 additional amino acids at one or both ends of the reference amino acid sequence. In some embodiments the additional amino acid(s) provided at one or both ends (*i.e.* the N-terminal and C-terminal ends) of the reference sequence correspond to the positions at the ends of the reference sequence in the context of the amino acid sequence of HER2.

In some embodiments the antigen-binding moiety is capable of binding to a peptide/polypeptide which is bound by an antibody comprising the VH and VL sequences of trastuzumab (*i.e.* an antigen-binding molecule comprising a VH having the amino acid sequence of SEQ ID NO:14, and a VL having the amino acid sequence of SEQ ID NO:22).

In some embodiments the antigen-binding moiety is capable of binding to a peptide/polypeptide which is bound by an antibody comprising the VH and VL sequences of a HER2-binding antibody described hereinabove.

In some embodiments, the antigen-binding moiety comprises the heavy chain CDRs and the light chain CDRs of a HER2-binding antibody described hereinabove. In some embodiments, the antigen-binding moiety comprises the VH and VL of a HER2-binding antibody described hereinabove. In some embodiments, the antigen-binding moiety comprises the heavy chain polypeptide (*i.e.* comprising VH, CH1, CH2 and CH3 region sequences) and light chain polypeptide (*i.e.* comprising VL and CL region sequences) of a HER2-binding antibody described hereinabove.

In some embodiments, the antigen-binding moiety comprises the heavy chain CDRs and the light chain CDRs of trastuzumab. In some embodiments, the antigen-binding moiety comprises the VH and VL of trastuzumab. In some embodiments, the antigen-binding moiety comprises the heavy chain polypeptide (*i.e.* comprising VH, CH1, CH2 and CH3 region sequences) and light chain polypeptide (*i.e.* comprising VL and CL region sequences) of trastuzumab.

In some embodiments, the antigen-binding moiety comprises:
a VH region incorporating the following CDRs:

HC-CDR1 having the amino acid sequence of SEQ ID NO:15
HC-CDR2 having the amino acid sequence of SEQ ID NO:16
HC-CDR3 having the amino acid sequence of SEQ ID NO:17,
or a variant thereof in which 1 or 2 or 3 amino acids in HC-CDR1, and/or in which 1 or 2 or 3 amino acids in HC-CDR2, and/or in which 1 or 2 or 3 amino acids in HC-CDR3 are substituted with another amino acid; and
a VL region incorporating the following CDRs:
LC-CDR1 having the amino acid sequence of SEQ ID NO:23
LC-CDR2 having the amino acid sequence of SEQ ID NO:24
LC-CDR3 having the amino acid sequence of SEQ ID NO:25;
or a variant thereof in which 1 or 2 or 3 amino acids in LC-CDR1, and/or in which 1 or 2 or 3 amino acids in LC-CDR2, and/or in which 1 or 2 or 3 amino acids in LC-CDR3 are substituted with another amino acid.

In some embodiments, the antigen-binding moiety comprises:
a VH region comprising an amino acid sequence having at least 60% sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:14; and
a VL region comprising an amino acid sequence having at least 60% sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:22.

In some embodiments, an antigen-binding moiety comprises, or consists of:
(i) one or more (*e.g.* two) polypeptides comprising, or consisting of, an amino acid sequence having at least 70% sequence identity, more preferably one of ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:12; and
(ii) one or more (*e.g.* two) polypeptides comprising, or consisting of, an amino acid sequence having at least 70% sequence identity, more preferably one of ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:13.

In some embodiments, an antigen-binding molecule of the present disclosure (*e.g.* an antigen-binding moiety thereof) comprises an Fc region. As used herein, an 'Fc region' refers to a polypeptide complex formed by interaction between two polypeptides, each polypeptide comprising the CH2-CH3 region of an immunoglobulin (Ig) heavy chain constant sequence.

Herein, a 'CH2 domain' refers to an amino acid sequence corresponding to the CH2 domain of an immunoglobulin (Ig). The CH2 domain is the region of an Ig formed by positions 231 to 340 of the immunoglobulin constant domain, according to the EU numbering system described in Edelman *et al.*, Proc Natl Acad Sci USA (1969) 63(1): 78-85. A 'CH3 domain' refers to an amino acid sequence

corresponding to the CH3 domain of an immunoglobulin (Ig). The CH3 domain is the region of an Ig formed by positions 341 to 447 of the immunoglobulin constant domain, according to the EU numbering system described in Edelman *et al.*, Proc Natl Acad Sci USA (1969) 63(1): 78-85. A 'CH2-CH3 region' refers to an amino acid sequence corresponding to the CH2 and CH3 domains of an immunoglobulin (Ig). The CH2-CH3 region is the region of an Ig formed by positions 231 to 447 of the immunoglobulin constant domain, according to the EU numbering system described in Edelman *et al.*, Proc Natl Acad Sci USA (1969) 63(1): 78-85.

In some embodiments, a CH2 domain, CH3 domain and/or a CH2-CH3 region according to the present disclosure corresponds to the CH2 domain/CH3 domain/CH2-CH3 region of an IgG (*e.g.* IgG1, IgG2, IgG3, IgG4), IgA (*e.g.* IgA1, IgA2), IgD, IgE or IgM. In some embodiments, the CH2 domain, CH3 domain and/or a CH2-CH3 region corresponds to the CH2 domain/CH3 domain/CH2-CH3 region of a human IgG (*e.g.* hIgG1, hIgG2, hIgG3, hIgG4), hIgA (*e.g.* hIgA1, hIgA2), hIgD, hIgE or hIgM. In some embodiments, the CH2 domain, CH3 domain and/or a CH2-CH3 region corresponds to the CH2 domain/CH3 domain/CH2-CH3 region of a human IgG1 allotype (*e.g.* G1m1, G1m2, G1m3 or G1m17). In some embodiments, the CH2 domain, CH3 domain and/or a CH2-CH3 region corresponds to the CH2 domain/CH3 domain/CH2-CH3 region of human IgG1 allotype G1m3.

Fc regions provide for interaction with Fc receptors and other molecules of the immune system to bring about functional effects. Fc-mediated effector functions are reviewed *e.g.* in Jefferis *et al.*, Immunol Rev 1998 163:59-76 (hereby incorporated by reference in its entirety), and are brought about through Fc-mediated recruitment and activation of immune cells (*e.g.* macrophages, dendritic cells, neutrophils, basophils, eosinophils, platelets, mast cells, NK cells and T cells) through interaction between the Fc region and Fc receptors expressed by the immune cells, recruitment of complement pathway components through binding of the Fc region to complement protein C1q, and consequent activation of the complement cascade. Fc-mediated functions include Fc receptor binding, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), formation of the membrane attack complex (MAC), cell degranulation, cytokine and/or chemokine production, and antigen processing and presentation.

In some embodiments, an antigen-binding molecule according to the present disclosure comprises one or more (*e.g.* two) polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:38 or 39. In some embodiments, the antigen-binding molecule comprises an Fc region comprising one or more (e.g. two) polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, e.g. one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:38 or 39.

Modifications to antibody Fc regions that influence Fc-mediated functions are known in the art, such as those described *e.g.* in Wang *et al.*, Protein Cell (2018) 9(1):63-73, which is hereby incorporated by reference in its entirety. Exemplary Fc region modifications known to influence antibody effector function are summarised in Table 1 of Wang *et al.*, Protein Cell (2018) 9(1):63-73. In some embodiments, the antigen-binding molecule of the present disclosure comprises an Fc region comprising modification to increase or reduce an Fc-mediated function as compared to an antigen-binding molecule comprising the corresponding unmodified Fc region. Where an Fc region/CH2/CH3 is described as comprising modification(s) 'corresponding to' reference substitution(s), equivalent substitution(s) in the homologous Fc/CH2/CH3 are contemplated.

In some embodiments, the antigen-binding molecule of the present disclosure comprises an Fc region comprising modification. In some embodiments, the antigen-binding molecule of the present disclosure comprises an Fc region comprising modification in one or more of the CH2 and/or CH3 regions.

In some embodiments, the Fc region comprises modification to reduce/prevent an Fc-mediated function (*e.g.* ADCC, ADCP, CDC). In some embodiments, the Fc region comprises modification to reduce/prevent ADCC. In some embodiments, the Fc region comprises modification to reduce/prevent CDC. In some embodiments, the Fc region comprises modification to reduce/prevent binding to an Fc receptor. In some embodiments, the Fc region comprises modification to reduce/prevent binding to an Fcγ receptor. In some embodiments, the Fc region comprises modification to reduce/prevent glycosylation of the amino acid residue corresponding to N297.

In some embodiments, the Fc region comprises modification at the amino acid residue corresponding to N297. In some embodiments, the Fc region comprises modification corresponding to N297A or N297Q or N297G as described in Leabman *et al.*, Mabs. (2013) 5:896–903. Substitution of 'N297' with 'A', 'G' or 'Q' is known to eliminate glycosylation, and thereby reduce Fc binding to C1q and Fcγ receptors, and thus also reducing CDC and ADCC. In some embodiments, the Fc region comprises modification corresponding to N297A.

In some embodiments, an antigen-binding molecule according to the present disclosure comprises one or more (*e.g.* two) polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, *e.g.* one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:49 or 50. In some embodiments, the antigen-binding molecule comprises an Fc region comprising one or more (e.g. two) polypeptides comprising an amino acid sequence having at least 60% amino acid sequence identity, e.g. one of ≥70%, ≥75%, ≥80%, ≥85%, ≥86%, ≥87%, ≥88%, ≥89%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:49 or 50.

An antigen-binding molecule according to the present disclosure may comprise a HER2-binding moiety according to any embodiment described hereinabove, and a linker-payload moiety as described hereinbelow.

Linker-payload moieties

Aspects and embodiments of the present disclosure relate to antigen-binding molecules comprising a linker-payload moiety. As used herein, a linker-payload moiety refers to a moiety comprising one or more payload moieties, and a linker moiety for linking the payload moiety(/ies) to the antigen-binding region of the antigen-binding molecule.

A payload moiety according to the present disclosure comprises or consist of a cytotoxic agent. Payload moieties are described *e.g.* in Parslow *et al.*, Biomedicines. 2016 Sep; 4(3):14, Goundry and Parker, Org. Process Res. Dev. (2022) 26, 8, 2121–2123, Fu *et al.*, Signal Transduction and Targeted Therapy (2022) 7:93, Wang *et al.*, Acta Pharmaceutica Sinica B (2023) 13 (10): 4025-4059 and Conilh *et al.*, J. Hematol. & Oncol. (2023) 16:3, all of which are hereby incorporated by reference in their entirety.

In particular, the present disclosure relates to antigen-binding molecules comprising at least one linker-payload moiety, wherein the antigen-binding molecule comprises (a) a payload moiety which is a DNA damage response (DDR) inhibitor, and (b) a payload moiety which is a DNA topoisomerase I (TOP1) inhibitor.

Hereinbelow, for conciseness, ‘a payload moiety which is a DDR inhibitor’ may be referred to simply as a ‘DDR inhibitor moiety’, and similarly ‘a payload moiety which is a TOP1 inhibitor’ may be referred to simply as a ‘TOP1 inhibitor moiety’.

The DNA Damage Response (DDR) is a complex network of mechanisms for detecting and repair DNA damage, in order to preserve genomic stability. The DDR is reviewed *e.g.* in Groelly *et al.*, Nature Reviews Cancer (2023) 23:78–94 and Molinaro *et al.*, Cancers (Basel). (2021) 13(15): 3819, both of which are hereby incorporated by reference in their entirety.

The detection of DNA damage and initiation of repair pathways is mediated by proteins such as ATM (Ataxia-Telangiectasia Mutated) and ATR (Ataxia-Telangiectasia and Rad3-Related). ATM is a protein kinase activated by double-strand breaks in DNA, and which initiates downstream signaling. ATR is activated by DNA damage and replication stress, and in particular responds to single-strand breaks and stalled DNA replication forks. CHK1 and CHK2 (Checkpoint Kinases 1 and 2) are downstream effectors of ATM and ATR, and phosphorylate various target proteins to stop cell cycle progression, and facilitate DNA repair. PARP (Poly ADP-Ribose Polymerase) is involved in repairing single-strand DNA breaks, helping to recruit repair factors and the formation of repair complexes at the sites of DNA damage. DNA-PK (DNA-Dependent Protein Kinase) helps bring broken DNA ends together for non-homologous end-joining (NHEJ), for repairing double-strand breaks. The DDR is facilitated by cell cycle regulation through WEE1 and PLK1 (Polo-Like Kinase 1). WEE1 is a kinase that phosphorylates and inhibits CDKs (Cyclin-

Dependent Kinases), thereby delaying cell cycle progression and allowing more time for DNA damage repair prior to cell division. PLK1 regulates the cell cycle checkpoint and promotes repair processes, through phosphorylation of Polθ. RAD51 is an ATPase involved in DNA repair. Ubiquitin-specific proteases (USPs) modulate the DDR by influencing the ubiquitination of proteins involved in the DDR. Protein kinase membrane associated tyrosine/threonine 1 (PKMYT1) regulates cell cycle and participates in DDR-related signaling. Aurora-A may contribute to the G2 DNA damage checkpoint through PLK1 and CDC25B activation, and is important in the mitotic DNA damage response.

Most cancerous cells have a greater dependency on the DDR than non-cancerous cells. DDR inhibitors and their use for the treatment of cancers is described *e.g.* in Cheng *et al.*, Eur J Med Chem. (2022) 230:114109, Wang *et al.*, Front Immunol. (2022) 13:854730 and Choi and Lee, Int J Mol Sci. (2022) 23(3):1701, all of which are hereby incorporated by reference in their entirety.

In some embodiments, a DDR inhibitor moiety according to the present disclosure is, or comprises, a DDR inhibitor selected from:

(a) a PARP inhibitor (*e.g.* olaparib, rucaparib, niraparib, talazoparib, veliparib, pamiparib, simmiparib, senaparib, SC-10914, 2X-121, AMXI-5001, JPI-547, AZD5305, IDX-1197, TQB-3823, HWH-340, AsiDNA, STP-1002, RBN-2397, fluzoparib, NMS-03305293, AZD9574);

(b) an ATM inhibitor (*e.g.* CP-466722, KU-55933, KU-60019, KU-59403, AZ31, AZ32, AZD0156, AZD1390, XRD-0394, M4076, M3541, WSD-0628, SYH-2051, IMP-08, SP-1161, INT-6C4/5C4);

(c) an ATR inhibitor (*e.g.* M6620 (berzosertib), M4344 (VX-803), AZD6738 (ceralasertib), BAY1895344 (elimusertib), RP3500 (camonsertib), ATRN119, ART380, IMP9064, HRS2398, M1774, IMP9064, SC0245, LF0397, NU6027);

(d) a WEE1 inhibitor (*e.g.* adavosertib, Debio 0123, PD0166285, PD0407824, AZD1775, ZN-c3, IMP7068, SY4835, SCO191, IMP7068);

(e) a CHK1/2 inhibitor (*e.g.* CBP-501, prexasertib, MK-8776, GDC-0575, SRA-737, PF-00477736, AZD7762, LY2603618 (rabusertib), LY2880070,, XL884, BEBT260, MU380, NU7441, KU-5778);

(f) a DNA-PK inhibitor (*e.g.* CC-115, LY-3023414, AsiDNA, M3814 (nedisertib, peposertib), VX-984 (M9831), BR-101801, XRD-0394, SL901, XZP-6877, IMP-11, ZL-2201, BR-2006, AZD7648, NU7441);

(g) a PLK1 inhibitor (*e.g.* BI-6727 (volasertib), PCM-075 (onvansertib) , CYC140 (plogosertib));

(h) a Polθ inhibitor (*e.g.* ART4215, ART6043, novobiocin, RP-6685, RP-3467);

(i) a RAD51 inhibitor (*e.g.* CYT0851);

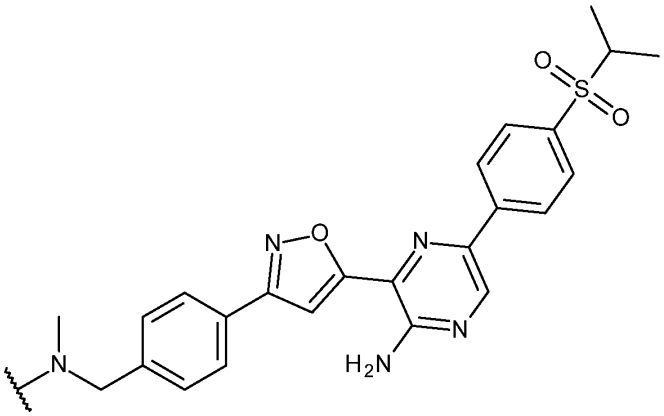
(j) an inhibitor of a ubiquitin-specific protease (USP) family enzyme (*e.g.* an inhibitor of USP11, USP7, USP4, USP37, USP39, USP45, USP24 and/or USP1; *e.g.* KSQ-4279);

(k) a PKMYT1 inhibitor (*e.g.* RP6306); and

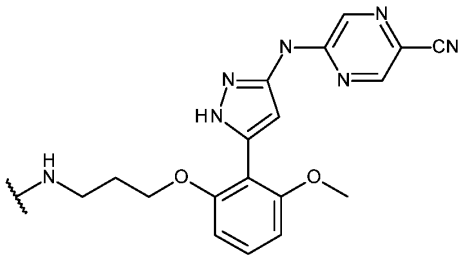
(l) an Aurora-A inhibitor (*e.g.* alisertib, WJ05129 (JS112), JAB-2485).

In some embodiments, the DDR inhibitor moiety is, or comprises, ceralasertib.

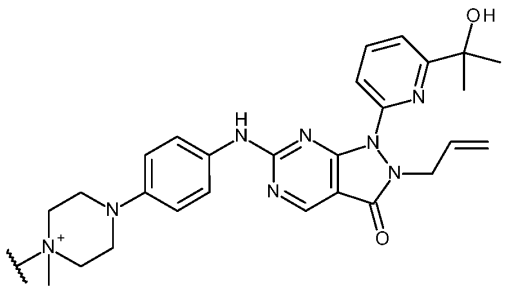
In some embodiments, the DDR inhibitor moiety is, or comprises, berzosertib:



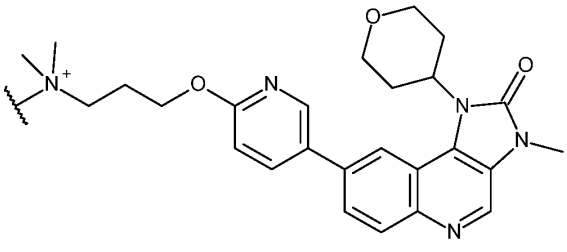
In some embodiments, the DDR inhibitor moiety is, or comprises, prexasertib:



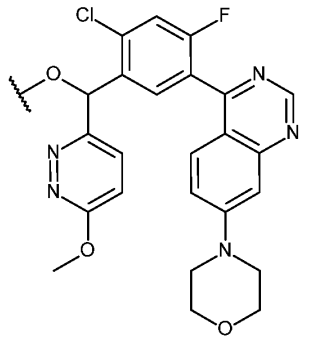
In some embodiments, the DDR inhibitor moiety is, or comprises, adavosertib, which can be linked as follows, as well as through other positions:



In some embodiments, the DDR inhibitor moiety is, or comprises, AZD0156, which can be linked as follows, as well as through other positions:



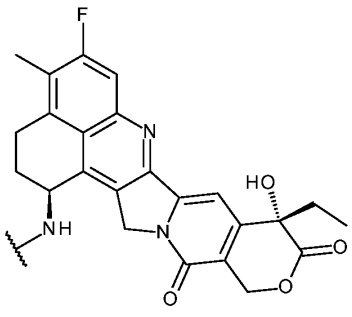
In some embodiments, the DDR inhibitor moiety is, or comprises, nedisertib:



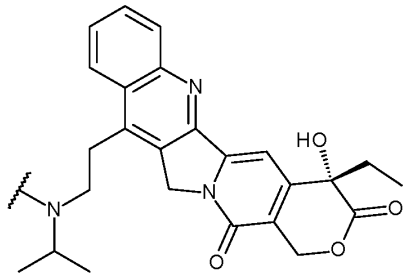
During DNA replication and transcription, significant torsional strain is placed on the DNA helix, and this is relieved through the action of DNA topoisomerases I and II (TOP1 and TOP2), which cleave the DNA strand and allow it to untwist, before resealing the breaks (see *e.g.* Delgado *et al.*, Biochem J. (2018) 475(2): 373–398). DNA topoisomerase inhibitors block the resealing step, resulting in DNA fragmentation and cell death. DNA topoisomerase I inhibitors and their use for the treatment of cancers is described *e.g.* in Pommier, Chem Rev. (2009) 109(7): 2894-2902, Li *et al.*, Am J Cancer Res. (2017) 7(12): 2350-2394 and Thomas and Pommier, Clin Cancer Res. (2019) 25(22): 6581-6589, all of which are hereby incorporated by reference in their entirety.

In some embodiments, a TOP1 inhibitor moiety according to the present disclosure is, or comprises, a TOP1 inhibitor selected from: camptothecin or a derivative thereof, exatecan, exatecan mesylate (DX-8951f), *N*-glycyl-exatecan, SN-38, DXd(1), DXd(2), irinotecan, etirinotecan, FL118, topotecan, gimatecan, belotecan, deruxtecan, belotecan, rubitecan, lurtotecan, diflomotecan, karenitecan, Silat can, namitecan, elomotecan, DRF-1042, delimotecan, NSC606985, chimmitecan, ZBH-1205, Genz-644282, non-CPT1, indotecan (LMP-400), indimitecan (LMP-776) , AZ14170132, SHR9265, Ed-04, KL610023, A1.9, ZD06519, P1003, P1021, VIP126, ZBH-01 and LMP-744.

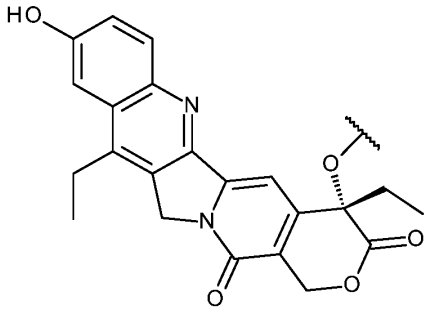
In some embodiments, the TOP1 inhibitor moiety is, or comprises, exatecan:



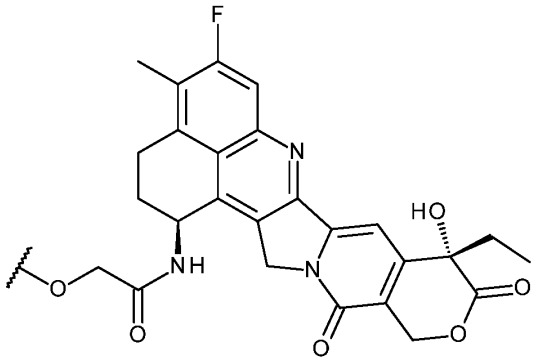
In some embodiments, the TOP1 inhibitor moiety is, or comprises, belotecan:



In some embodiments, the TOP1 inhibitor moiety is, or comprises, SN38:



In some embodiments, the TOP1 inhibitor moiety is, or comprises, DXd:



A linker moiety according to the present disclosure may be any moiety suitable for linking the payload moiety to the antigen-binding region of the antigen-binding molecule of the present disclosure. Accordingly, they generally comprise a group enabling connection to the payload moiety, a group connecting conjugation to the antigen-binding region of the antigen-binding molecule, and a linker core.

Linker moieties are described *e.g.* in Su *et al.*, Acta Pharmaceutica Sinica B (2021) 11(12): 3889-3907, Fu *et al.*, Signal Transduction and Targeted Therapy (2022) 7:93,

A linker moiety according to the present disclosure may be a cleavable linker moiety or a non-cleavable moiety.

Cleavable linkers typically utilise differences between the environment of systemic circulation and that in cancer cells/the tumor microenvironment to release the payload moiety in a targeted manner. Cleavable linkers include chemical cleavage linkers (*e.g.* acid-cleavable linkers, GSH-cleavable linkers, Fe(II)-cleavable linkers) and enzyme cleavage linkers (*e.g.* cathepsin-cleavable linkers, glycosidase-cleavable linkers, phosphatase-cleavable linkers, sulfatase-cleavable linkers).

In some embodiments, a linker moiety according to the present disclosure is a chemical cleavage linker. In some embodiments, a linker moiety according to the present disclosure is an enzyme cleavage linker. In some embodiments, a linker moiety is an acid-cleavable linker, *e.g.* comprising a hydrazone group (*e.g.* a 6-maleimidocaproylhydrazone linker or a (4-(4-acetylphenoxy)butanoic acid) hydrazaone linker), a carbonate group or a silyl ether group. In some embodiments, a linker moiety is a GSH-cleavable linker, *e.g.* comprising a disulfide group. In some embodiments, a linker moiety is a Fe(II)-cleavable linker, *e.g.* comprising a 1,2,4-trioxolane group. In some embodiments, a linker moiety is a cathepsin-cleavable linker, *e.g.* comprising a dipeptide (*e.g.* a valine-citrulline linker, a phenylalanine–lysine linker or a valine-alanine linker), a triglycyl peptide (CX) or a cBu-Cit group. In some embodiments, the linker moiety is GGFG (Glycine-Glycine-Phenylalanine-Glycine). In some embodiments, a linker moiety is a glucuronidase-cleavable linker, *e.g.* comprising a β-glucuronide group. In some embodiments, a linker moiety is a glycosidase-cleavable linker, *e.g.* comprising a β-galactoside group. In some embodiments, a linker moiety is a phosphatase-cleavable linker, *e.g.* comprising a pyrophosphate group. In some embodiments, a linker moiety is a sulfatase-cleavable linker, *e.g.* comprising an arylsulfate group. In some embodiments, a linker moiety is a photo-responsive linker, *e.g.* comprising a heptamethine cyanine fluorophore group, an O-nitrobenzyl group or a PC4AP group. In some embodiments, a linker moiety is a biorthogonal cleavable linker, *e.g.* comprising a dsProc group.

Non-cleavable linkers remain inert in common chemical and enzymatic environments in the body, with the payload moiety being released following processing of the ADC by cellular lysosomal proteases. Non-cleavable linkers include linkers comprising thioether or maleimidocaproyl groups.

In some embodiments, a linker moiety is a thioether linker. In some embodiments, a linker moiety is a maleimidocaproyl linker, *e.g.* comprising a 2-(maleimidomethyl)-1,3-dioxane (MD) group or a Mal-PAB group. In some embodiments, a linker moiety comprises a polyethylene glycol (PEG) group and an alkyne, triazole or piperazine group.

In some embodiments, a linker-payload moiety according to the present disclosure has an amino (-NH2) group for linkage to the antigen-binding moiety, for example by enzymatic conjugation. In some of these embodiments, enzymatic conjugation with microbial transglutaminase may be used to conjugate the linker-payload moiety to the antigen-binding moiety.

In some embodiments, a linker moiety further comprises a spacer moiety. Spacer moieties are sometimes required sue to the bulky nature of payload moieties. Commonly employed spacer moieties include para-

aminobenzyl carbamate (PABC), hemiaminal groups, PEG groups, polar acyl sulfamide groups, polar carbamoyl sulfamide groups and HydraSpace (described *e.g.* in Verkade *et al.*, Antibodies (Basel) (2018) 7(1):12 and WO 2016/053107 A1, both of which are hereby incorporated by reference in their entirety). PABC is commonly employed as a spacer moiety in cathepsin-cleavable dipeptide linkers, β-glucuronidase-cleavable linkers, β-galactosidase-cleavable linkers and phosphatase cleavable linkers. In some embodiments, para-aminobenzyl (PAB) is used as a spacer group.

In some embodiments, an antigen-binding molecule according to the present disclosure comprises a linker-payload moiety, which in turn comprises both a DDR inhibitor moiety and a TOP1 inhibitor moiety. That is, in some embodiments, the antigen-binding molecule comprises a linker-payload moiety comprising: (a) a DDR inhibitor moiety, and (b) a TOP1 inhibitor moiety. In some embodiments, the DDR inhibitor moiety and TOP1 inhibitor moieties are provided in the same linker-payload moiety. In some embodiments, the DDR inhibitor moiety and the TOP1 inhibitor moiety are connected to the antigen-binding moiety of the antigen-binding molecule via the same linker moiety.

Approaches for the attachment of multiple payload moieties to a single linker moiety are described *e.g.* in Yamazaki, *et al.*, Nat Commun. (2021) 12(1): 3528, Kumar *et al.*, Bioorg Med Chem Lett (2018) 28 (23-24): 3617-3621, Levengood, *et al.*, Angew Chem Int Ed Engl (2017) 56(3): 733-737 and Wang *et al.*, Acta Pharmaceutica Sinica B (2023) 13(10): 4025-4059, all of which are hereby incorporated by reference in their entirety.

In some embodiments, the DDR inhibitor and TOP1 inhibitor payload moieties are provided in the same linker-payload moiety. In some embodiments, the DDR inhibitor and TOP1 inhibitor payload moieties are connected to the antigen-binding moiety of the antigen-binding molecule through the same linker moiety.

In some embodiments, the DDR inhibitor and TOP1 inhibitor payload moieties are connected to the antigen-binding moiety of the antigen-binding molecule through a branched linker moiety. The linker may comprise of multiple branches to allow for DAR flexibility.

In some embodiments, the DDR inhibitor and TOP1 inhibitor payload moieties are connected to the antigen-binding moiety of the antigen-binding molecule through a linker moiety that has increased hydrophilicity.

In some embodiments, the DDR inhibitor and TOP1 inhibitor payload moieties are connected to the antigen-binding moiety of the antigen-binding molecule through a branched hydrophilic linker moiety.

In some embodiments, the DDR inhibitor moiety and the TOP1 inhibitor moiety are connected to the linker moiety of the linker-payload moiety via orthogonal functional groups. In some embodiments, the linker-payload moiety comprises a trifunctional linker moiety providing for linkage of an antigen0binding moiety to two different payload moieties. Such approaches to the production of a linker-payload moiety

comprising two different payloads are described *e.g.* in Yamazaki, *et al.*, Nat Commun. (2021) 12(1): 3528 and Kumar *et al.*, Bioorg Med Chem Lett (2018) 28 (23-24): 3617-3621.

Kumar *et al.*, Bioorg Med Chem Lett (2018) 28 (23-24): 3617-3621 describes a branched linker moiety comprising:

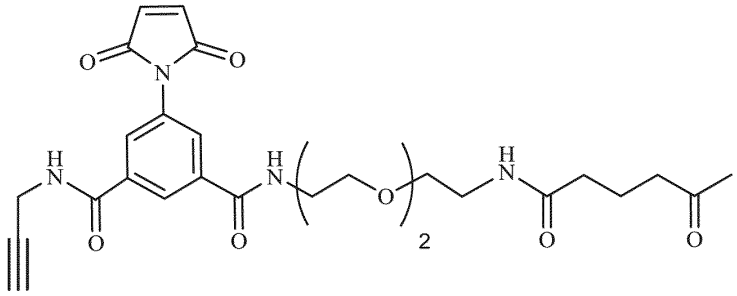
(a) a group for connection to an antigen-binding moiety (which is a self-stabilizing N-aryl maleimide); and

(b) two orthogonal functional groups for linking payload moieties, which are:

(i) an alkyne group, for incorporation of a payload moiety via copper-mediated azide-alkyne cycloaddition (CuAAC), and

(ii) a ketone group, for incorporation of a payload moiety via for aminooxy reaction resulting in oxime linkage.

The branched linker moiety described in Kumar *et al.*, Bioorg Med Chem Lett (2018) 28 (23-24): 3617-3621 has the following structure:



Accordingly, in some embodiments a linker-payload moiety according to the present disclosure comprises a linker moiety comprising: (i) a moiety derived from a group for connection to the antigen-binding moiety (*e.g.* a self-stabilizing N-aryl maleimide group), (ii) a moiety derived from an alkyne group suitable for incorporating a first payload moiety via CuAAC (i.e. a divalent triazole), and (iii) a moiety derived from a ketone group suitable for incorporating a second payload moiety via aminooxy reaction resulting in oxime linkage (i.e. an oxime) . In accordance with such embodiments, in some cases the first payload moiety is a DDR inhibitor moiety as described herein, and the second payload moiety is a TOP1 inhibitor moiety as described herein. In some embodiments, the first payload moiety is a TOP1 inhibitor moiety as described herein, and the second payload moiety is a DDR inhibitor moiety as described herein.

In some embodiments, an antigen-binding molecule according to the present disclosure comprises a linker-payload moiety comprising: (i) a first payload moiety conjugated to the linker moiety via a CuAAC reaction between an azide group and an alkyne group, and (ii) a second payload moiety conjugated to the linker moiety oxime linkage between an alkoxyamine or hydrazide group, and a ketone group. In accordance with such embodiments, in some cases the first payload moiety is a DDR inhibitor moiety as described herein, and the second payload moiety is a TOP1 inhibitor moiety as described herein. In some embodiments, the first payload moiety is a TOP1 inhibitor moiety as described herein, and the second payload moiety is a DDR inhibitor moiety as described herein.

Yamazaki, *et al.*, Nat Commun. (2021) 12(1): 3528 describes a branched linker moiety comprising:

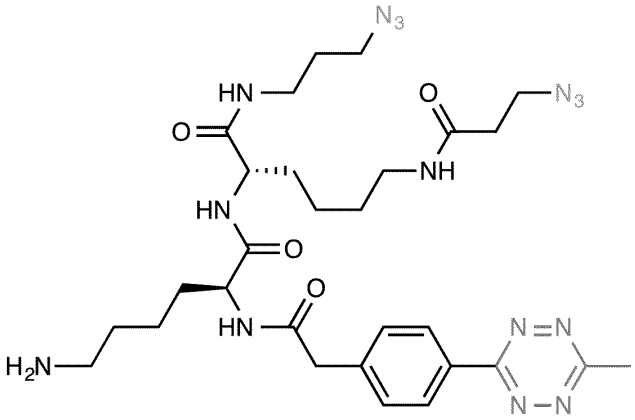
(a) a group for connection to an antibody (which is lysine-based); and

(b) two orthogonal functional groups for linking payload moieties, which are:

(i) one or two azide groups for incorporation of a payload moiety via strain-promoted azide-dibenzocyclooctyne (DBCO) cycloaddition; and

(ii) a methyltetrazine group for incorporation of a payload moiety via trans-cyclooctene (TCO) cycloaddition.

The branched linker moiety described in Yamazaki, *et al.*, Nat Commun. (2021) 12(1): 3528 has the following structure:

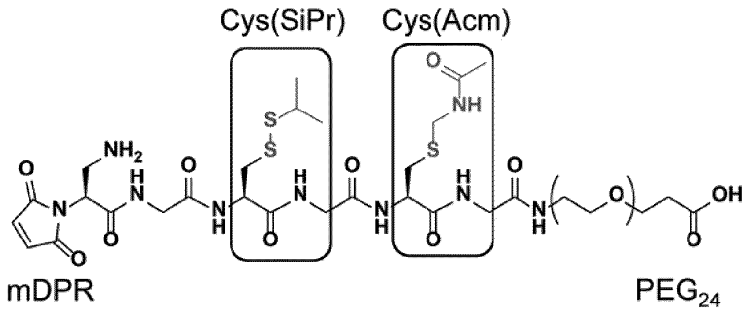


Accordingly, in some embodiments a linker-payload moiety according to the present disclosure comprises a linker moiety comprising: (i) a moiety derived from a group for connection to the antigen-binding moiety (*e.g.* a lysine-based group), (ii) one or two moieties derived from azide groups suitable for incorporating a first payload moiety via DBCO cycloaddition, and (iii) a moiety derived from a methyltetrazine group for incorporation of a second payload moiety via TCO cycloaddition. In accordance with such embodiments, in some cases the first payload moiety is a DDR inhibitor moiety as described herein, and the second payload moiety is a TOP1 inhibitor moiety as described herein. In some embodiments, the first payload moiety is a TOP1 inhibitor moiety as described herein, and the second payload moiety is a DDR inhibitor moiety as described herein.

In some embodiments, an antigen-binding molecule according to the present disclosure comprises a linker-payload moiety comprising: (i) a first payload moiety conjugated to the linker moiety via a DBCO cycloaddition reaction between a DBCO group and an azide group; and (ii) a second payload moiety conjugated to the linker moiety via a TCO cycloaddition reaction between a TCO group and a methyltetrazine group. In accordance with such embodiments, in some cases the first payload moiety is a DDR inhibitor moiety as described herein, and the second payload moiety is a TOP1 inhibitor moiety as described herein. In some embodiments, the first payload moiety is a TOP1 inhibitor moiety as described herein, and the second payload moiety is a DDR inhibitor moiety as described herein.

In some embodiments, the DDR inhibitor moiety and the TOP1 inhibitor moiety are connected to a linker moiety of the linker-payload moiety via cysteine groups. Such an approach to the production of a linker-payload moiety comprising two different payloads is described *e.g.* in Levengood, *et al.*, Angew Chem Int Ed Engl (2017) 56(3): 733-737.

Levengood, *et al.*, Angew Chem Int Ed Engl (2017) 56(3): 733-737 describes a linker-payload moiety comprising two different payload moieties, constructed by sequential deprotection of orthogonally-protected cysteines. Each payload is connected to a maleimide group (for example with a cleavable linker), and the maleimide undergoes a Michael reaction with the deprotected cysteines. The branched linker moiety described in Levengood, *et al.*, Angew Chem Int Ed Engl (2017) 56(3): 733-737 has the following structure:



Accordingly, in some embodiments a linker-payload moiety according to the present disclosure comprises: (i) a first payload moiety conjugated to the linker-payload moiety via reduction of a cysteine residue bearing a protecting disulfide group (*e.g.* a S-(tert-butyl) disulfide group or S-(isopropyl) disulfide group), and subsequent incorporation of the first payload moiety via thiol-maleimide reaction; and (ii) a second payload moiety conjugated to the linker-payload moiety via reduction of a cysteine residue bearing a protecting acetamidomethyl group, and subsequent incorporation of the first payload moiety via thiol-maleimide reaction. In accordance with such embodiments, in some cases the first payload moiety is a DDR inhibitor moiety as described herein, and the second payload moiety is a TOP1 inhibitor moiety as described herein. In some embodiments, the first payload moiety is a TOP1 inhibitor moiety as described herein, and the second payload moiety is a DDR inhibitor moiety as described herein.

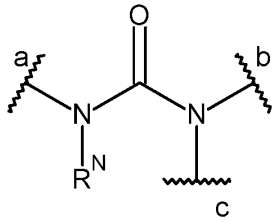
In some of these embodiments, the linker-payload moiety comprises:

(a) an amino group for conjugation to an antigen-binding moiety;

(b) at least one first payload comprising moiety clicked to a first click group, where the first payload comprising moiety comprises a DDR inhibitor moiety;

(c) at least one second payload comprising moiety clicked to a second click group, where the second payload comprising moiety comprises a TOP1 inhibitor moiety;

(d) the branching group:



wherein R^N is selected from H and -(C_{1-5} alkylene)-C(O)OH, where one CH_2 unit may be replaced by -O-,
a indicates where the amino group is linked to the branching group;
b indicates where the at least one first click group is linked to the branching group;
c indicates where the at least one second click group is linked to the branching group.

In some embodiments, the DDR inhibitor moiety and the TOP1 inhibitor moiety of the antigen-binding molecule of the present disclosure are connected to the antigen-binding moiety of the antigen-binding molecule via different linker moieties.

In some embodiments, an antigen-binding molecule according to the present disclosure comprises: (a) a linker-payload moiety comprising a DDR inhibitor moiety, and (b) a linker-payload moiety comprising a TOP1 inhibitor moiety. That is, in some embodiments, the antigen-binding molecule comprises at least two linker-payload moieties, wherein one of the linker-payload moieties comprises a DDR inhibitor moiety, and wherein another of the linker-payload moieties comprises a TOP1 inhibitor moiety.

Approaches for the attachment of multiple payload moieties to an antigen-binding moiety via multiple linker moieties are described *e.g.* in Świderska *et al.*, Intl J Mol Sci (2018) 19: 2098, Nilchan *et al.*, Antib. Ther. (2019) 2:71-78 and Wang *et al.*, Acta Pharmaceutica Sinica B (2023) 13(10): 4025-4059, all of which are hereby incorporated by reference in their entirety.

Świderska *et al.*, Intl J Mol Sci (2018) 19: 2098 describes an approach in which (i) a first linker-payload moiety (specifically maleimide-Val-Cit-PAB-α-amanitin) is conjugated to a cysteine residue of a polypeptide via thiol-maleimide reaction, and in which (ii) a second linker-payload moiety (specifically an azide linked to MMAE) is conjugated to the same polypeptide via CuAAC-mediated conjugation to the alkyne group of an engineered N-propargyl-L-lysine (PrK) residue.

Accordingly, in some embodiments an antigen-binding molecule according to the present disclosure comprises: (i) a first linker-payload moiety conjugated to the antigen-binding moiety via thiol-maleimide reaction between a cysteine residue of the antigen-binding moiety and a maleimide group of the linker-payload moiety, and (ii) a second linker-payload moiety conjugated via CuAAC reaction between an azide group of the linker-payload moiety to the alkyne group of an N-propargyl-L-lysine residue of the antigen-binding moiety. In accordance with such embodiments, in some cases the first linker-payload moiety comprises a DDR inhibitor moiety as described herein, and the second linker-payload moiety comprises a TOP1 inhibitor moiety as described herein. In some embodiments, the first linker-payload moiety

comprises a TOP1 inhibitor moiety as described herein, and the second linker-payload moiety comprises a DDR inhibitor moiety as described herein.

Nilchan *et al.*, Antib. Ther. (2019) 2:71-78 describes a dual conjugation approach in which (i) a first linker-payload moiety is conjugated to an antigen-binding moiety via selenoether conjugation between a selenocysteine residue of the antigen-binding moiety and an iodoacetamide group of the linker-payload moiety, and in which (ii) a second linker-payload moiety is conjugated to the same antigen-binding moiety via reaction between a cysteine residue of the antigen-binding moiety and a methylsulfone phenyloxadiazole (MSODA) group of the linker-payload moiety.

Accordingly, in some embodiments an antigen-binding molecule according to the present disclosure comprises: (i) a first linker-payload moiety conjugated to the antigen-binding moiety via selenoether conjugation between a selenocysteine residue of the antigen-binding moiety and an iodoacetamide group of the linker-payload moiety, and (ii) a second linker-payload moiety conjugated via reaction between a cysteine residue of the antigen-binding moiety and a MSODA group of the linker-payload moiety. In accordance with such embodiments, in some cases the first linker-payload moiety comprises a DDR inhibitor moiety as described herein, and the second linker-payload moiety comprises a TOP1 inhibitor moiety as described herein. In some embodiments, the first linker-payload moiety comprises a TOP1 inhibitor moiety as described herein, and the second linker-payload moiety comprises a DDR inhibitor moiety as described herein.

A further aspect of the present disclosure provides a DDR inhibitor moiety linked to click group, wherein the click group is suitable for conjugation to a corresponding click group in a linker moiety, and wherein the linker moiety is conjugated, or is suitable for conjugation, to an antigen-binding moiety.

A further aspect of the present disclosure provides a TOP1 inhibitor moiety linked to click group, wherein the click group is suitable for conjugation to a corresponding click group in a linker moiety, and wherein the linker moiety is conjugated, or is suitable for conjugation, to an antigen-binding moiety.

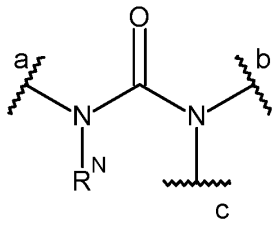
In accordance with such aspects, in some embodiments, the click group is selected from:
(i) an azide group;
(ii) an alkyne;
(iii) a tetrazine or tetrazine derivative group;
(iv) a cyclooctyne, cyclooctyne derivative, or cyclooctyne analogue group; and
(v) a strained alkene.

The tri-functional linking groups of the present disclosure are of a modular design which allows the number of payloads attached to the linker and thus the antigen-binding moieties to be readily varied, which has been shown to be important in the development of clinically relevant antibody drug conjugates. Furthermore, the use of click groups to attach payload containing moieties to the branching group allows for a wide range of payload types to be conjugated. Where orthogonal click moieties are used, two

different payload moieties can be connected, including those with different, and possibly complimentary, modes of action.

The tri-functional linking groups of the present disclosure have a hydrophilic branching group which may lead to a reduction in toxicities and an improvement in biophysical, stability and pharmacokinetic properties.

As described above, in some aspects the linker-payload moiety may comprise:
(a) an amino group for conjugation to an antigen-binding moiety;
(b) at least one first payload comprising moiety clicked to a first click group, where the first payload comprising moiety comprises a DDR inhibitor moiety;
(c) at least one second payload comprising moiety clicked to a second click group, where the second payload comprising moiety comprises a TOP1 inhibitor moiety;
(d) the branching group:



wherein R^N is selected from H and -(C_{1-5} alkylene)-C(O)OH, where one CH_2 unit may be replaced by -O-,
a indicates where the amino group is linked to the branching group;
b indicates where the at least one first click group is linked to the branching group;
c indicates where the at least one second click group is linked to the branching group.

In some embodiments, R^N is H.
In some embodiments, R^N is -(C_{1-5} alkylene)-C(O)OH, where one CH_2 unit may be replaced by -O-.
In some embodiments, R^N is -(C_{1-5} alkylene)-C(O)OH.
In some embodiments, R^N is -$CH_2CH_2OCH_2CH_2C(O)OH$.
In some embodiments, R^N is $CH_2C(O)OH$.

The amino group may be linked to the branching group by a first spacer group. The first spacer group (A1) may comprise:
(i) a C_{1-7} alkylene group; and/or
(ii) a PEG 1 to 12 group.

A1 may be of the formula:
-$(CH_2)_{xa}$-$(C_2H_4O)_{xb}$-$(CH_2)_{xc}$-,
where xa is 0 or 1, xb is 0-12, and xc is 0 to 6, wherein at least one of xa and xb is 1.

In some embodiments, xb is 0, and xa+xc are from 1 to 7, such as 5 (i.e. A1 is -$(CH_2)_5$-).

In other embodiments, xb is from 1 to 12, xa is 0 and xc is 0 or 1.

In other embodiments, xb is from 1 to 12. In some of these embodiments, xb is from 1 to 6. In some of these embodiments, xb is from 1 to 3, i.e. 1, 2 or 3.

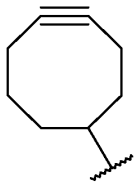
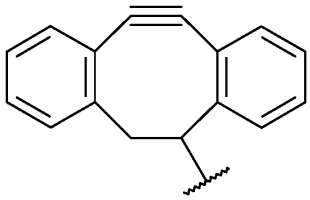
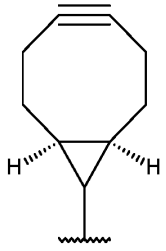
In some embodiments where xb is 1 to 12, xc is 1 to 6, or 1 to 2. In some of these embodiments, xc is 1. In some of these embodiments, xc is 2.

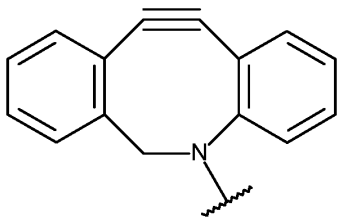
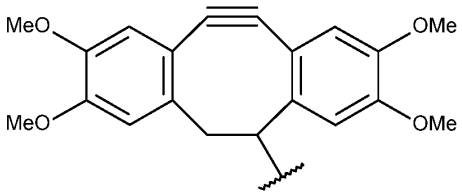
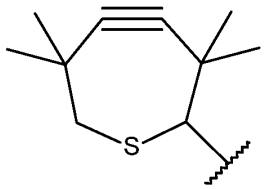
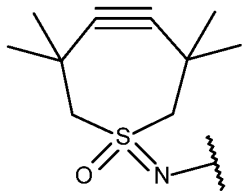
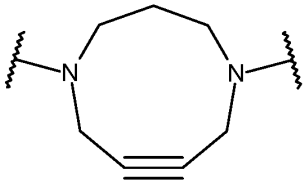
In some embodiments, xa is 0, xb is 1 to 6 and xc is 2. In some of these embodiments, A1 is $-(C_2H_4)-O-(C_2H_4)-$. In some of these embodiments, A1 is $-(C_2H_4O)_3-(C_2H_4)-$.

The first and second click groups may be selected from either member of the following click-group pairs:

	First member	*Second member*	*Click Reaction*
(i)	Azide ($-N_3$)	Alkyne (-CCH)	Copper-catalysed azide-alkyne cycloaddition
(ii)	Azide ($-N_3$)	Cyclooctyne, Cyclooctyne derivatives and Cyclooctyne analogues	Strain-promoted [3+2] cycloaddition
(iii)	Tetrazine and tetrazine derivatives	Strained alkene	Inverse-Electron-Demand Diels–Alder (IEDDA) reaction

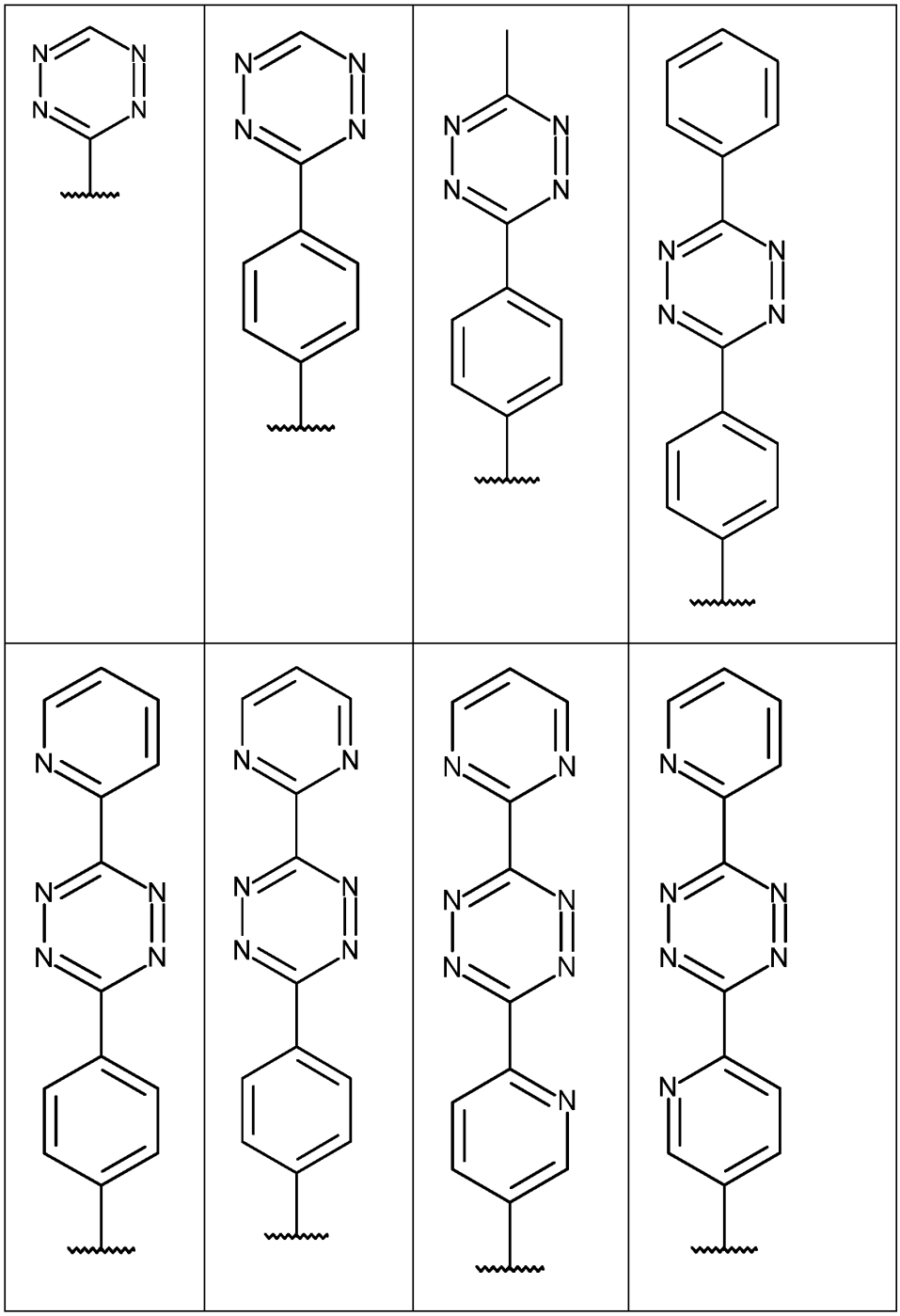
Cyclooctyne, cyclooctyne derivatives and cyclooctyne analogues for use in the present disclosure include:

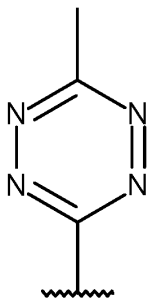
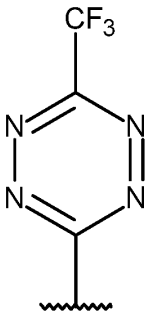
Cyclooctyne	
Dibenzocyclooctyne (DIBO)	
Achiral bicyclo[6.1.0]nonyne (BCN)	

Dibenzoazacyclooctyne (DIBAC/DBCO)	
2,3,6,7-tetramethoxy-DIBO (TMDIBO)	
3,3,6,6-tetramethylthiacycloheptyne (TMTH)	
Tetramethylthiocycloheptyne sulfoximine (TMTHSI)	
Diazacyclononyne (DACN)	

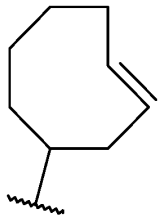
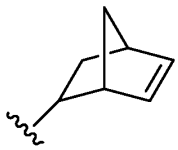
These groups can alternatively be called cyclic alkynes.

Tetrazine and tetrazine derivatives for use in the present disclosure include:

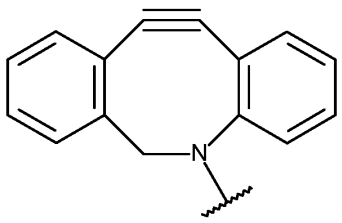


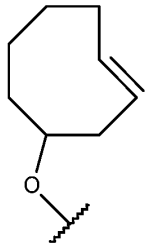
			

Strained alkenes for use in the present disclosure may have the structure:

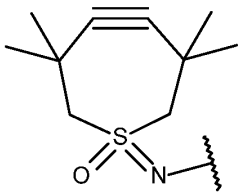
Cylcooctene	
Norbornene	
1-Methylcyclopropene (1-MCP)	

In some embodiment, the click group may be a dibenzoazacyclooctyne (DIBAC) group or a 1-ethylhept-6-enoxy group:

Dibenzoazacyclooctyne (DIBAC/DBCO)	

Trans-cyclooctene (TCO)/ 1-ethylhept-6-enoxy	

In some embodiments, the click group may be Tetramethylthiocycloheptyne sulfoximine (TMTHSI):

Tetramethylthiocycloheptyne sulfoximine (TMTHSI)	

The link between the payload moiety (*e.g.* the DDR inhibitor moiety or the TOP1 inhibitor moiety) and the click group according to the present disclosure may be a cleavable linker moiety or a non-cleavable moiety, *e.g.* as described hereinabove.

In some embodiments of the tri-functional linking group, the first and second click groups are the same. In other embodiments of the tri-functional linking group, the first and second click groups are selected from orthogonal click-group pairs.

In some embodiments of the tri-functional linking group, the first and/or second click group is azide.
In some embodiments of the tri-functional linking group, the first and/or second click group is tetrazine or a tetrazine derivative.
In some embodiments of the tri-functional linking group, the first and/or second click group is an alkyne (-CCH).
In some embodiments of the tri-functional linking group, the first and/or second click group is cyclooctyne or a cyclooctyne derivative.
In some embodiments of the tri-functional linking group, the first and/or second click group is norbonene or a norbonene derivative.
In some embodiments of the tri-functional linking group, the first and/or second click group is methylcyclopropene (1-MCP).

In some embodiments of the tri-functional linking group, the first click group is azide and the second click group is tetrazine or a tetrazine derivative.
In some embodiments of the tri-functional linking group, the first click group is azide and the second click group is cyclooctene.
In some embodiments of the tri-functional linking group, the first click group is azide and the second click group is norbonene or a norbonene derivative.

In some embodiments of the tri-functional linking group, the first click group is azide and the second click group is methylcyclopropene (1-MCP).

In some embodiments of the tri-functional linking group, the first click group is alkyne (-CCH) and the second click group is tetrazine or a tetrazine derivative.

In some embodiments of the tri-functional linking group, the first click group is alkyne (-CCH) and the second click group is cyclooctene.

In some embodiments of the tri-functional linking group, the first click group is alkyne (-CCH) and the second click group is norbonene or a norbonene derivative.

In some embodiments of the tri-functional linking group, the first click group is alkyne (-CCH) and the second click group is methylcyclopropene (1-MCP).

In some embodiments of the tri-functional linking group, the first click group is cyclooctyne or a cyclooctyne derivative and the second click group is tetrazine or a tetrazine derivative.

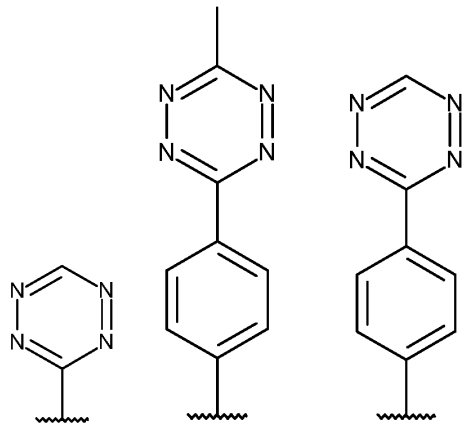
In some embodiments of the tri-functional linking group, the first click group is cyclooctyne or a cyclooctyne derivative and the second click group is cyclooctene.

In some embodiments of the tri-functional linking group, the first click group is cyclooctyne or a cyclooctyne derivative and the second click group is norbonene or a norbonene derivative.

In some embodiments of the tri-functional linking group, the first click group is cyclooctyne or a cyclooctyne derivative and the second click group is methylcyclopropene (1-MCP).

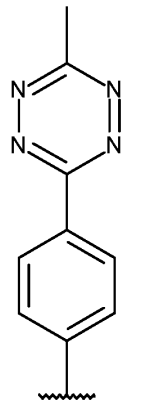
In some embodiments of the tri-functional linking group, the second click group is phenyl-tetrazine.

In some embodiments of the tri-functional linking group, the second click group is selected from the following groups:



, , or .

In some embodiments of the tri-functional linking group, the second click group is:

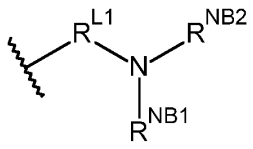


.

The at least one first click group may be linked to the branching group by a second spacer group (B1). In some embodiments, the second spacer group is branched, such that two first click groups are linked to the branching group. In other embodiments, the second spacer group is not branched, such that a single first click group is linked to the branching group.

The at least one second click group may be linked to the branching group by a third spacer group (B2). In some embodiments, the third spacer group is branched, such that two second click groups are linked to the branching group. In other embodiments, the third spacer group is not branched, such that a single second click group is linked to the branching group.

In some embodiments of the tri-functional linking group, the second spacer group (B1) is of formula (B1-1):



(B1-1)

R^{L1} is $-(C_2H_4O)_{xl3}-(CH2)_{xd}-(C(=O))_{xl4}-$

where xl3 is 0 to 4, xd is 0 to 3, xl4 is 0 or 1,

R^{NB1} is $-(C_2H_4O)_{xe1}-(CH2)_{xf1}-(NH)_{xg1}-(C(=O)CH_2)_{xh1}-$

where xe1 is 0 to 4, xf1 is 0 to 2, xg1 is 0 or 1, and xh1 is 0 or 1,

R^{NB2} is H or $-(C_2H_4O)_{xe2}-(CH_2)_{xf2}-(NH)_{xg2}-(C(=O)CH_2)_{xh2}-$

where xe2 is 0 to 4, xf2 is 0 to 2, xg2 is 0 or 1, and xh2 is 0 or 1.

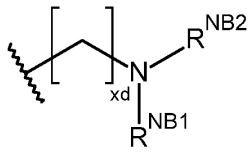
In some embodiments, xl3 is 0-2. In some embodiments, xl3 is 0. In some embodiments, xl3 is 1. In some embodiments, xl3 is 2.

In some embodiments, xl4 is 0. In some embodiments, xl4 is 1.

In some embodiments, xe1 is 2-4. In some embodiments, xe1 is 2. In some embodiments, xe1 is 3. In some embodiments, xe1 is 4.

In some embodiments, xe2 is 2-4. In some embodiments, xe2 is 2. In some embodiments, xe2 is 3. In some embodiments, xe2 is 4.

In some of these embodiments of the tri-functional linking group, the second spacer group (B1) is of formula (B1-2):



(B1-2)

where xd is 0-3,

R^{NB1} is $-(C_2H_4O)_{xe1}-(CH_2)_{xf1}-(NH)_{xg1}-(C(=O)CH_2)_{xh1}-$

where xe1 is 0 or 1, xf1 is 0 to 2, xg1 is 0 or 1, and xh1 is 0 or 1,

R^{NB2} is H or $-(C_2H_4O)_{xe2}-(CH_2)_{xf2}-(NH)_{xg2}-(C(=O)CH_2)_{xh2}-$

where xe2 is 0 or 1, xf2 is 0 to 2, xg2 is 0 or 1, and xh2 is 0 or 1.

In some embodiments, R^{NB2} is H. In some embodiments, R^{NB2} is $-(C_2H_4O)_{xe2}-(CH_2)_{xf2}-(NH)_{xg2}-(C(=O)CH_2)_{xh2}-$. In other embodiments, R^{NB2} is the same as R^{NB1}.

In some embodiments, xd is 0-2. In some embodiments, xd is 0-1. In some embodiments, xd is 0. In some embodiments, xd is 1. In some embodiments, xd is 2. In some embodiments, xd is 3. In some embodiments, xd is 0 or 2.

In some embodiments, xe1 is 0. In some embodiments, xe1 is 1.

In some embodiments, xf1 is 0-1. In some embodiments, xf1 is 0. In some embodiments, xf1 is 1. In some embodiments, xf1 is 2. In some embodiments, xf1 is 0 or 2.

In some embodiments, xg1 is 0. In some embodiments, xg1 is 1.

In some embodiments, xh1 is 0. In some embodiments, xh1 is 1.

In some embodiments, xe2 is 0. In some embodiments, xe2 is 1.

In some embodiments, xf2 is 0-1. In some embodiments, xf2 is 0. In some embodiments, xf2 is 1. In some embodiments, xf2 is 2. In some embodiments, xf2 is 0 or 2.

In some embodiments, xg2 is 0. In some embodiments, xg2 is 1.

In some embodiments, xe1 is 1, xf1 is 2, xg1 is 0, and xh1 is 0.

In some embodiments, xe1 is 0, xf1 is 0, xg1 is 0, and xh1 is 1.

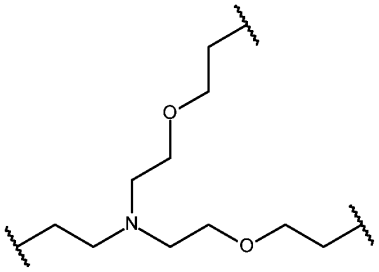
In some embodiments, xe1 is 1, xf1 is 2, xg1 is 1, and xh1 is 1.

In some embodiments, xe2 is 1, xf2 is 2, xg2 is 0, and xh2 is 0.

In some embodiments, xe2 is 0, xf2 is 0, xg2 is 0, and xh2 is 1.

In some embodiments, xe2 is 1, xf2 is 2, xg2 is 1, and xh2 is 1.

In some embodiments of the tri-functional linking group, the second spacer group (B1) is selected from the groups containing: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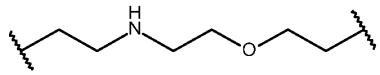
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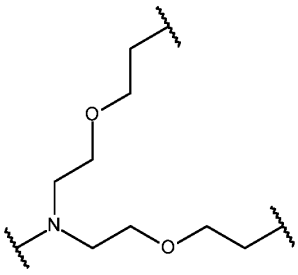
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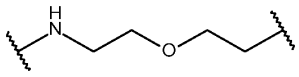
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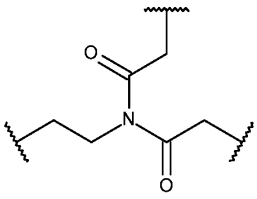
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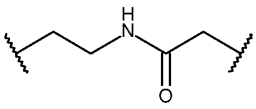
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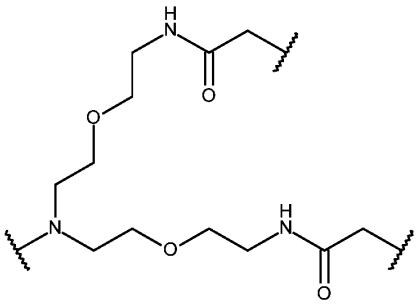
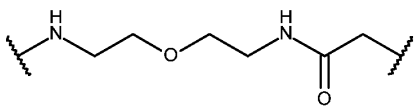
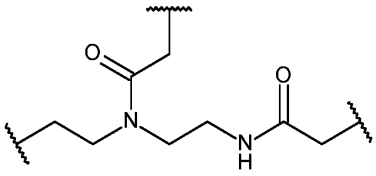
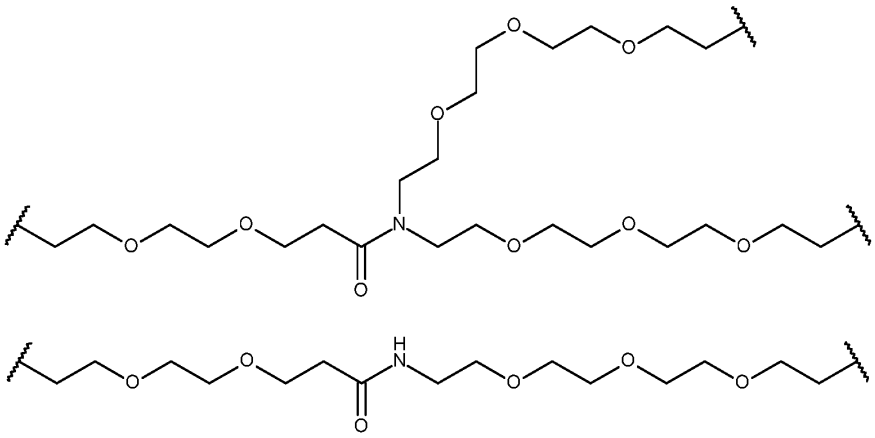
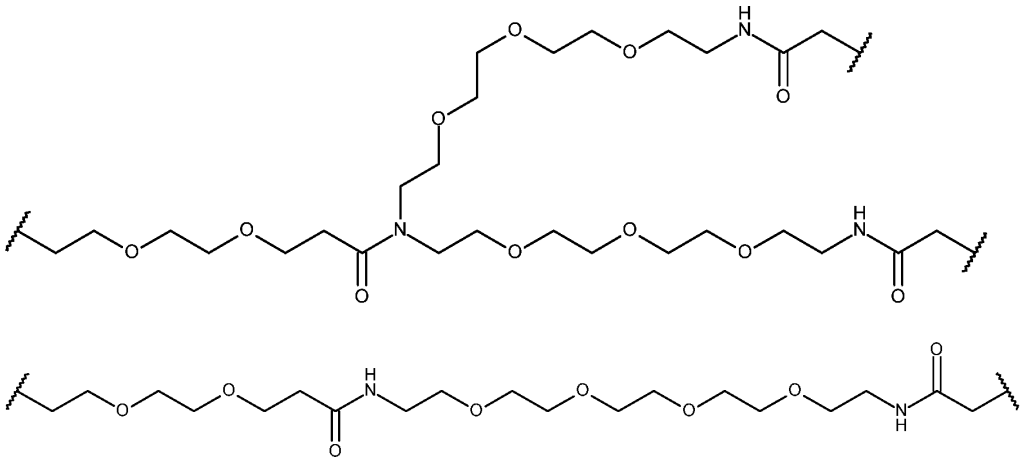


, and .

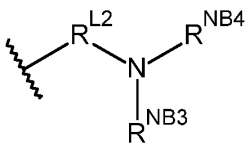
In some embodiments of the tri-functional linking group, the second spacer group (B1) is selected from the groups containing:







In some embodiments of the tri-functional linking group, the second spacer group (B2) is of formula (B2-1):



(B2-1)

R^{L2} is $-(C_2H_4O)_{xl5}-(CH2)_{xi}-(C(=O))_{xl6}-$

where xl6 is 0 to 4, xi is 0 to 3, xl6 is 0 or 1,

R^{NB3} is $-(C_2H_4O)_{xj1}-(CH2)_{xk1}-(NH)_{xl1}-(C(=O)CH_2)_{xm1}-$

where xj1 is 0 to 4, xk1 is 0 to 2, xl1 is 0 or 1, and xm1 is 0 or 1,

R^{NB4} is H or $-(C_2H_4O)_{xj2}-(CH_2)_{xk2}-(NH)_{xl2}-(C(=O)CH_2)_{xm2}-$
where xj2 is 0 to 4, xk2 is 0 to 2, xl2 is 0 or 1, and xm2 is 0 or 1.

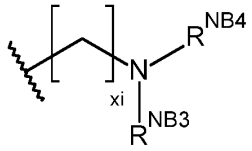
In some embodiments, xl5 is 0-2. In some embodiments, xl5 is 0. In some embodiments, xl5 is 1. In some embodiments, xl5 is 2.

In some embodiments, xl6 is 0. In some embodiments, xl6 is 1.

In some embodiments, xj1 is 2-4. In some embodiments, xj1 is 2. In some embodiments, xj1 is 3. In some embodiments, xj1 is 4.

In some embodiments, xj2 is 2-4. In some embodiments, xj2 is 2. In some embodiments, xj2 is 3. In some embodiments, xj2 is 4.

In some embodiments of the tri-functional linking group, the third spacer group (B2) is of formula (B2-2):



(B2-2)

where xi is 0-3,
R^{NB3} is $-(C_2H_4O)_{xj1}-(CH_2)_{xk1}-(NH)_{xl1}-(C(=O)CH_2)_{xm1}-$
where xj1 is 0 or 1, xk1 is 0 to 2, xl1 is 0 or 1, and xm1 is 0 or 1,
R^{NB4} is H or $-(C_2H_4O)_{xj2}-(CH_2)_{xk2}-(NH)_{xl2}-(C(=O)CH_2)_{xm2}-$
where xj2 is 0 or 1, xk2 is 0 to 2, xl2 is 0 or 1, and xm2 is 0 or 1.

In some embodiments, R^{NB4} is H. In some embodiments, R^{NB4} is $-(C_2H_4O)_{xj2}-(CH_2)_{xk2}-(NH)_{xl2}-(C(=O)CH_2)_{xm2}-$. In other embodiments, R^{NB4} is the same as R^{NB3}.

In some embodiments, xi is 0-2. In some embodiments, xi is 0-1. In some embodiments, xi is 0. In some embodiments, xi is 1. In some embodiments, xi is 2. In some embodiments, xi is 3. In some embodiments, xi is 0 or 2.

In some embodiments, xj1 is 0. In some embodiments, xj1 is 1.

In some embodiments, xk1 is 0-1. In some embodiments, xk1 is 0. In some embodiments, xk1 is 1. In some embodiments, xk1 is 2. In some embodiments, xk1 is 0 or 2.

In some embodiments, xl1 is 0. In some embodiments, xl1 is 1.

In some embodiments, xm1 is 0. In some embodiments, xm1 is 1.

In some embodiments, xj2 is 0. In some embodiments, xj2 is 1.

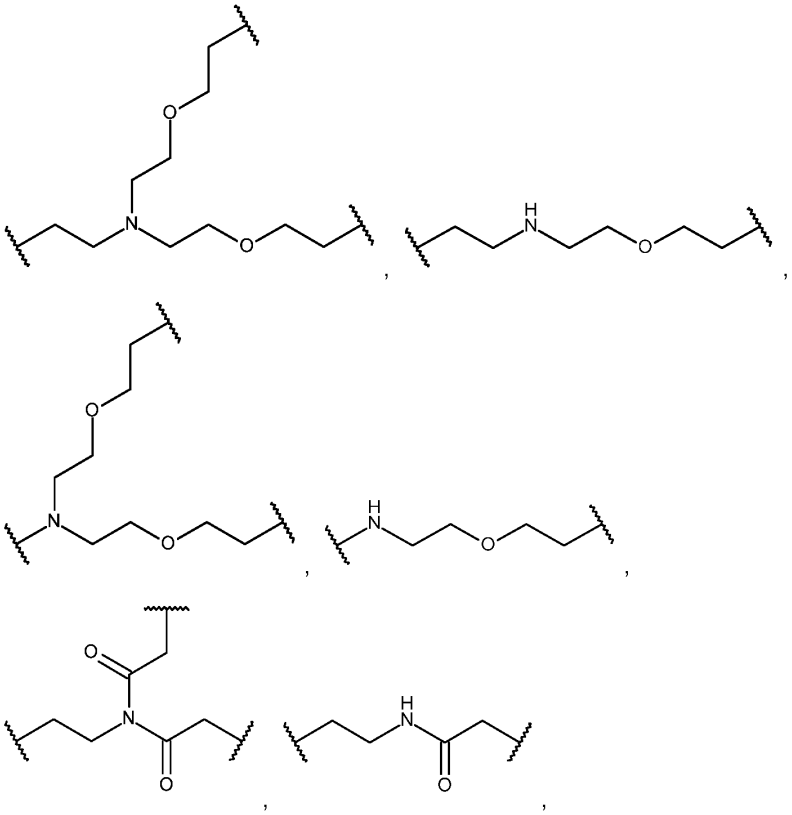
In some embodiments, xk2 is 0-1. In some embodiments, xk2 is 0. In some embodiments, xk2 is 1. In some embodiments, xk2 is 2. In some embodiments, xk2 is 0 or 2.

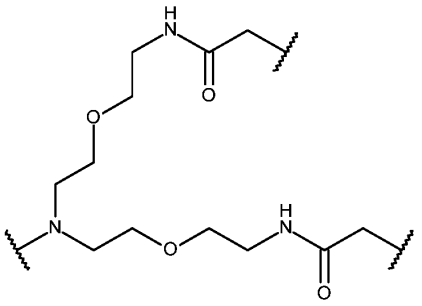
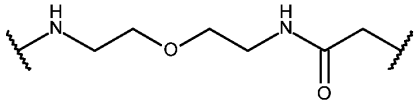
In some embodiments, xl2 is 0. In some embodiments, xl2 is 1.

In some embodiments, xj1 is 1, xk1 is 2, xl1 is 0, and xm1 is 0.
In some embodiments, xj1 is 0, xk1 is 0, xl1 is 0, and xm1 is 1.
In some embodiments, xj1 is 1, xk1 is 2, xl1 is 1, and xm1 is 1.

In some embodiments, xj2 is 1, xk2 is 2, xl2 is 0, and xm2 is 0.
In some embodiments, xj2 is 0, xk2 is 0, xl2 is 0, and xm2 is 1.
In some embodiments, xj2 is 1, xk2 is 2, xl2 is 1, and xm2 is 1.

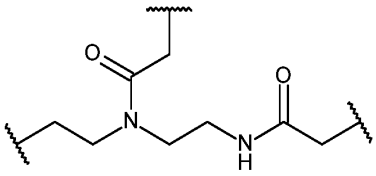
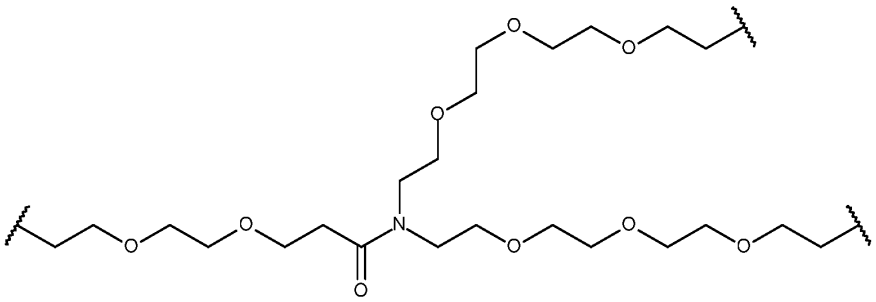
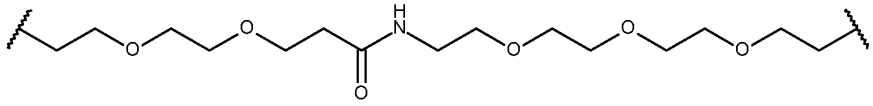
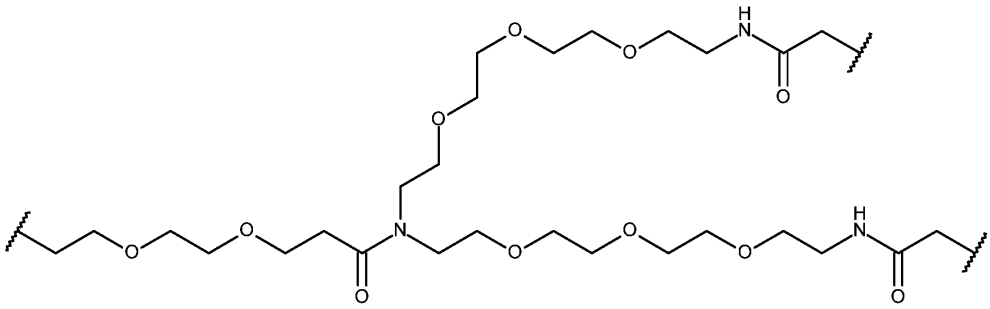
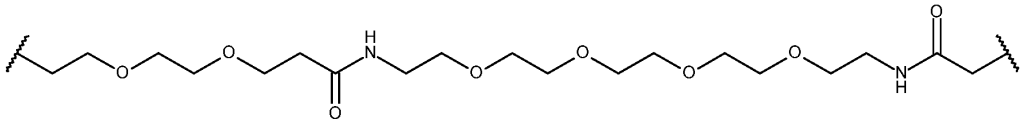
In some embodiments of the tri-functional linking group, the third spacer group (B2) is selected from the groups containing:



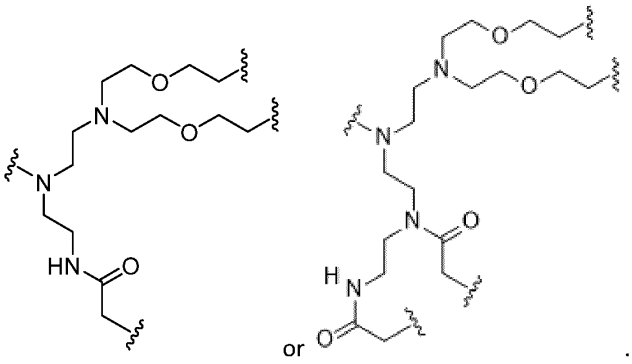
, and .

In some embodiments of the tri-functional linking group, the third spacer group (B2) is selected from the groups containing:

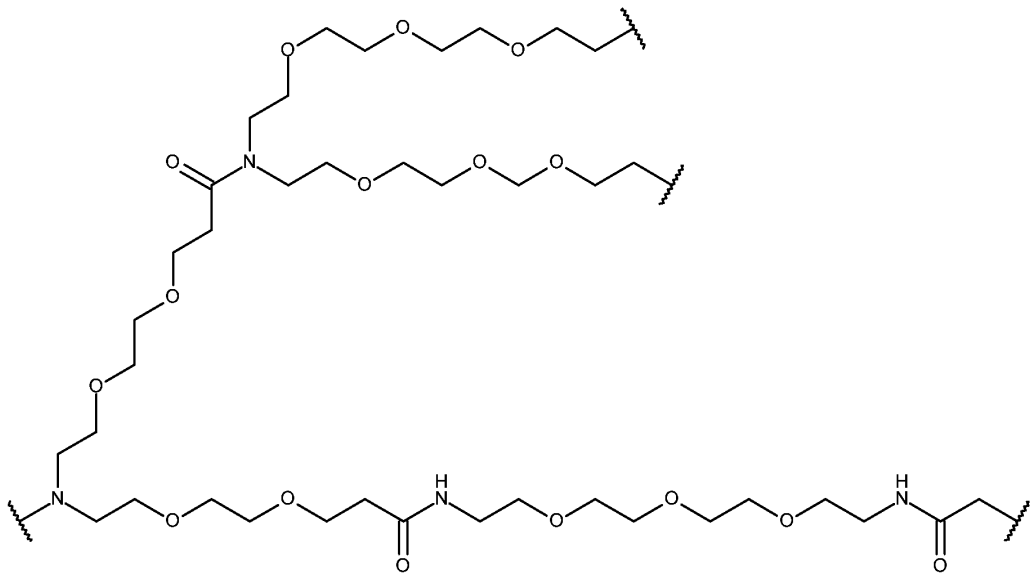






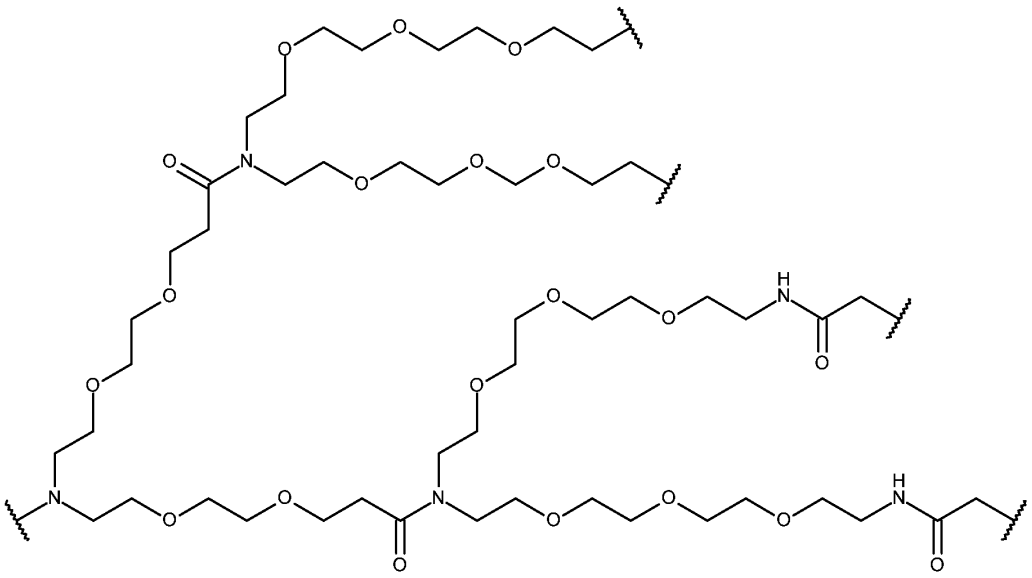
In some embodiments of the tri-functional linking group, the second spacer group (B1) is the same as the third spacer group (B2). In some embodiments of the tri-functional linking group, the second spacer group (B1) is different to the third spacer group (B2).

In some embodiments of the tri-functional linking group, the second spacer group (B1) and the third spacer group (B2), together with the nitrogen atom to which they are attached form one of the flowing groups:



In some embodiments of the tri-functional linking group, the second spacer group (B1) and the third spacer group (B2), together with the nitrogen atom to which they are attached form one of the flowing groups:





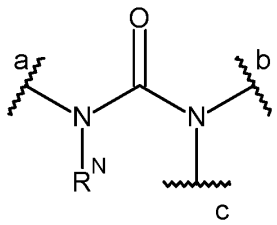
The present disclosure provides a linker comprising:

(a) an amino group conjugated to an antigen-binding moiety;

(b) at least one first payload comprising moiety clicked to a first click group;

(c) at least one second payload comprising moiety clicked to a second click group;

(d) the branching group:



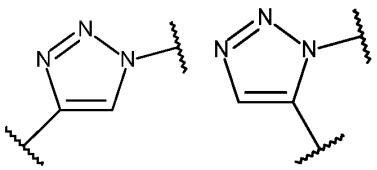
wherein R^N is selected from H and -(C_{1-5} alkylene)-C(O)OH, where one CH_2 unit may be replaced by -O-,

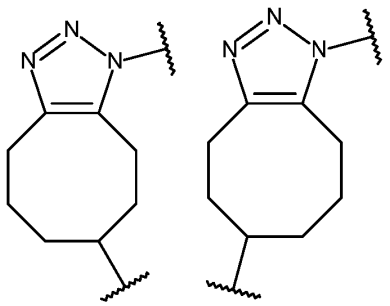
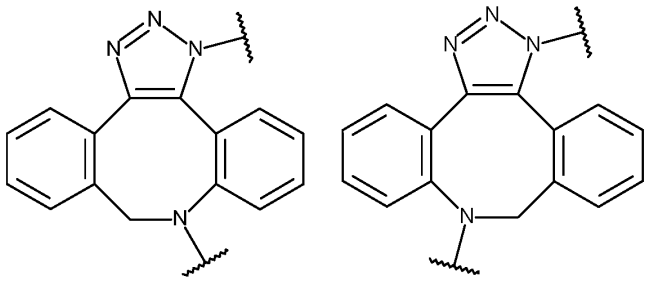
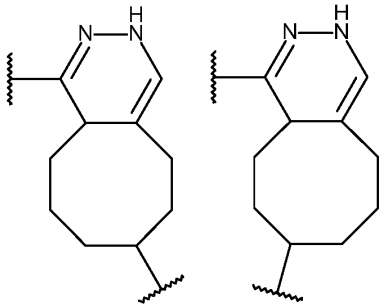
a indicates where the amino group is linked to the branching group;

b indicates where the at least one first click group is linked to the branching group;

c indicates where the at least one second click group is linked to the branching group.

Examples of the clicked group are shown below:

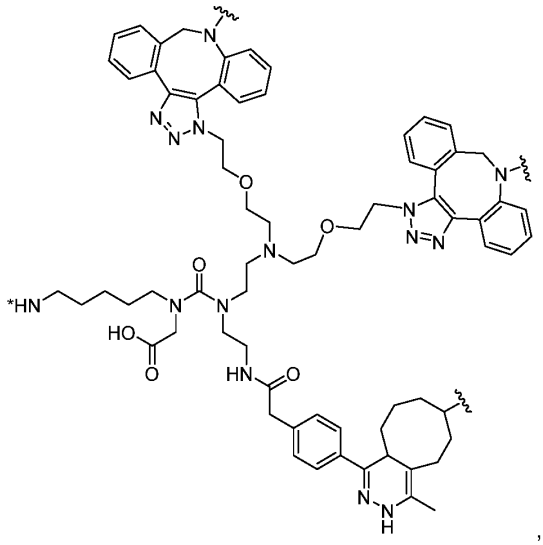
	First member	*Second member*	*Clicked group*
(i)	Azide (-N_3)	Alkyne (-CCH)	

(ii)	Azide (-N_3)	Cyclooctyne	
(ii)	Azide (-N_3)	DBCO	
(iv)	Tetrazine	Cylcooctene	

As shown in the table above, the reaction between the first and second members of the click group pairs can result in two isomeric products, i.e. a mixture. The present disclosure includes both isomeric forms when only one is shown.

The amino group may be linked to the branching group by a first spacer group (A1) as defined above.
The at least one first click group may be linked to the branching group by a second spacer group (B1) as defined above.
The at least one second click group may be linked to the branching group by a third spacer group (B2) as defined above.

In some embodiments, the linker comprises one of the following groups:



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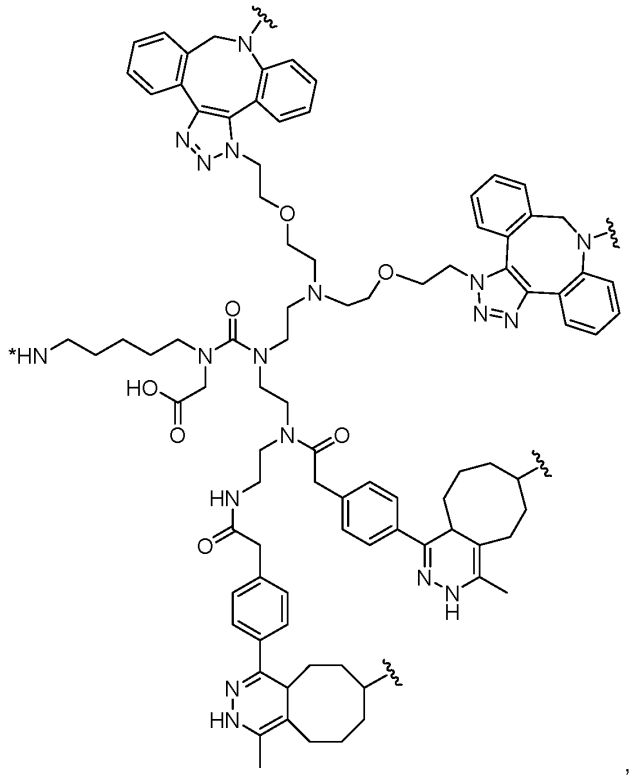

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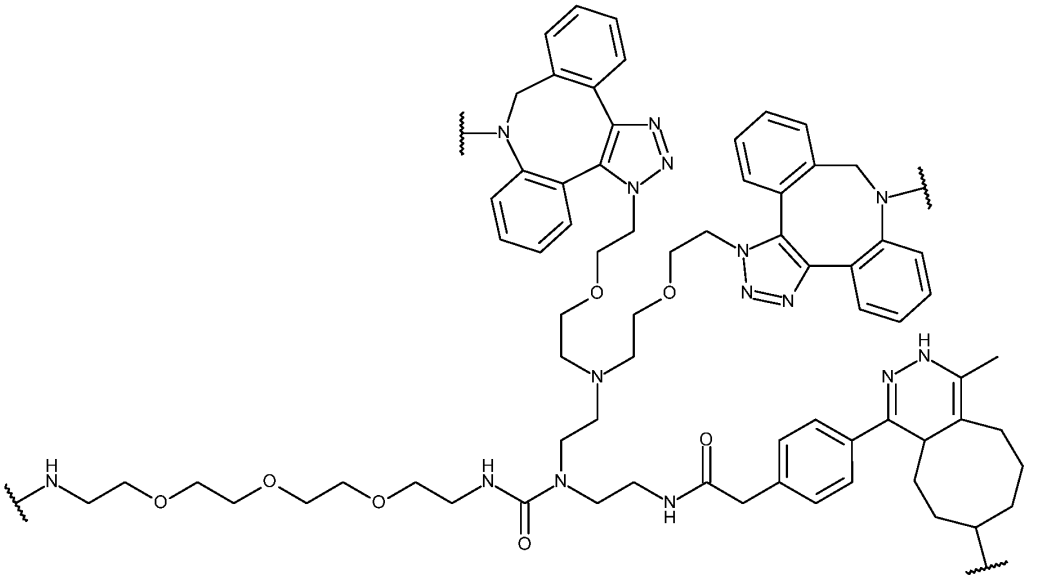

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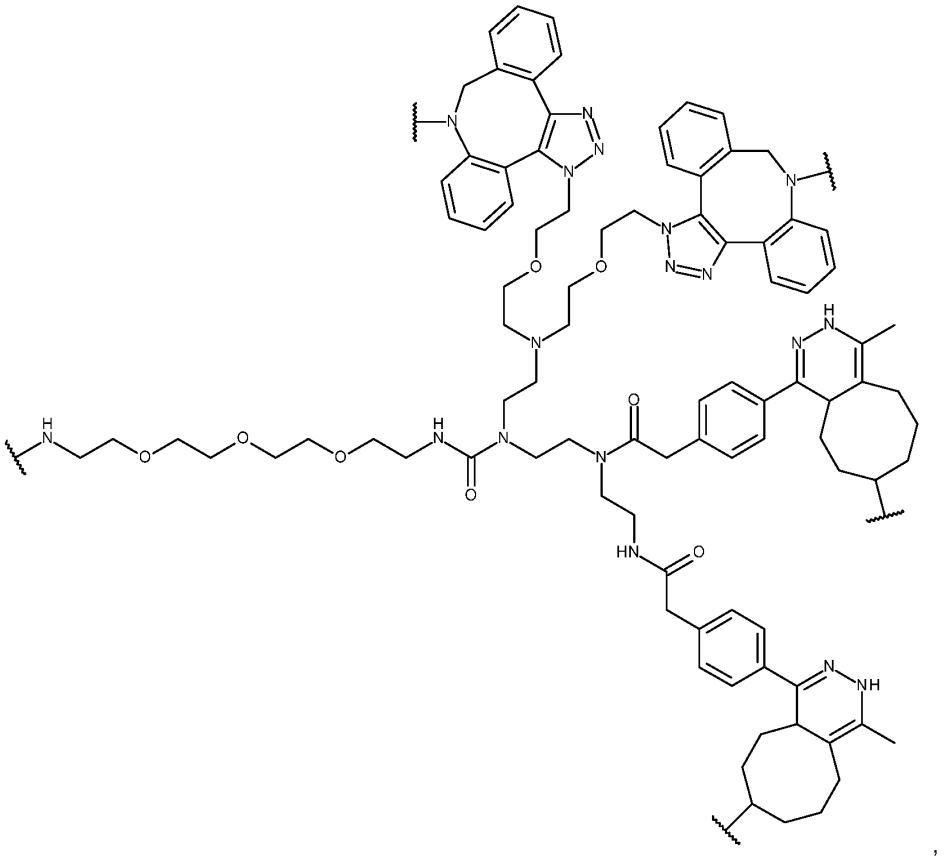

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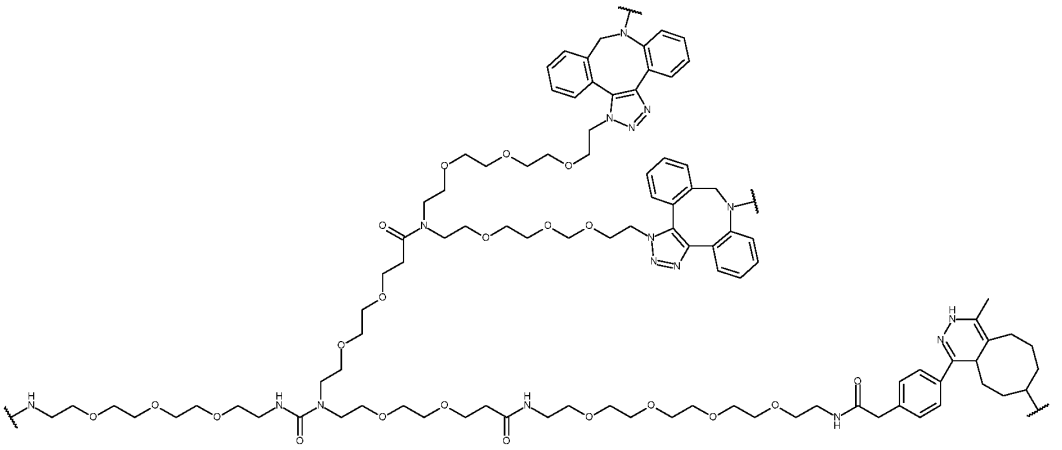

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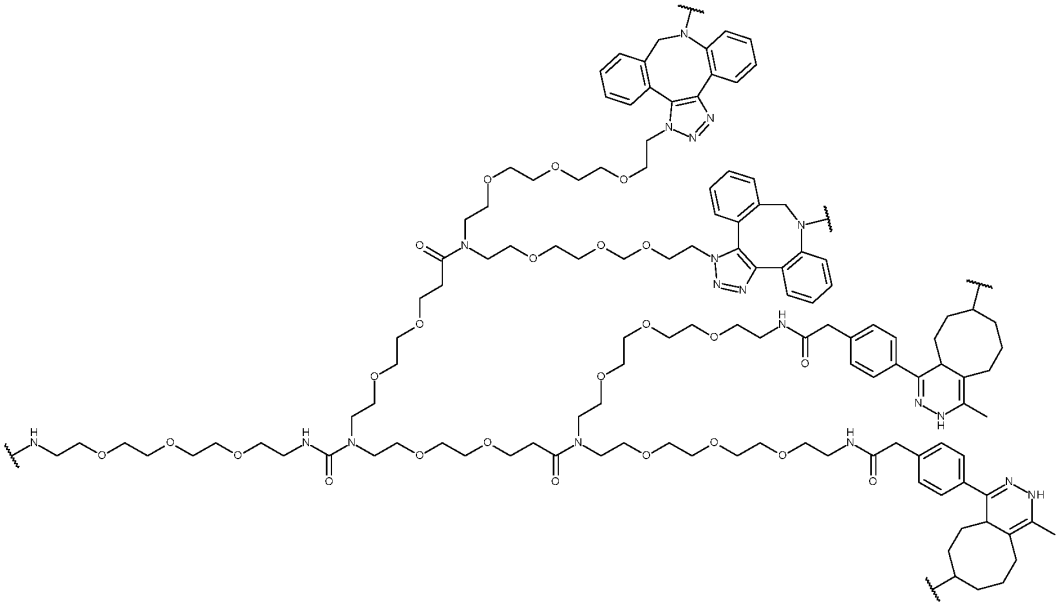

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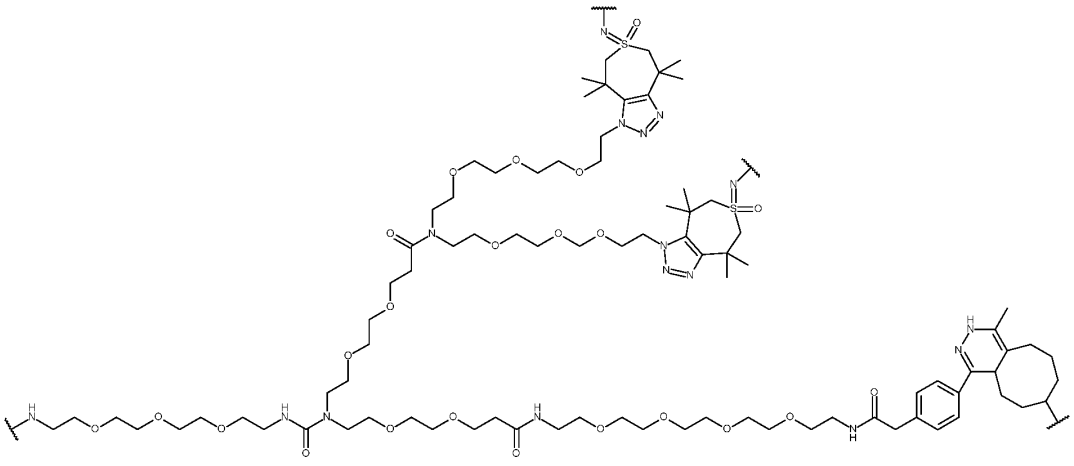
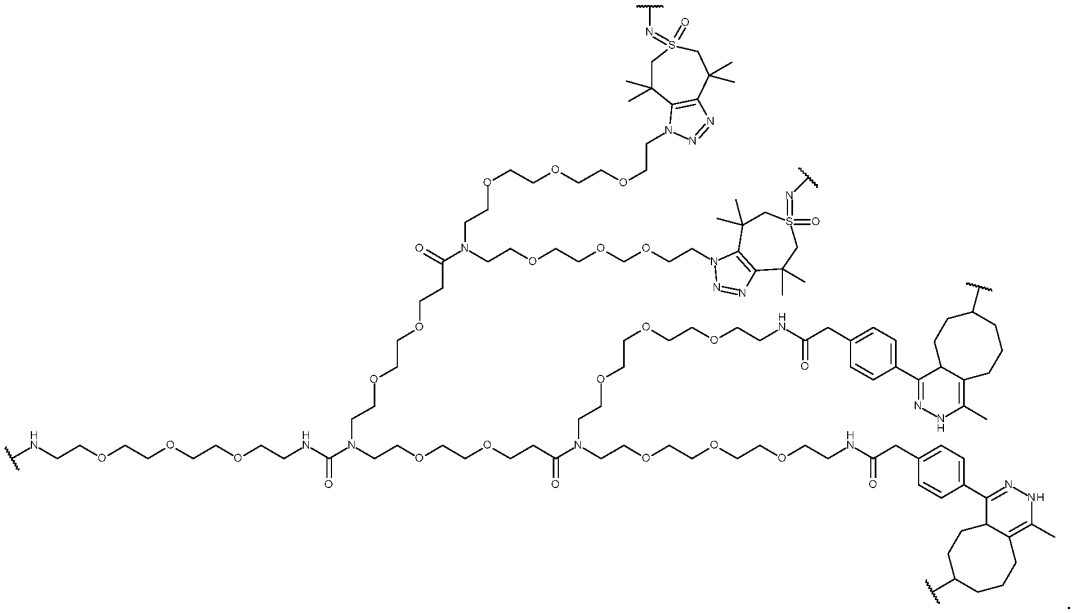


,

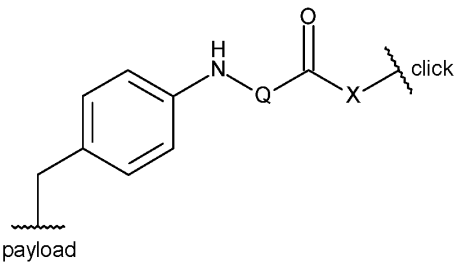
or



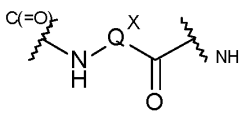
The link between the payload moiety (*e.g.* the DDR inhibitor moiety or the TOP1 inhibitor moiety) and the click group according to the present disclosure may be a cleavable linker moiety or a non-cleavable moiety, *e.g.* as described hereinabove.

In some embodiments, there is a functional group to connect the click group. This functional group may be selected from carbonyl (C=O) and oxy (O). Where the click group is DBCO or TMTHSI, the functional group may be carbonyl. Where the click group is TCO, the functional group may be oxy.

In some embodiments, the linker the link between the payload moiety and the click moiety comprises:

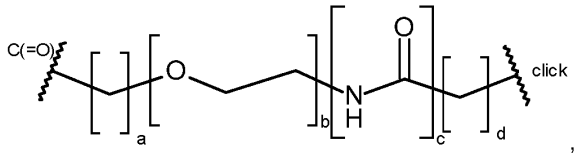


Q is:



, where Q^X is such that Q is an amino-acid residue, a dipeptide residue or a tripeptide residue;

X is:



,

where a = 0 to 5, b = 0 to 8, c = 0 or 1, d = 0 to 5;

a may be 0, 1, 2, 3, 4 or 5. In some embodiments, a is 0 to 3. In some of these embodiments, a is 0 or 1. In further embodiments, a is 1.

b may be 0, 1, 2, 3, 4, 5, 6, 7 or 8. In some embodiments, b is 0 to 6. In some of these embodiments, b is 0 to 4, and may be 0, 1, 2, 3 or 4. In further embodiments, b is 3.

c may be 0 or 1. In some of these embodiments, c is 1.

d may be 0, 1, 2, 3, 4 or 5. In some embodiments, d is 0 to 3. In some of these embodiments, d is 0, 1 or 2. In further embodiments, d is 2. In other further embodiments, d is 1. In other further embodiments, d is 0.

In some embodiments of X, a is 0, c is 1 and d is 2, and b may be from 0 to 8. In some of these embodiments, b is 0, 4 or 8.

In one embodiment, Q is an amino acid residue. The amino acid may a natural amino acids or a non-natural amino acid.

In one embodiment, Q is selected from: Phe, Lys, Val, Ala, Cit, Leu, Ile, Arg, Ser, and Trp, where Cit is citrulline. In one, Q is a serine derivative (see WO2018/234636A1).

In one embodiment, Q comprises a dipeptide residue. The amino acids in the dipeptide may be any combination of natural amino acids and non-natural amino acids. In some embodiments, the dipeptide comprises natural amino acids. Where the linker is a cathepsin labile linker, the dipeptide is the site of action for cathepsin-mediated cleavage. The dipeptide then is a recognition site for cathepsin.

In one embodiment, Q is selected from:

^{CO}-Phe-Lys-^{NH},

^{CO}-Val-Ala-^{NH},

^{CO}-Val-Lys-^{NH},

^{CO}-Ala-Lys-^{NH},

^{CO}-Val-Cit-^{NH},

^{CO}-Phe-Cit-^{NH},

^{CO}-Leu-Cit-^{NH},

^{CO}-Ile-Cit-^{NH},

^{CO}-Phe-Arg-^{NH}

^{CO}-Gly-Cit-^{NH},

^{CO}-Gly-Ala-^{NH}, and

^{CO}-Trp-Cit-^{NH};

where Cit is citrulline.

In some of these embodiments, Q is selected from:

^{CO}-Phe-Lys-^{NH},

^{CO}-Val-Ala-^{NH},

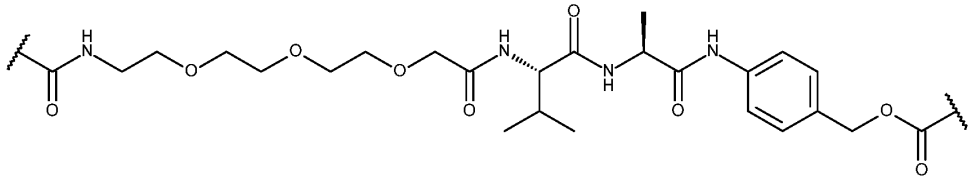
^{CO}-Val-Lys-^{NH},

^{CO}-Ala-Lys-^{NH},

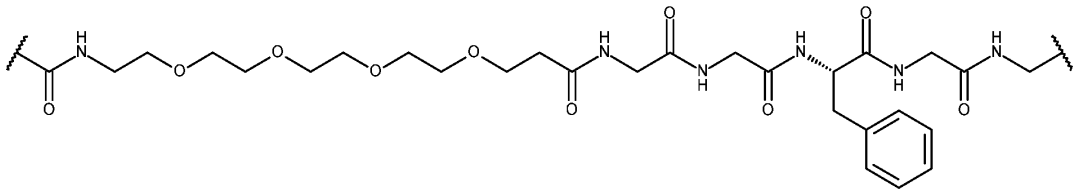
^{CO}-Val-Cit-^{NH}.

In further embodiments, Q is selected from ^{CO}-Phe-Lys-^{NH}, ^{CO}-Val-Cit-^{NH} and ^{CO}-Val-Ala-^{NH}.

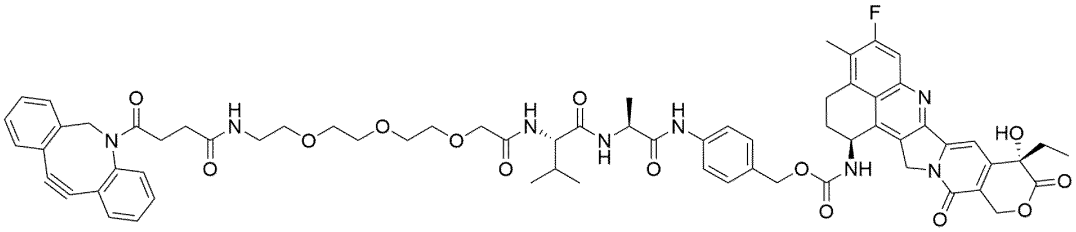
In some embodiments, the link between the payload moiety (*e.g.* the DDR inhibitor moiety or the TOP1 inhibitor moiety) and the click group comprises: PABC, a cathepsin-cleavable dipeptide, and a PEG2 to PEG4 (*e.g.* a PEG3) group. In some of these embodiments, the link between the payload moiety and the click group comprises, or is:



In some embodiments, the link between the payload moiety comprises GGFG (Glycine-Glycine-Phenylalanine-Glycine). This may be directly linked to the payload or linked via a CH_2 group. In some of these embodiments, the link between the payload moiety and the click group comprises, or is:

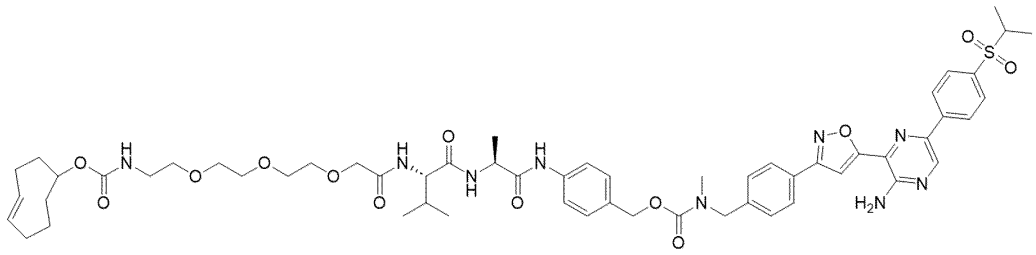


In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



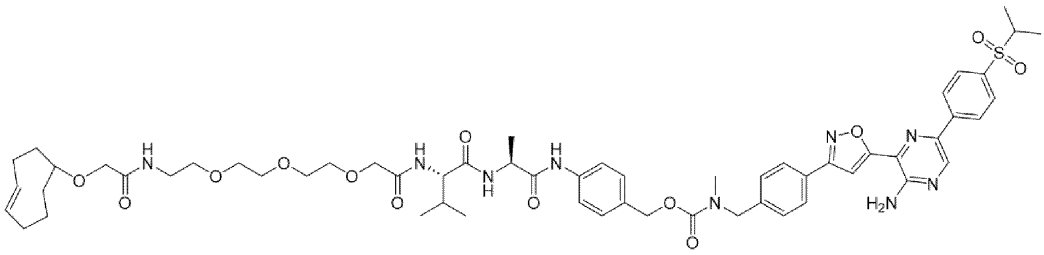
LP-1

In some embodiments, the TOP1 inhibitor moiety linked to click group has the structure:



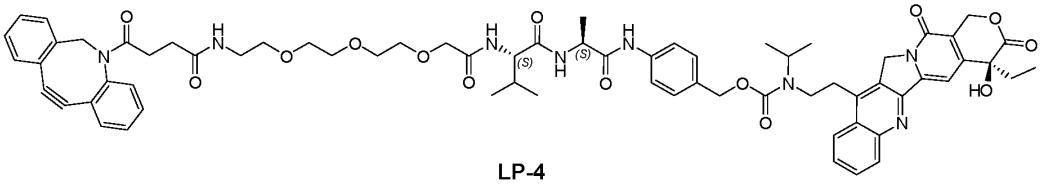
LP-2

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



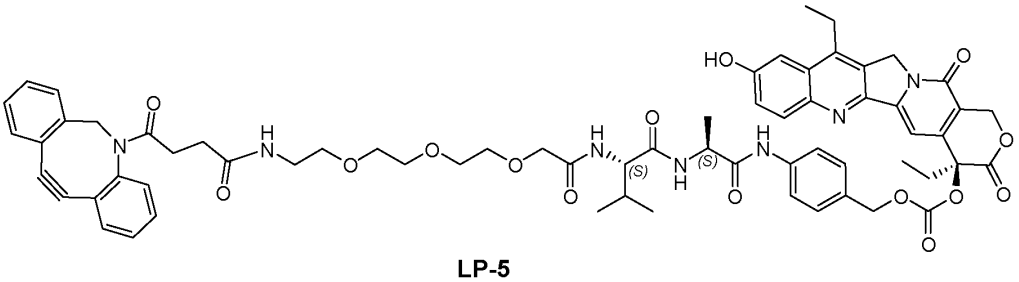
LP-3

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



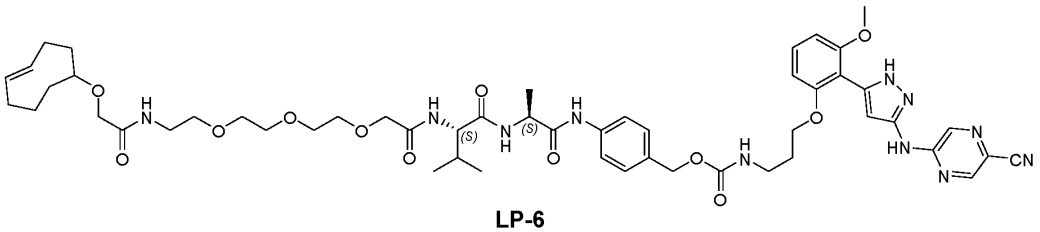
LP-4

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



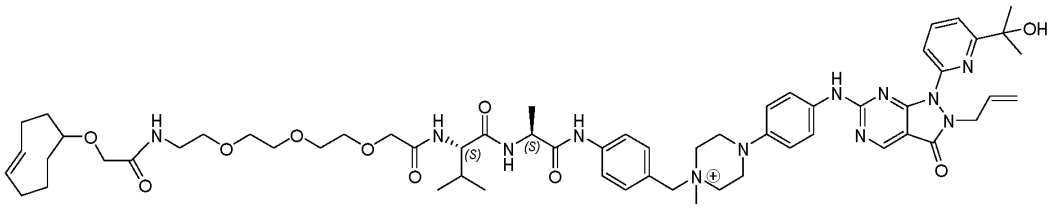
LP-5

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



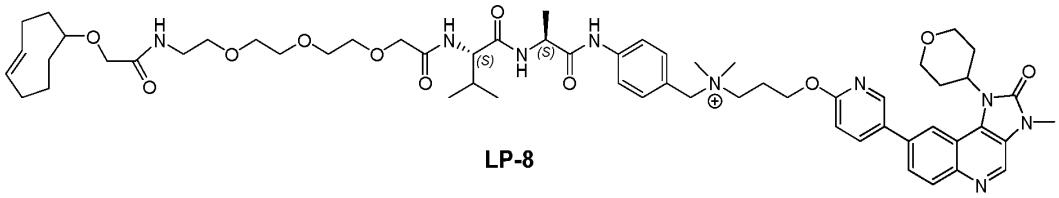
LP-6

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



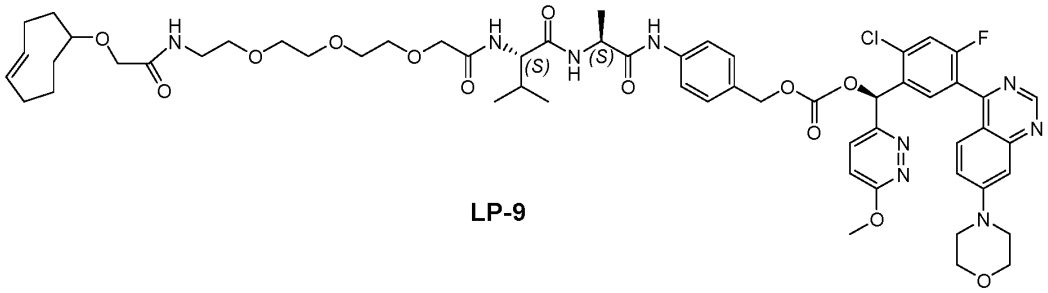
LP-7

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



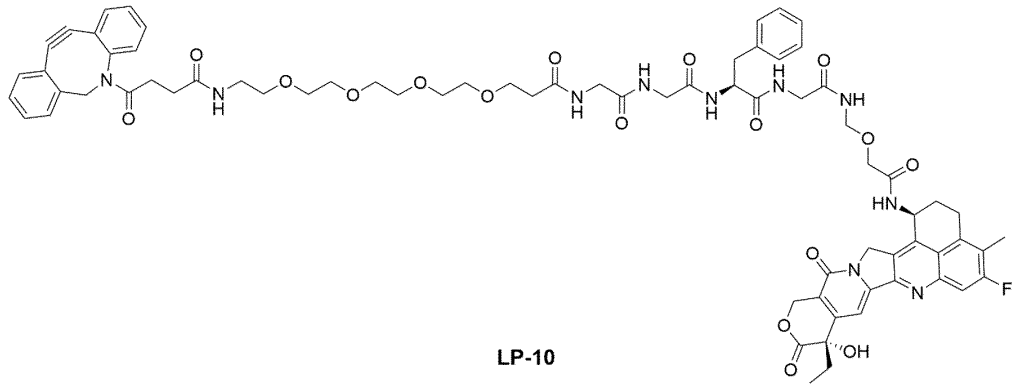
LP-8

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



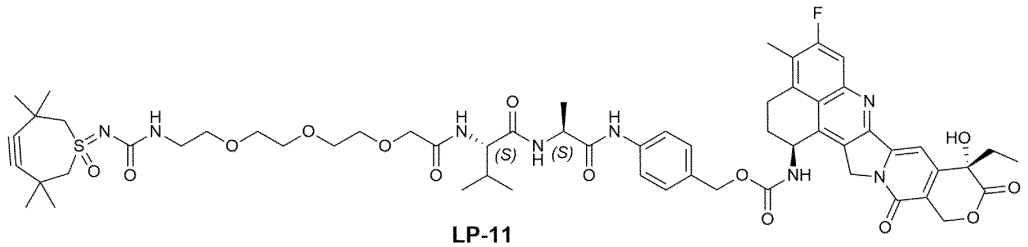
LP-9

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



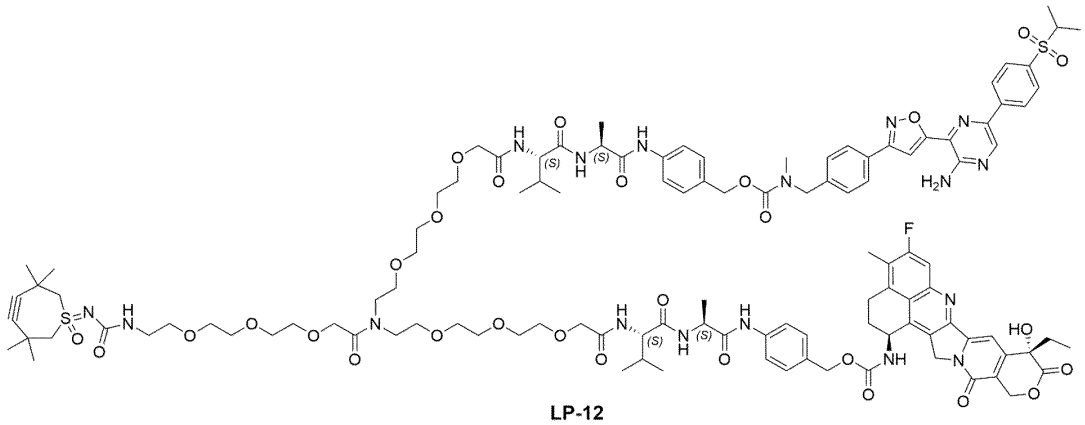
LP-10

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



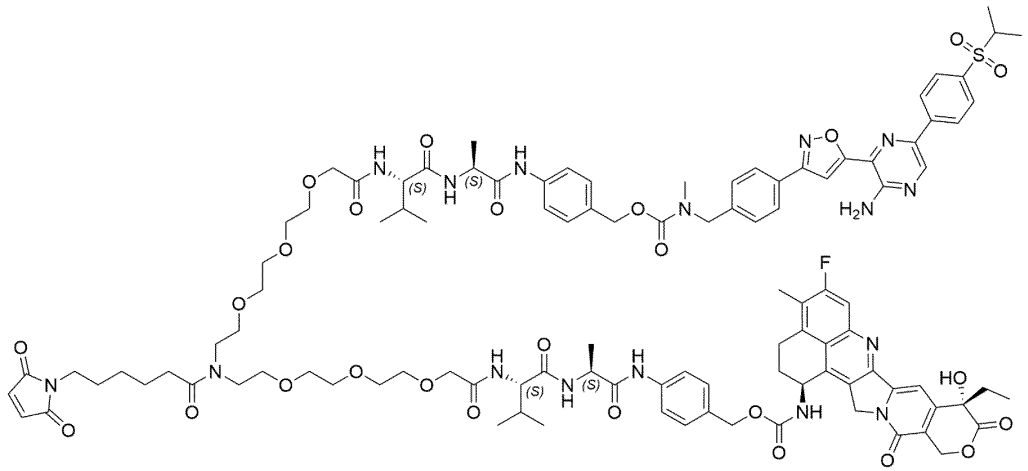
LP-11

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



LP-12

In some embodiments, the DDR inhibitor moiety linked to click group has the structure:



LP-13

Functional properties of the antigen-binding molecules

The antigen-binding molecules described herein may be characterised by reference to certain functional properties. In some embodiments, an antigen-binding molecule described herein may possess one or more of the following properties:

binds to cells expressing HER2;

inhibits proliferation of HER2-expressing cells;
increases killing of cells expressing HER2;
inhibits tumor growth and/or reduces tumor size/volume (*e.g.* of a HER2-expressing cancer);
increases survival of subjects having a cancer (*e.g.* a HER2-expressing cancer).

It will be appreciated that a given antigen-binding molecule may display more than one of the properties recited in the preceding paragraph. A given antigen-binding molecule may be evaluated for the properties recited in the preceding paragraph using suitable assays. For example, the assays may be *e.g. in vitro* assays, optionally cell-based assays or cell-free assays. In some embodiments, the assays may be *e.g. in vivo* assays, *i.e.* performed in non-human animals. In some embodiments, the assays may be *e.g. ex vivo* assays, *i.e.* performed using cells/tissue/an organ obtained from a subject.

Where assays are cell-based assays, they may comprise treating cells with an antigen-binding molecule in order to determine whether the antigen-binding molecule displays one or more of the recited properties. Assays may employ species labelled with detectable entities in order to facilitate their detection. Assays may comprise evaluating the recited properties following treatment of cells separately with a range of quantities/concentrations of a given antigen-binding molecule (*e.g.* a dilution series).

Analysis of the results of such assays may comprise determining the concentration at which 50% of the maximal level of the relevant activity is attained. The concentration of a given agent at which 50% of the maximal level of the relevant activity is attained may be referred to as the 'half-maximal effective concentration' of the agent in relation to the relevant activity, which may also be referred to as the 'EC_{50}'. Depending on the property, the EC_{50} may also be referred to as the 'half-maximal inhibitory concentration' or 'IC_{50}', this being the concentration of the agent at which 50% of the maximal level of inhibition of a given property is observed.

In some embodiments, the antigen-binding molecule of the present disclosure binds to HER2 in a region which is accessible to an antigen-binding molecule (*i.e.*, an extracellular antigen-binding molecule) when HER2 is expressed at the cell surface (*i.e.* in or at the cell membrane). In some embodiments, the antigen-binding molecule binds to HER2 expressed at the cell surface of a cell expressing HER2. In some embodiments, the antigen-binding molecule binds to HER2-expressing cells.

The ability of an antigen-binding molecule to bind to a given cell type can be analysed by contacting cells with the antigen-binding molecule, and detecting antigen-binding molecule bound to the cells, *e.g.* after a washing step to remove unbound antigen-binding molecule. The ability of an antigen-binding molecule to bind to HER2-expressing cells can be analysed by methods such as flow cytometry and immunofluorescence microscopy.

In some embodiments, the antigen-binding molecule inhibits proliferation of HER2-expressing cells (*e.g.* HER2-expressing cancer cells). The ability of an antigen-binding molecule to inhibit proliferation of a given cell type can be analysed by contacting cells with the antigen-binding molecule, and subsequently

evaluating proliferation of the cells (*i.e.* after a period of time sufficient for an effect on cell proliferation to be observed). Cell proliferation can be evaluated *e.g.* by detecting changes in number of cells over time, or by *in vitro* analysis of incorporation of ^{3}H-thymidine or by CFSE dilution assay, *e.g.* as described in Fulcher and Wong, Immunol Cell Biol (1999) 77(6): 559-564, hereby incorporated by reference in entirety.

In some embodiments, the antigen-binding molecule of the present invention is capable of inhibiting proliferation of HER2-expressing cells to less than 1 times, *e.g.* ≤0.99 times, ≤0.95 times, ≤0.9 times, ≤0.85 times, ≤0.8 times, ≤0.75 times, ≤0.7 times, ≤0.65 times, ≤0.6 times, ≤0.55 times, ≤0.5 times, ≤0.45 times, ≤0.4 times, ≤0.35 times, ≤0.3 times, ≤0.25 times, ≤0.2 times, ≤0.15 times, ≤0.1 times, ≤0.05 times, or ≤0.01 times the level of proliferation of HER2-expressing cells observed in the absence of the antigen-binding molecule (or in the presence of an appropriate control antigen-binding molecule known not to influence proliferation of HER2-expressing cells), in a given assay.

In some embodiments, the antigen-binding molecule described herein inhibits proliferation of cells expressing human HER2 with an IC_{50} of 100 nM or less, preferably one of ≤50 nM, ≤40 nM, ≤30 nM, ≤20 nM, ≤10 nM, ≤5 nM, ≤4 nM, ≤3 nM, ≤2 nM, ≤1 nM, ≤900 pM, ≤800 pM, ≤700 pM, ≤600 pM or ≤500 pM.

In some embodiments, the antigen-binding molecule according to the present disclosure potentiates (*i.e.* upregulates, enhances) cell killing of cells comprising/expressing HER2.

In some embodiments, an antigen-binding molecule according to the present disclosure may inhibit growth or reduce metastasis of a cancer comprising cells comprising/expressing HER2. In some embodiments, an antigen-binding molecule may potentiate (*i.e.* upregulate, enhance) cell killing of cells comprising/expressing HER2. In some embodiments, an antigen-binding molecule may inhibit growth of cells of a cancer, or may inhibit growth of a tumor, comprising cells comprising/expressing HER2. In some embodiments, an antigen-binding molecule may inhibit metastasis of a cancer/tumor comprising cells comprising/expressing HER2.

Cell killing can be investigated, for example, using any of the methods reviewed in Zaritskaya *et al.*, Expert Rev Vaccines (2011), 9(6):601-616, hereby incorporated by reference in its entirety. Examples of *in vitro* assays of cytotoxicity/cell killing assays include release assays such as the ^{51}Cr release assay, the lactate dehydrogenase (LDH) release assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) release assay, ATP release assay using Cell Titre Glo, and the calcein-acetoxymethyl (calcein-AM) release assay. These assays measure cell killing based on the detection of factors released from lysed cells.

In some embodiments an antigen-binding molecule according to the present disclosure is capable of reducing the number/proportion of cells expressing HER2. In some embodiments, an antigen-binding molecule according to the present disclosure is capable of depleting/enhancing depletion of such cells.

In some embodiments, an antigen-binding molecule of the present disclosure displays anticancer activity. In some embodiments, the antigen-binding molecule increases killing of cancer cells. In some embodiments, the antigen-binding molecule causes a reduction in the number of cancer cells *in vivo*, *e.g.* as compared to an appropriate control condition. The cancer may be a cancer as described herein, *e.g.* a cancer expressing/overexpressing HER2.

In some embodiments, an antigen-binding molecule according to the present disclosure reduces/inhibits growth of a cancer and/or of a tumor of a cancer. In some embodiments, an antigen-binding molecule reduces tissue invasion by cells of a cancer. In some embodiments, an antigen-binding molecule reduces metastasis of a cancer. In some embodiments, an antigen-binding molecule displays anticancer activity. In some embodiments, an antigen-binding molecule reduces the growth/proliferation of cancer cells. In some embodiments, an antigen-binding molecule reduces the survival of cancer cells. In some embodiments, an antigen-binding molecule increases the killing of cancer cells. In some embodiments, an antigen-binding molecule of the present disclosure causes a reduction in the number of cancer cells *e.g. in vivo*. The cancer may be a cancer comprising cells expressing HER2.

An antigen-binding molecule of the present disclosure may be analysed for the properties described in the preceding paragraph in appropriate assays. Such assays include *e.g. in vivo* models.

In some embodiments, administration of an antigen-binding molecule according to the present disclosure may cause one or more of: inhibition of the development/progression of the cancer, a delay to/prevention of onset of the cancer, a reduction in/delay to/prevention of tumor growth, a reduction in/delay to/prevention of tissue invasion, a reduction in/delay to/prevention of metastasis, a reduction in the severity of one or more symptoms of the cancer, a reduction in the number of cancer cells, a reduction in the cancer burden, a reduction in tumor size/volume, and/or an increase in survival of subjects having the cancer (*e.g.* progression free survival or overall survival), *e.g.* as determined in an appropriate model.

It will be appreciated that the properties recited in the preceding paragraph are evaluated after a period of time sufficient for an effect associated with treatment using the antigen-binding molecule to be observed. Tumor growth may be monitored by investigating tumor volume over time. Tumor growth may be evaluated by measuring tumor volume (*e.g.* in mm^3) over time.

In some embodiments, an antigen-binding molecule of the present disclosure is capable of reducing tumor size/volume (*e.g.* the mean tumor size/volume for the treatment group in an *in vivo* model, *e.g.* of a HER2-expressing cancer) to less than 1 times, *e.g.* ≤0.99 times, ≤0.95 times, ≤0.9 times, ≤0.85 times, ≤0.8 times, ≤0.75 times, ≤0.7 times, ≤0.65 times, ≤0.6 times, ≤0.55 times, ≤0.5 times, ≤0.45 times, ≤0.4 times, ≤0.35 times, ≤0.3 times, ≤0.25 times, ≤0.2 times, ≤0.15 times, ≤0.1 times, ≤0.05 times, or ≤0.01 times the tumor size/volume observed at the same time point in the absence of treatment with the antigen-binding molecule (or following treatment with an appropriate control antigen-binding molecule known not to influence tumor growth), in a given assay. In some embodiments, evaluation of tumor size/volume for the purposes of such comparison is performed after more than 5 days, *e.g.* one of ≥10

days, ≥15 days, ≥20 days, ≥25 days, ≥30 days, ≥35 days, ≥40 days, ≥35 days, ≥50 days, ≥55 days, ≥60 days, ≥65 days, ≥70 days, ≥75 days, ≥80 days, ≥85 days, ≥90 days, ≥95 days or ≥100 days following administration of the first dose of the antigen-binding molecule, in the relevant model.

In some embodiments, an antigen-binding molecule of the present disclosure achieves a level of tumor growth inhibition (*e.g.* expressed as % tumor growth inhibition, *e.g.* calculated relative to tumor growth observed on treatment with an appropriate control antigen-binding molecule) which is greater than 1 times, *e.g.* one of ≥1.01 times, ≥1.02 times, ≥1.03 times, ≥1.04 times, ≥1.05 times, ≥1.1 times, ≥1.2 times, ≥1.3 times, ≥1.4 times, ≥1.5 times, ≥1.6 times, ≥1.7 times, ≥1.8 times, ≥1.9 times, ≥2 times, ≥3 times, ≥4 times, ≥5 times, ≥6 times, ≥7 times, ≥8 times, ≥9 times or ≥10 times the level of tumor growth inhibition observed at the same time point in the absence of treatment with the antigen-binding molecule (or following treatment with an appropriate control antigen-binding molecule known not to influence tumor growth), in a given assay. In some embodiments, evaluation of tumor growth inhibition for the purposes of such comparison is performed after more than 5 days, *e.g.* one of ≥10 days, ≥15 days, ≥20 days, ≥25 days, ≥30 days, ≥35 days, ≥40 days, ≥35 days, ≥50 days, ≥55 days, ≥60 days, ≥65 days, ≥70 days, ≥75 days, ≥80 days, ≥85 days, ≥90 days, ≥95 days or ≥100 days following administration of the first dose of the antigen-binding molecule, in the relevant model.

In some embodiments, an antigen-binding molecule of the present disclosure is capable of increasing median survival of subjects having a cancer (*e.g.* in an *in vivo* model, *e.g.* of a HER2-expressing cancer) to greater than 1 times, *e.g.* one of ≥1.01 times, ≥1.02 times, ≥1.03 times, ≥1.04 times, ≥1.05 times, ≥1.1 times, ≥1.2 times, ≥1.3 times, ≥1.4 times, ≥1.5 times, ≥1.6 times, ≥1.7 times, ≥1.8 times, ≥1.9 times, ≥2 times, ≥3 times, ≥4 times, ≥5 times, ≥6 times, ≥7 times, ≥8 times, ≥9 times or ≥10 times the median survival observed in the absence of treatment with the antigen-binding molecule (or following treatment with an appropriate control antigen-binding molecule known not to influence survival of subjects having the cancer), in a given assay. Median survival may be expressed in days from the start of the experiment, for subjects in the relevant treatment groups.

Additional sequences

The antigen-binding molecules of the present disclosure and their constituent polypeptides may additionally comprise further amino acids or sequences of amino acids.

The polypeptides of the present disclosure may comprise one or more linker sequences between sequences of amino acids. Linker sequences are known to the skilled person, and are described, for example in Chen *et al.*, Adv Drug Deliv Rev (2013) 65(10): 1357-1369, which is hereby incorporated by reference in its entirety. In some embodiments, a linker sequence may be a flexible linker sequence. Flexible linker sequences allow for relative movement of the amino acid sequences which are linked by the linker sequence. Flexible linkers are known to the skilled person, and several are identified in Chen *et al.*, Adv Drug Deliv Rev (2013) 65(10): 1357-1369. Flexible linker sequences often comprise high proportions of glycine and/or serine residues.

In some embodiments, the linker sequence comprises at least one glycine residue and/or at least one serine residue. In some embodiments, the linker sequence comprises or consists of glycine and serine residues. In some embodiments, the linker sequence has the structure: (GxS)n or (GxS)nGm; wherein G = glycine, S = serine, x = 3 or 4, n = 2, 3, 4, 5 or 6, and m = 0, 1, 2 or 3. In some embodiments, the linker sequence comprises one or more (*e.g.* 1, 2, 3, 4, 5 or 6) copies (*e.g.* in tandem) of the sequence motif G_4S. In some embodiments, the linker sequence comprises or consists of $(G_4S)_4$ or $(G_4S)_6$. In some embodiments, the linker sequence has a length of 1-2, 1-3, 1-4, 1-5, 1-10, 1-15, 1-20, 1-25, or 1-30 amino acids.

The antigen-binding molecules of the present disclosure and their constituent polypeptides may comprise amino acid sequence(s) to facilitate expression, folding, trafficking, processing, purification or detection of the antigen-binding molecule/polypeptide. For example, antigen-binding molecules and polypeptides of the present disclosure may additionally comprise a sequence of amino acids forming a detectable moiety, *e.g.* as described hereinbelow.

The antigen-binding molecules of the present disclosure and their constituent polypeptides may additionally comprise a signal peptide (also known as a leader sequence or signal sequence). Signal peptides normally consist of a sequence of 5-30 hydrophobic amino acids, which form a single alpha helix. Secreted proteins and proteins expressed at the cell surface often comprise signal peptides. Signal peptides are known for many proteins, and are recorded in databases such as GenBank, UniProt and Ensembl, and/or can be identified/predicted *e.g.* using amino acid sequence analysis tools such as SignalP (Petersen *et al.*, 2011 Nature Methods 8: 785-786) or Signal-BLAST (Frank and Sippl, 2008 Bioinformatics 24: 2172-2176).

The signal peptide may be present at the N-terminus of the polypeptide, and may be present in the newly synthesised polypeptide. The signal peptide provides for efficient trafficking of the polypeptide. Signal peptides are often removed by cleavage, and thus are not comprised in the mature polypeptide.

Signal peptides are known for many proteins, and are recorded in databases such as GenBank, UniProt, Swiss-Prot, TrEMBL, Protein Information Resource, Protein Data Bank, Ensembl, and InterPro, and/or can be identified/predicted *e.g.* using amino acid sequence analysis tools such as SignalP (Petersen *et al.*, 2011 Nature Methods 8: 785-786) or Signal-BLAST (Frank and Sippl, 2008 Bioinformatics 24: 2172-2176).

Labels and conjugates

In some embodiments, the antigen-binding molecules of the present disclosure and their constituent polypeptides comprise a detectable moiety.

In some embodiments, a detectable moiety is a fluorescent label, phosphorescent label, luminescent label, immuno-detectable label (*e.g.* an epitope tag), radiolabel, chemical, nucleic acid or enzymatic label.

An antigen-binding molecule or a constituent polypeptide thereof may be covalently or non-covalently labelled with the detectable moiety.

Fluorescent labels include *e.g.* fluorescein, rhodamine, allophycocyanin, eosine and NDB, green fluorescent protein (GFP), chelates of rare earths such as europium (Eu), terbium (Tb) and samarium (Sm), tetramethyl rhodamine, Texas Red, 4-methyl umbelliferone, 7-amino-4-methyl coumarin, Cy3, and Cy5. Radiolabels include radioisotopes such as Hydrogen^{3}, Sulfur^{35}, Carbon^{14}, Phosphorus^{32}, Iodine^{123}, Iodine^{125}, Iodine^{126}, Iodine^{131}, Iodine^{133}, Bromine^{77}, Technetium^{99m}, Indium^{111}, Indium^{113m}, Gallium^{67}, Gallium^{68}, Ruthenium^{95}, Ruthenium^{97}, Ruthenium^{103}, Ruthenium^{105}, Mercury^{207}, Mercury^{203}, Rhenium^{99m}, Rhenium^{101}, Rhenium^{105}, Scandium^{47}, Tellurium^{121m}, Tellurium^{122m}, Tellurium^{125m}, Thulium^{165}, Thuliuml^{167}, Thulium^{168}, Copper^{67}, Fluorine^{18}, Yttrium^{90}, Palladium^{100}, Bismuth^{217} and Antimony^{211}. Luminescent labels include as radioluminescent, chemiluminescent (*e.g.* acridinium ester, luminol, isoluminol) and bioluminescent labels. Immuno-detectable labels include haptens, peptides/polypeptides, antibodies, receptors and ligands such as biotin, avidin, streptavidin or digoxigenin. Nucleic acid labels include aptamers.

In some embodiments, an antigen-binding molecule or a constituent polypeptide thereof comprises a radionuclide. Such conjugates may be called radionuclide drug conjugates (RDCs) or radioimmunoconjugates (RICs). In some embodiments, an antigen-binding molecule or a constituent polypeptide thereof comprises a chelator group that is capable of chelating a radionuclide. Radionuclides include radioisotopes such as those listed above, or others such as Lutetium^{177}, Actinium^{225} and Strontium^{90}.

In some embodiments, an antigen-binding molecule or a constituent polypeptide thereof comprises an epitope tag, *e.g.* a His, (*e.g.* 6XHis), FLAG, c-Myc, StrepTag, haemagglutinin, E, calmodulin-binding protein (CBP), glutathione-s-transferase (GST), maltose-binding protein (MBP), thioredoxin, S-peptide, T7 peptide, SH2 domain, avidin, streptavidin, and haptens (*e.g.* biotin, digoxigenin, dinitrophenol), optionally at the N- or C- terminus of the antigen-binding molecule/polypeptide.

In some embodiments, an antigen-binding molecule or a constituent polypeptide thereof polypeptide comprises a moiety having a detectable activity, *e.g.* an enzymatic moiety. Enzymatic moieties include *e.g.* luciferases, glucose oxidases, galactosidases (*e.g.* beta-galactosidase), glucorinidases, phosphatases (*e.g.* alkaline phosphatase), peroxidases (*e.g.* horseradish peroxidase) and cholinesterases.

Nucleic acids and vectors

The present disclosure provides a nucleic acid, or a plurality of nucleic acids, encoding an antigen-binding molecule/antigen-binding polypeptide complex or a constituent polypeptide thereof according to the present disclosure. In some embodiments, the nucleic acid(s) comprise or consist of DNA and/or RNA.

An antigen-binding molecule/antigen-binding polypeptide complex or a constituent polypeptide thereof according to the present disclosure may be produced within a cell by translation of RNA encoding the polypeptide(s). An antigen-binding molecule/antigen-binding polypeptide complex or a constituent polypeptide thereof may be produced within a cell by transcription from nucleic acid encoding the polypeptide(s), and subsequent translation of the transcribed RNA.

In some embodiments, the nucleic acid(s) may be, or may be comprised/contained in, a vector, or a plurality of vectors. A 'vector' as used herein is a nucleic acid molecule used as a vehicle to transfer exogenous nucleic acid into a cell.

Accordingly, the present disclosure also provides a vector, or plurality of vectors, comprising the nucleic acid or plurality of nucleic acids according to the present disclosure. The vector may facilitate delivery of the nucleic acid(s) encoding a polypeptide according to the present disclosure to a cell. The vector may be an expression vector comprising elements required for expressing a polypeptide according to the present disclosure. The vector may comprise elements facilitating integration of the nucleic acid(s) into the genomic DNA of cell into which the vector is introduced.

Nucleic acids and vectors according to the present disclosure may be provided in purified or isolated form, *i.e.* from other nucleic acid, or naturally-occurring biological material.

A vector may be a vector for expression of the nucleic acid in the cell (*i.e.* an expression vector). Such vectors may include a promoter sequence operably linked to a nucleotide sequence encoding an antigen-binding molecule or polypeptide according to the present disclosure. A vector may also include a termination codon (*i.e.* 3' in the nucleotide sequence of the vector to the nucleotide sequence encoding the polypeptide(s)) and expression enhancers. Any suitable vectors, promoters, enhancers and termination codons known in the art may be used to express a peptide or polypeptide from a vector according to the present disclosure.

The term 'operably linked' may include the situation where nucleic acid encoding a polypeptide according to the present disclosure and regulatory nucleic acid sequence(s) (*e.g.* a promoter and/or enhancers) are covalently linked in such a way as to place the expression of the nucleic acid encoding a polypeptide under the influence or control of the regulatory nucleic acid sequence(s) (thereby forming an expression cassette). Thus, a regulatory sequence is operably linked to the selected nucleic acid sequence if the regulatory sequence is capable of effecting transcription of the nucleic acid sequence. The resulting transcript(s) may then be translated into the desired polypeptide(s).

Vectors contemplated in connection with the present disclosure include DNA vectors, RNA vectors, plasmids (*e.g.* conjugative plasmids (*e.g.* F plasmids), non-conjugative plasmids, R plasmids, col plasmids, episomes), viral vectors (*e.g.* retroviral vectors, *e.g.* gammaretroviral vectors (*e.g.* murine Leukemia virus (MLV)-derived vectors, *e.g.* SFG vector), lentiviral vectors, adenovirus vectors, adeno-associated virus vectors, vaccinia virus vectors and herpesvirus vectors), transposon-based vectors, and

artificial chromosomes (*e.g.* yeast artificial chromosomes), *e.g.* as described in Maus *et al.*, Annu Rev Immunol (2014) 32:189-225 and Morgan and Boyerinas, Biomedicines (2016) 4:9, which are both hereby incorporated by reference in their entirety. In some embodiments, a vector according to the present disclosure is a lentiviral vector.

In some embodiments, the vector may be a eukaryotic vector, *i.e.* a vector comprising the elements necessary for expression of protein from the vector in a eukaryotic cell. In some embodiments, the vector may be a mammalian vector, *e.g.* comprising a cytomegalovirus (CMV) or SV40 promoter to drive protein expression.

Constituent polypeptides of an antigen-binding molecule/antigen-binding polypeptide complex according to the present disclosure may be encoded by different nucleic acids of the plurality of nucleic acids, or by different vectors of the plurality of vectors.

Producing the antigen-binding molecules

Antigen-binding molecules according to the present disclosure may be prepared according to methods for the production of antibody-drug conjugates known to the skilled person.

Antigen-binding moieties according to the present disclosure may be prepared by chemical synthesis, *e.g.* liquid or solid phase synthesis. For example, peptides/polypeptides can be synthesised using the methods described in, for example, Chandrudu *et al.*, Molecules (2013), 18: 4373-4388, which is hereby incorporated by reference in its entirety.

Alternatively, antigen-binding moieties according to the present disclosure may be produced by recombinant expression. Molecular biology techniques suitable for recombinant production of polypeptides are well known in the art, such as those set out in Green and Sambrook, Molecular Cloning: A Laboratory Manual (4th Edition), Cold Spring Harbor Press, 2012, and in Nat Methods. (2008); 5(2): 135-146 both of which are hereby incorporated by reference in their entirety. Methods for the recombinant production of antigen-binding polypeptides are also described in Frenzel *et al.*, Front Immunol. (2013); 4: 217 and Kunert and Reinhart, Appl Microbiol Biotechnol. (2016) 100: 3451–3461, both of which are hereby incorporated by reference in their entirety.

In some cases, the antigen-binding moieties of the present disclosure are comprised of more than one polypeptide chain. In such cases, production of the antigen-binding moiety may comprise transcription and translation of more than one polypeptide, and subsequent association of the polypeptide chains to form the antigen-binding moiety.

For recombinant production according to the present disclosure, any cell suitable for the expression of polypeptides may be used. The cell may be a prokaryote or eukaryote. In some embodiments, the cell is a prokaryotic cell, such as a cell of archaea or bacteria. In some embodiments, the bacteria may be Gram-negative bacteria such as bacteria of the family Enterobacteriaceae, for example *Escherichia coli*.

In some embodiments, the cell is a eukaryotic cell such as a yeast cell, a plant cell, insect cell or a mammalian cell, *e.g.* a cell described hereinabove. In some cases, the cell is not a prokaryotic cell because some prokaryotic cells do not allow for the same folding or post-translational modifications as eukaryotic cells. In addition, very high expression levels are possible in eukaryotes and proteins can be easier to purify from eukaryotes using appropriate tags. Specific plasmids may also be utilised which enhance secretion of the protein into the media.

In some embodiments polypeptides may be prepared by cell-free-protein synthesis (CFPS), *e.g.* according to a system described in Zemella *et al.* Chembiochem (2015) 16(17): 2420-2431, which is hereby incorporated by reference in its entirety.

Production of antigen-binding moieties may involve culture or fermentation of a eukaryotic cell modified to express the polypeptide(s) of interest. The culture or fermentation may be performed in a bioreactor provided with an appropriate supply of nutrients, air/oxygen and/or growth factors. Secreted proteins can be collected by partitioning culture media/fermentation broth from the cells, extracting the protein content, and separating individual proteins to isolate secreted polypeptide(s). Culture, fermentation and separation techniques are well known to those of skill in the art, and are described, for example, in Green and Sambrook, Molecular Cloning: A Laboratory Manual (4th Edition; incorporated by reference herein above). Bioreactors include one or more vessels in which cells may be cultured. Culture in the bioreactor may occur continuously, with a continuous flow of reactants into, and a continuous flow of cultured cells from, the reactor. Alternatively, the culture may occur in batches. The bioreactor monitors and controls environmental conditions such as pH, oxygen, flow rates into and out of, and agitation within the vessel such that optimum conditions are provided for the cells being cultured.

Following culturing the cells that express the polypeptide(s), the polypeptide(s) of interest may be isolated. Any suitable method for separating proteins from cells known in the art may be used. In order to isolate the polypeptide, it may be necessary to separate the cells from nutrient medium. If the polypeptide(s) are secreted from the cells, the cells may be separated by centrifugation from the culture media that contains the secreted polypeptide(s) of interest. If the polypeptide(s) of interest collect within the cell, protein isolation may comprise centrifugation to separate cells from cell culture medium, treatment of the cell pellet with a lysis buffer, and cell disruption *e.g.* by sonification, rapid freeze-thaw or osmotic lysis.

It may then be desirable to isolate the polypeptide(s) of interest from the supernatant or culture medium, which may contain other protein and non-protein components. A common approach to separating protein components from a supernatant or culture medium is by precipitation. Proteins of different solubilities are precipitated at different concentrations of precipitating agent such as ammonium sulfate. For example, at low concentrations of precipitating agent, water soluble proteins are extracted. Thus, by adding different increasing concentrations of precipitating agent, proteins of different solubilities may be distinguished. Dialysis may be subsequently used to remove ammonium sulfate from the separated proteins. Other methods for distinguishing different proteins are known in the art, for example ion exchange

chromatography and size chromatography. These may be used as an alternative to precipitation or may be performed subsequently to precipitation.

Once the polypeptide(s) of interest have been isolated from culture it may be desired or necessary to concentrate the polypeptide(s). A number of methods for concentrating proteins are known in the art, such as ultrafiltration or lyophilisation.

Antigen-binding polypeptides/polypeptide complexes according to the present disclosure may be conjugated to linker-payload moieties according to the present disclosure for the production of antigen-binding molecules according to the present disclosure by any suitable techniques, which are well known to the skilled person and routinely employed in the art.

General methods for the conjugation of antigen-binding polypeptides/polypeptide complexes to linker-payload moieties are described *e.g.* in Chudasama *et al.*, Nature Chemistry, (2016), 8:114-119, Baah *et al.*, Molecules. (2021) 26(10): 2943, and Walsh *et al.*, Chem. Soc. Rev. (2021) 50:1305-1353, all of which are hereby incorporated by reference in their entirety. Conjugation of antigen-binding moieties and linker-payload moieties and purification of antigen-binding molecules produced by such conjugation is described *e.g.* in Beck *et al.*, (2017) Nat Rev Drug Discov 16: 315-337; Peters and Brown Biosci Rep (2015) 35: art:e00225; McCombs and Owen, The AAPS Journal (2015) 17: 339-351; Jackson, Org Process Res Dev (2016) 20: 852-866; and Olivier and Hurvitz, Antibody-Drug Conjugates: Fundamentals, Drug Development, and Clinical, (2016) Wiley.

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Antigen-binding moieties according to the present disclosure may be conjugated to linker-payload moieties according to the present disclosure by any suitable techniques, which are well known to the skilled person and routinely employed in the art. Such methods are described *e.g.* in Chudasama *et al.*, Nature Chemistry, (2016), 8:114-119, Baah *et al.*, Molecules. (2021) 26(10): 2943, and Walsh *et al.*, Chem. Soc. Rev. (2021) 50:1305-1353, all of which are hereby incorporated by reference in their entirety.

Conjugation of antigen-binding moieties and linker-payload moieties and the purification of antigen-binding molecules produced by such conjugation can be performed *e.g.* as described in in Beck *et al.*, (2017) Nat Rev Drug Discov 16: 315-337; Peters and Brown Biosci Rep (2015) 35: art:e00225; McCombs

and Owen, The AAPS Journal (2015) 17: 339-351; Jackson, Org Process Res Dev (2016) 20: 852-866; or Olivier and Hurvitz, Antibody-Drug Conjugates: Fundamentals, Drug Development, and Clinical Outcome to Target Cancer, (2016) Wiley, all of which are hereby incorporated by reference in their entirety. Other relevant disclosures relating to conjugation and linkers are: Tsuchikama and An, Protein Cell. (2018) 9(1): 33-46, Khongorzul *et al.*, Mol Cancer Res (2020) 18 (1): 3-19 and Drago *et al.*, Nature Reviews Clinical Oncology (2021) 18: 327-344, all of which are hereby incorporated by reference in their entirety.

Lysine amide coupling

Lysine-based conjugation is one of the most widely used non-specific conjugation strategies. Such conjugation occurs on reactive amine side chains of lysine residues due to their good nucleophilicity. Immunoglobulin scaffolds contains over 80 lysine residues, most of which are exposed on the surface of the molecule. Among the surface lysine residues, more than 20 have been shown as highly solvent-accessible and can serve as potential ADC conjugation sites. Lysine conjugation follows two main strategies that result in the formation of a stable amide or amidine bond between the protein and the drug-linker complex. Generally speaking, activated esters on the drug-linker complexes, often O-succinimide reagents such as N-hydroxysuccinimidyl (NHS) or sulfo-NHS esters, react with the antibody lysine residues and achieve the conjugation via amide bonds. On the other hand, stable amidine bonds can be generated on an antibody by the reaction of imido ester compounds, such as Traut's reagent, with antibody lysine residues.

A one-step conjugation of a drug-linker moiety containing an amine-reactive group to the antibody via amide bonds is known, as well as two-step conjugation, where in the first step, a small bi-functional reagent containing both an amine- and a thiol-reactive functional groups is reacted with the available lysine ε-amino groups to serve as a chemical adaptor, leaving free thiol-reactive groups on the antibody. In the second step, the payload drugs or drug-linker complexes are attached to the thiol-reactive groups introduced previously to form the ADC. The two-step approach is often used when the drug/drug-linker complex contains a thiol-reactive module or as an alternative route when introducing an amine-reactive module into the drug or drug-linker complex is proven to be difficult. Four small adaptors commonly used in the two-step conjugation: SPDB disulfide, MCC (maleimidomethyl cyclohexane-1-carboxylate), sulfo-SPDB, and Hydrazine.

Cysteine coupling

Cysteine modification occurs most commonly by 1,4-conjugate addition to *N*-substituted maleimides. Maleimides are particularly attractive reagents due to their synthetic accessibility and rapid reaction rates with cysteine under mild conditions. The resulting thiosuccinimide conjugates are inherently unstable, due to their propensity towards retro-Michael addition. This instability can be mitigated by forcing post-conjugation hydrolysis of the thiosuccinimide, creating a stable chemical linkage. Accordingly, a number of "self-hydrolysing" maleimides have now been developed, with ring-opening catalysed by adjacent functional groups such as primary amine, polyethylene glycol (PEG) and *N*-aryl amongst the most

promising. Other reagents including α-halocarbonyls, palladium oxidative-addition complexes, ethynylphosphonamidates, vinylphosphonites and ethynylbenziodoxolones.

Some non-maleimide cysteine conjugations are summarised in Kang, *et al.*, Chem Sci (2021) 12, 13613-13647 (doi: 10.1039/D1SC02973H), and include the use of:

(i) alkynyl carboxylic acid derivatives;
(ii) 5-methylene pyrrolone (5MP);
(iii) 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB);
(iv) phenyloxadiazole sulfone (PODS);
(v) aza-dibenzocyclooctyne (DBCO);
(vi) phosphonamidite;
(vii) 3-arylpropionitrile (APN);
(viii) perfluoroarene;
(ix) ethynylbenziodoxolone (EBX);
(x) bicyclo[1.1.0]butane (BCB) carboxylic amide; and
(xi) allenamide.

Another possible approach is described in Cheng, *et al.*, Front. Oncol. 12:951589 (doi: 10.3389/fonc.2022.951589) where 2-methylsulfonyl pyrimidine is used instead of a maleimide.

Genetic modification of the number of accessible cysteine residues on an antibody surface is a method to achieve site-selective and homogeneous modification. For example, in THIOMABs, the engineered cysteine is installed on an anti-MUC16 antibody by mutation of heavy chain alanine 114 (HC-A114). Other approaches have engineered antibodies to contain cysteine mutations at D265C, S239C, E269C, K326C or A327C, or to insert additional cysteines before and after positions HC-S239, HC-A114, and LC-V205.

Non-natural amino acid incorporation by genetic engineering

Site-specific incorporation of non-canonical amino acids (ncAAs) into antibodies results in an efficient approach to the site-specific modification of antibodies, and therefore homogeneous ADCs.

NcAAs bearing unique functionalities, such as ketones, azides, cyclopropenes or diene functional groups, have been developed and incorporated into antibodies. Such ncAAs include p-acetylphenylalanine (pAcF), which has a ketone side chain which can participate in oxime ligation reactions; Nε-(1-methylcycloprop-2-enecarboxamido)-lysine (CpK), which has a cyclopropene side chain which can participate in IEDDA reactions; para-azidomethyl phenylalanine (pAMF), which has a an azide side chain which can undergo click reactions; spiro[2.4]hepta-4,6-diene-lysine (SCpHK), which has a spiro[2.4]hepta-4,6-diene side chain which can participate in Diels–Alder reactions; and N6-(2-azidoethoxy)-carbonyl-L-lysine (AzK), which has a an azide side chain which can undergo click reactions.

Azide-containing ncAAs can undergo rapid CuAAC or SPAAC reactions under physiological conditions. *para*-azidophenylalanine (pAzF) can undergo reactions with, for example, cyclooctyne-functionalised

linkers and dibenzylcyclooctyne (DBCO)-functionalised linkers. A cyclopropene derivative of lysine (N ϵ-[((2-methylcycloprop-2-en-1-yl)methoxy)carbonyl]-l-lysine; CypK) can undergo a rapid and efficient inverse-electron demand Diels–Alder (IEDDA) reaction with a tetrazine-functionalised linker.

Cyclopentadiene-containing ncAAs, spiro[2.4]hepta-4,6-diene-lysine (SCpHK) and cyclopentadiene-lysine (CpHK), can undergo irreversible Diels–Alder cycloadditions with maleimide-modified drugs.

Enzymatic conjugation

Enzymes can be used to achieve site-selective antibody modification due to their high specificity and mild reaction conditions. Enzymes can either directly attach a payload to a specific amino acid sequence or introduce a reactive functionality on the antibody that can be further functionalised with the desired payload.

Transpeptidation using sortase

Sortase-mediated antibody conjugation (SMAC) technology is an additional enzymatic ligation approach. SMAC-technology uses S. aureus sortase A, which is a transpeptidase that cleaves the amide bond between threonine and glycine residues in the LPXTG (X = any amino acid) pentapeptide motif, and subsequently catalyses the attachment of glycine-functionalised payloads to the newly generated C-terminus. The sortase recognition motif and a Strep II tag, which is used to aid removal of unreacted antibody, were fused to the light and heavy chain C-terminus of different antibodies. Sortase-mediated conjugation can then be used to attach a series of penta-glycine tagged payloads.

Transpeptidation using microbial transglutaminase

The use of bacterial transglutaminases is a powerful approach for site-specific incorporation of the payload into the antibody. A transglutaminase derived from Streptomyces mobaraensis catalyzes transpeptidation where a primary amine-containing linker is covalently attached to the primary amide side chain of a specific glutamine (Q295) within deglycosylated antibodies, resulting in ADCs with a defined DAR arising from the conjugation of 2 linker-payloads (one conjugation site per heavy chain). An N297Q mutation prior to this conjugation provides two more reaction sites (resulting in the conjugation of 4-linker-payloads). An alternative version using a peptide sequence-specific transglutaminase. This enzyme recognizes and utilizes LLQG motif that is genetically incorporated, resulting in site-specific antibody-drug conjugation. Another advantage of this LLQG-specific bacterial transglutaminase is that conjugation sites can be flexibly laid by inserting this short peptide motif within the antibody structure. Further alternative approaches allow for the use of transglutaminase without deglycosylation.

The DAR will depend on the number of payloads per linker-payload moieties conjugated.

N-Glycan engineering

Asn297 (N297) within the Fc domain and the N-glycan on this residue are conserved in all IgG classes, making these components attractive reaction sites for broadly applicable ADC conjugation. Incorporation of an aldehyde group on the N-glycan terminus using β-1,4-galactosyltransferase (GalT) and α-2,6-

sialyltransferase (SialT) introduce a sialic acid on each N-glycan terminus, which is subsequently converted into an aldehyde group using $NaIO_4$ under mild oxidation conditions. The aldehyde groups generated can then used to conjugate aminooxyfunctionalized payloads.

Another approach is to incorporate non-natural saccharides possessing orthogonal reaction handles into the antibody. A technology based on this strategy is the GlycoConnect in which the glycan chain at Asn297 is trimmed using the endoglycosidase Endo S2 and then azide groups are introduced using a mutant galactosyl transferase GalT(Y289L) and N-azidoacetylgalactosamine (GalNAz). The azide handles can be used for a strain-promoted click reaction with payloads.

In some embodiments, the linker-payload terminates in an amino group which is conjugated to the antigen-binding molecule using transglutaminase.

In some embodiments, the method further comprises purifying/isolating the antigen-binding molecule (*i.e.* from unreacted precursors and/or by-products). In some embodiments, the antigen-binding molecule may be purified/isolated by chromatography, *e.g.* size-exclusion chromatography.

The present disclosure also provides an antigen-binding molecule obtained or obtainable by the methods of the present disclosure.

Compositions

The present disclosure provides a composition comprising an antigen-binding molecule according to the present disclosure.

The antigen-binding molecules described herein may be formulated as pharmaceutical compositions or medicaments for clinical use and may comprise a pharmaceutically-acceptable carrier, diluent, excipient or adjuvant. Thus, the present disclosure provides a pharmaceutical composition/medicament comprising an antigen-binding molecule described herein.

The pharmaceutical compositions/medicaments of the present disclosure may comprise one or more pharmaceutically-acceptable carriers (*e.g.* liposomes, micelles, microspheres, nanoparticles), diluents/excipients (*e.g.* starch, cellulose, a cellulose derivative, a polyol, dextrose, maltodextrin, magnesium stearate), adjuvants, fillers, buffers, preservatives (*e.g.* vitamin A, vitamin E, vitamin C, retinyl palmitate, selenium, cysteine, methionine, citric acid, sodium citrate, methyl paraben, propyl paraben), anti-oxidants (*e.g.* vitamin A, vitamin E, vitamin C, retinyl palmitate, selenium), lubricants (*e.g.* magnesium stearate, talc, silica, stearic acid, vegetable stearin), binders (*e.g.* sucrose, lactose, starch, cellulose, gelatin, polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), xylitol, sorbitol, mannitol), stabilisers, solubilisers, surfactants (*e.g.*, wetting agents), masking agents or colouring agents (*e.g.* titanium oxide).

The term ‘pharmaceutically-acceptable’ as used herein pertains to compounds, ingredients, materials, compositions, dosage forms, *etc.*, which are, within the scope of sound medical judgment, suitable for use

in contact with the tissues of the subject in question (*e.g.* a human subject) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, diluent, excipient, adjuvant, filler, buffer, preservative, anti-oxidant, lubricant, binder, stabiliser, solubiliser, surfactant, masking agent, colouring agent, flavouring agent or sweetening agent of a composition according to the present disclosure must also be 'acceptable' in the sense of being compatible with the other ingredients of the formulation. Suitable carriers, diluents, excipients, adjuvants, fillers, buffers, preservatives, anti-oxidants, lubricants, binders, stabilisers, solubilisers, surfactants, masking agents, colouring agents, flavouring agents or sweetening agents can be found in standard pharmaceutical texts, for example, Remington's 'The Science and Practice of Pharmacy' (Ed. A. Adejare), 23rd Edition (2020), Academic Press.

Pharmaceutical compositions and medicaments of the present disclosure may be formulated for topical, parenteral, systemic, intracavitary, intravenous, intra-arterial, intramuscular, intrathecal, intraocular, intraconjunctival, intratumoral, subcutaneous, intradermal, intrathecal, oral or transdermal routes of administration. In some embodiments, a pharmaceutical composition/medicament may be formulated for administration by injection or infusion, or administration by ingestion.

Suitable formulations may comprise the antigen-binding molecule provided in a sterile or isotonic medium. Medicaments and pharmaceutical compositions may be formulated in fluid, including gel, form. Fluid formulations may be formulated for administration by injection or infusion (*e.g.* via catheter) to a selected region of the human or animal body.

In some embodiments, the pharmaceutical compositions/medicament is formulated for injection or infusion, *e.g.* into a blood vessel, tissue/organ of interest, or a tumor.

The present disclosure also provides methods for the production of pharmaceutically useful compositions, such methods of production may comprise one or more steps selected from:

producing an antigen-binding molecule described herein;

isolating/purifying an antigen-binding molecule described herein; and/or

mixing an antigen-binding molecule described herein with a pharmaceutically-acceptable carrier, adjuvant, excipient or diluent.

For example, a further aspect of the present disclosure relates to a method of formulating or producing a medicament or pharmaceutical composition for use in the treatment of a disease/condition (*e.g.* a disease/condition described herein), the method comprising formulating a pharmaceutical composition or medicament by mixing an antigen-binding molecule described herein with a pharmaceutically-acceptable carrier, adjuvant, excipient or diluent.

Therapeutic and prophylactic applications

The antigen-binding molecules and compositions described herein find use in therapeutic and prophylactic intervention for disease, *e.g.* cancers.

It will be appreciated that the antigen-binding molecules and compositions of the present disclosure may be used for the treatment/prevention of any disease/condition that would derive therapeutic or prophylactic benefit from a reduction in the level of expression or activity of HER2, or a reduction in the number or activity of cells comprising/expressing HER2.

For example, the disease/condition may be a disease/condition in which HER2, or cells expressing/overexpressing HER2 are pathologically-implicated, *e.g.* a disease/condition in which an increased level/activity of HER2, or an increase in the number/proportion of cells comprising/expressing HER2 is positively associated with the onset, development or progression of the disease/condition, and/or severity of one or more symptoms of the disease/condition. In some embodiments, an increased level/activity of HER2, or an increase in the number/proportion of cells comprising/expressing HER2 may be a risk factor for the onset, development or progression of the disease/condition.

The present disclosure provides an antigen-binding molecule or composition described herein for use in a method of medical treatment or prophylaxis. Also provided is an antigen-binding molecule or composition described herein for use in a method of treating or preventing a cancer (*e.g.* a cancer described herein). Also provided is the use of an antigen-binding molecule or composition described herein in the manufacture of a medicament for treating or preventing a cancer (*e.g.* a cancer described herein). Also provided is a method of treating or preventing a cancer (*e.g.* a cancer described herein) in a subject, comprising administering to a subject a therapeutically or prophylactically effective amount of an antigen-binding molecule or composition described herein.

The methods may be effective to reduce the development or progression of a cancer, alleviation of the symptoms of a cancer or reduction in the pathology of a cancer. The methods may be effective to prevent progression of the cancer, *e.g.* to prevent worsening of, or to slow the rate of development of, the cancer. In some embodiments, the methods may lead to an improvement in the cancer, *e.g.* a reduction in the symptoms of the cancer or reduction in some other correlate of the severity/activity of the cancer. In some embodiments, the methods may prevent development of the cancer to a later stage (*e.g.* a chronic stage or metastasis).

As used herein, a ‘cancer’ may be or comprise any unwanted cell proliferation (or any disease manifesting itself by unwanted cell proliferation), neoplasm or tumor. The cancer may be benign or malignant. The cancer may be primary or secondary (metastatic). A neoplasm or tumor may be any abnormal growth or proliferation of cells and may be located in any tissue. The cancer may be of tissues/cells derived from *e.g.* the adrenal gland, adrenal medulla, anus, appendix, bladder, blood, bone, bone marrow, brain, breast, cecum, central nervous system (including or excluding the brain) cerebellum, cervix, colon, duodenum, endometrium, epithelial cells (*e.g.* renal epithelia), gallbladder, biliary tract, oesophagus, glial cells, heart, ileum, jejunum, kidney, lacrimal glad, larynx, liver, lung, lymph, lymph node, lymphoblast, maxilla, mediastinum, mesentery, myometrium, nasopharynx, omentum, oral cavity, ovary, pancreas, parotid gland, peripheral nervous system, peritoneum, pleura, prostate, salivary gland,

sigmoid colon, skin, small intestine, soft tissues, spleen, stomach, testis, thymus, thyroid gland, tongue, tonsil, trachea, uterus, vulva, white blood cells.

Tumors to be treated may be nervous or non-nervous system tumors. Nervous system tumors may originate either in the central or peripheral nervous system, *e.g.* glioma, medulloblastoma, meningioma, neurofibroma, ependymoma, Schwannoma, neurofibrosarcoma, astrocytoma and oligodendroglioma. Non-nervous system cancers/tumors may originate in any other non-nervous tissue; examples include melanoma, mesothelioma, lymphoma, myeloma, leukemia, Non-Hodgkin's lymphoma (NHL), Hodgkin's lymphoma, chronic myelogenous leukemia (CML), acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), cutaneous T-cell lymphoma (CTCL), chronic lymphocytic leukemia (CLL), hepatoma, epidermoid carcinoma, prostate carcinoma, breast cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, thymic carcinoma, NSCLC, hematologic cancer and sarcoma.

In some embodiments, the cancer to be treated/prevented comprises cells expressing an EGFR family member (*e.g.* HER2, EGFR, HER3 or HER4), and/or cells expressing a ligand for an EGFR family member. In some embodiments, the cancer to be treated/prevented comprises cells expressing a mutant or wildtype version of an EGFR family member (*e.g.* HER2, EGFR, HER3 or HER4). In some embodiments, the cancer to be treated/prevented is a cancer which is positive for an EGFR family member. In some embodiments, the cancer comprises cells that overexpress an EGFR family member and/or a ligand for an EGFR family member. Overexpression can be determined by detection of a level of expression which is greater than the level of expression by equivalent non-cancerous cells/non-tumor tissue.

Expression may be determined by any suitable means. Expression may be gene expression or protein expression. Gene expression can be determined *e.g.* by detection of mRNA encoding HER2, for example by quantitative real-time PCR (qRT-PCR). Protein expression can be determined *e.g.* by antibody-based methods, for example by western blot, immunohistochemistry, immunocytochemistry, flow cytometry, or ELISA.

In some embodiments the cancer is a cancer in which HER2 is pathologically-implicated. That is, in some embodiments the cancer is a cancer which is caused or exacerbated by the expression of HER2, a cancer for which expression of HER2 is a risk factor and/or a cancer for which expression of HER2 is positively associated with onset, development, progression, severity or metastasis of the cancer. The cancer may be characterised by expression of HER2, *e.g.* the cancer may comprise cells (*e.g.* cells of tumor tissue) expressing HER2. Such cancers may be referred to as being positive for HER2. A cancer which is 'positive' for HER2 may be a cancer comprising cells expressing HER2 (*e.g.* at the cell surface). A cancer which is 'positive' for HER2 may overexpress HER2.

In some embodiments, the cancer to be treated/prevented comprises cells harboring a genetic variant (*e.g.* a mutation) which causes increased (gene and/or protein) expression and/or activity of HER2, relative to comparable cells harboring a reference allele not comprising the genetic variant (*e.g.* a non-

mutated, or 'wildtype' allele). The genetic variant may be or comprise insertion, deletion, substitution to, or larger-scale translocation/rearrangement of, the nucleotide sequence relative to the reference allele.

A mutation 'resulting in' increased expression of HER2 may be known or predicted to cause, or may be associated with, increased gene/protein expression of HER2. A mutation 'resulting in' increased activity of HER2 may be known or predicted to cause, or may be associated with, increased HER2-mediated signaling and/or EGFR-mediated signaling. Mutations resulting in increased expression and/or activity of HER2 may be referred to as 'activating' mutations.

A mutation which causes increased expression of HER2 may result in gene or protein expression of HER2 which is not expressed by, and/or not encoded by genomic nucleic acid of, an equivalent cell not harboring the mutation. That is, the expression of HER2 may be a result of the mutation, and thus 'increased expression' may be from no expression.

A mutation which causes increased expression of HER2 may result in increased gene or protein expression of HER2 which is expressed by, and/or which is encoded by genomic nucleic acid of, an equivalent cell not comprising the mutation. By way of illustration, a cell may comprise a mutation resulting in an increase in the level of transcription of nucleic acid encoding HER2 relative to the level of transcription of nucleic acid encoding HER2 by an equivalent cell not comprising the mutation.

In some embodiments, a mutation which causes increased expression of HER2 may cause an increase in gene expression of HER2 relative to an equivalent cell not comprising the mutation. In some embodiments, a mutation which causes increased expression of HER2 may cause an increase in protein expression of HER2 relative to an equivalent cell not comprising the mutation.

In some embodiments, a mutation which causes increased expression of HER2 may cause an increase in the level of HER2 on or at the cell surface of a cell comprising the mutation, relative to an equivalent cell not comprising the mutation.

Cells having increased expression of HER2 relative to the level of expression of HER2 by a reference cell (*e.g.* as a result of mutation) may be described as 'overexpressing' HER2, or having 'upregulated expression' of HER2. For example, a cancer comprising cells harboring a mutation resulting in increased expression of HER2 relative to equivalent cells lacking the mutation may be described as a cancer comprising cells displaying overexpression/upregulated expression of HER2. In some embodiments, the reference cell lacking the mutation may be a non-cancerous cell (*e.g.* of equivalent cell type) or a cancerous cell (*e.g.* of equivalent cancer type).

A mutation which causes increased activity of HER2 may result in an increase in HER2-mediated signaling relative to the level of HER2-mediated signaling by an equivalent cell not comprising the mutation.

In some embodiments, a cancer to be treated/prevented in accordance with the present disclosure may be characterised by an increase in the expression and/or activity of HER2 (*i.e.* gene and/or protein expression) in an organ/tissue/subject affected by the disease/condition *e.g.* as compared to normal organ/tissue/subject (*i.e.* in the absence of the disease/condition). In some embodiments, cells and/or a tumor of a cancer to be treated/prevented may be characterised by an increase in the expression and/or activity of HER2, *e.g.* as compared to the level of expression and/or activity observed in equivalent non-cancerous cells/non-tumor tissue.

A HER2-overexpressing cancer may overexpress HER2 as a consequence of amplification of the *ERBB2* gene.

In some embodiments, a cancer to be treated/prevented in accordance with the present disclosure is a *ERBB2*-amplified cancer.

ERBB2 amplification can be identified using techniques well known in the art, such as by immunohistochemical analysis, and analysis by *in situ* hybridisation (see *e.g.* Wesoła and Jeleń, Adv Clin Exp Med. (2015) 24(5):899-903). For example, *ERBB2* amplification can be evaluated by fluorescence *in situ* hybridisation, *e.g.* as described in Stocker *et al.*, PLoS One (2016) 11(7): e0159176. *ERBB2*-amplified cancers may comprise a ratio of *ERBB2* to centromere 17 (*CEP17*) ≥2 (*e.g.* ≥4, ≥8), as determined by *in situ* hybridisation.

HER2 and its association with and role in cancer is reviewed *e.g.* in Oh and Bang, Nat Rev Clin Oncol (2020) 17:33-48, Hudis, NEJM (2007) 357(1):39-51, Arteaga and Engelman, Cancer Cell. (2014) 25(3): 282-303 and Yan *et al.*, Cancer Metastasis Rev. (2015) 34(1):157-164 all of which are hereby incorporated by reference in their entirety.

ERBB2 amplification has been observed in various cancers including breast cancer, gastric cancer and esophageal cancer (Koboldt *et al.*, Nature. (2012) 490:61–70). Potentially activating (*i.e.* gain-of-function) mutations in HER2 have also been reported in cancers such as lobular breast cancer, lung cancer, gastric cancer, bladder cancer and endometrial cancer. Very high proportions of the following cancers express HER2 (*i.e.* are HER2-positive; see Table 2 of Yan *et al.*, Cancer Metastasis Rev. (2015) 34(1):157-164): bladder cancer, breast cancer, cervical cancer, cholangiocarcinoma, colorectal cancer, esophageal esophagogastric junction cancer, gallbladder cancer, gastric adenocarcinoma, gastrointestinal stromal tumor, glioblastoma multiforme, glioma, head and neck carcinoma, hepatocellular carcinoma, intestinal cancer, kidney cancer, lung cancer, non-small cell lung cancer, small cell lung cancer, melanoma, neuroendocrine tumor, oligodendroglioma, ovarian cancer, pancreatic adenocarcinoma, penile cancer, pituitary cancer, prostate cancer, sarcoma, solitary fibrous tumor, testicular cancer, thymic cancer, thyroid cancer and uterine cancer.

In some embodiments, a cancer is selected from: a cancer comprising cells expressing/overexpressing an EGFR family member, a cancer comprising cells expressing/overexpressing HER2, a cancer

comprising cells that do not overexpress an EGFR family member, a cancer comprising cells that do not overexpress HER2, a HER2-low cancer, a HR-positive cancer, a solid tumor, bladder cancer, breast cancer, HER2-positive breast cancer, metastatic HER2-positive breast cancer, HER2-low breast cancer, unresectable or metastatic HER2-low breast cancer, HR-positive breast cancer, triple-negative breast cancer, cervical cancer, gastric cancer, HER2-positive gastric cancer, locally-advanced or metastatic HER2-positive gastric cancer, cholangiocarcinoma, colorectal cancer, esophageal esophagogastric junction cancer, gallbladder cancer, gastric adenocarcinoma, gastroesophageal junction adenocarcinoma, HER2-positive gastric gastroesophageal junction adenocarcinoma, locally-advanced or metastatic HER2-positive gastric gastroesophageal junction adenocarcinoma, gastrointestinal stromal tumor, glioblastoma multiforme, glioma, head and neck carcinoma, hepatocellular carcinoma, intestinal cancer, kidney cancer, lung cancer, non-small cell lung cancer, unresectable or metastatic non-small cell lung cancer comprising activating mutation to *ERBB2*, small cell lung cancer, melanoma, neuroendocrine tumor, oligodendroglioma, ovarian cancer, pancreatic adenocarcinoma, penile cancer, pituitary cancer, prostate cancer, sarcoma, solitary fibrous tumor, testicular cancer, thymic cancer, thyroid cancer and uterine cancer.

In some embodiments, a cancer according to the present disclosure is a cancer for which fam-trastuzumab deruxtecan-nxki is an approved treatment. In some embodiments, a cancer is selected from: metastatic HER2-positive breast cancer, unresectable or metastatic HER2-low (*i.e.* IHC 1+ or IHC 2+, ISH-) breast cancer, unresectable or metastatic non-small cell lung cancer comprising activating mutation to *ERBB2*, and locally-advanced or metastatic HER2-positive gastric or gastroesophageal junction adenocarcinoma.

In some embodiments, a cancer is selected from: a cancer comprising cells that do not overexpress an EGFR family member (*e.g.* HER2, EGFR, HER3 or HER4), a cancer comprising cells that do not overexpress HER2, a HER2-low (*i.e.* IHC 1+ or IHC 2+, ISH-) cancer, HER2-low breast cancer, a hormone receptor (HR)-positive cancer (*i.e.* a cancer comprising cells expressing estrogen receptor (ER) and/or progesterone receptor (PR)), HR-positive breast cancer and triple-negative (*i.e.* HER2-negative, ER-negative and PR-negative) breast cancer.

Herein, a ‘HER2-low’ cancer refers to a cancer having an immunohistochemical (IHC) score for HER2 expression of 1+, or a cancer having an IHC score for HER2 expression of 2+ provided the cancer does not comprise *ERBB2* amplification as determined by *in situ* hybridization (ISH) analysis. IHC analysis and scoring of HER2 expression and ISH analysis of *ERBB2* amplification is described *e.g.* in Wolff *et al.*, J Clin Oncol. (2018) 36(20):2105-2122, which is hereby incorporated by reference in its entirety.

Herein, an ‘activating mutation to *ERBB2*’ may: increase transcription of *ERBB2*; increase the level of RNA encoded by *ERBB2*; decrease degradation of RNA encoded by *ERBB2*; increase the level of a protein encoded by *ERBB2*; increase (facilitate) normal splicing of pre-mRNA encoded by *ERBB2*; increase translation of mRNA encoding a protein encoded by *ERBB2*; increase (facilitate) normal post-translational processing of a protein encoded by *ERBB2*; increase (facilitate) normal trafficking of a

protein encoded by *ERBB2*; decrease degradation of a protein encoded by *ERBB2*; increase the level of a function of a protein encoded by *ERBB2*; and/or confer a protein encoded by *ERBB2* with a novel property.

In some embodiments, a cancer is selected from: a cancer comprising cells that do not overexpress an EGFR family member (*e.g.* HER2, EGFR, HER3 or HER4), a cancer comprising cells that do not overexpress HER2, a HER2-low (*i.e.* IHC 1+ or IHC 2+, ISH-) cancer, HER2-low breast cancer, a hormone receptor (HR)-positive cancer (*i.e.* a cancer comprising cells expressing estrogen receptor (ER) and/or progesterone receptor (PR)), HR-positive breast cancer and triple-negative (*i.e.* HER2-negative, ER-negative and PR-negative) breast cancer.

In some embodiments, the cancer may be a relapsed cancer. As used herein, a 'relapsed' cancer refers to a cancer which responded to a treatment (*e.g.* a first line therapy for the cancer), but which has subsequently re-emerged/progressed, *e.g.* after a period of remission. For example, a relapsed cancer may be a cancer whose growth/progression was inhibited by a treatment (*e.g.* a first line therapy for the cancer), and which has subsequently grown/progressed. A cancer that is relapsed with respect to given treatment may be described as having acquired resistance to such treatment.

In some embodiments, the cancer may be a refractory cancer. As used herein, a 'refractory' cancer refers to a cancer which has not responded to a treatment (*e.g.* a first line therapy for the cancer). For example, a refractory cancer may be a cancer whose growth/progression was not inhibited by a treatment (*e.g.* a first line therapy for the cancer). In some embodiments a refractory cancer may be a cancer for which a subject receiving treatment for the cancer did not display a partial or complete response to the treatment. A cancer that is refractory with respect to given treatment may be described as having intrinsic resistance to such treatment.

In some embodiments, the cancer is a cancer that is relapsed or refractory with respect to treatment with a DNA damage response (DDR) inhibitor. In some embodiments, the cancer is refractory with respect to treatment with a DDR inhibitor. In some embodiments, the cancer is relapsed with respect to treatment with a DDR inhibitor. In some embodiments, the cancer has intrinsic resistance to treatment with a DDR inhibitor. In some embodiments, the cancer has acquired resistance to treatment with a DDR inhibitor. In accordance with such embodiments, the DDR inhibitor may have been administered in the form of an antigen-binding molecule comprising a payload moiety comprising or consisting of the DDR inhibitor, or may have been administered in unconjugated form.

Herein, where a cancer is described as being relapsed/refractory/resistant, *etc.* with respect to a given intervention, it may be simply described as being 'relapsed/refractory/resistant to' the relevant intervention.

DDR inhibitors and their use for the treatment of cancers is described *e.g.* in Cheng *et al.*, Eur J Med Chem. (2022) 230:114109, Wang *et al.*, Front Immunol. (2022) 13:854730 and Choi and Lee, Int J Mol Sci. (2022) 23(3):1701, all of which are hereby incorporated by reference in their entirety. In some embodiments, a DDR inhibitor according to the present disclosure is selected from: a PARP inhibitor (*e.g.* olaparib, rucaparib, niraparib, talazoparib, veliparib, pamiparib, simmiparib, senaparib, SC-10914, 2X-121, AMXI-5001, JPI-547, AZD5305, IDX-1197, TQB-3823, HWH-340, AsiDNA, STP-1002, RBN-2397), an ATM inhibitor (*e.g.* CP-466722, KU-55933, KU-60019, KU-59403, AZ31, AZ32, AZD0156, AZD1390), an ATR inhibitor (*e.g.* M6620 (berzosertib), M4344 (VX-803), AZD6738 (ceralasertib), BAY1895344 (elimusertib)), a WEE1 inhibitor (*e.g.* adavosertib, Debio 0123, PD0166285, PD0407824, AZD1775), a CHK1/2 inhibitor (*e.g.* CBP-501, prexasertib, MK-8776, GDC-0575, SRA-737), a DNA-PK inhibitor (*e.g.* CC-115, LY-3023414, AsiDNA, M3814 (nedisertib), M9831 (VX-984)) and a PLK1 inhibitor (*e.g.* BI-6727 (volasertib), PCM-075 (onvansertib)).

In some embodiments, the cancer is a cancer that is relapsed or refractory with respect to treatment with a DNA topoisomerase I (TOP1) inhibitor. In some embodiments, the cancer is refractory with respect to treatment with a TOP1 inhibitor. In some embodiments, the cancer is relapsed with respect to treatment with a TOP1 inhibitor. In some embodiments, the cancer has intrinsic resistance to treatment with a TOP1 inhibitor. In some embodiments, the cancer has acquired resistance to treatment with a TOP1 inhibitor. In accordance with such embodiments, the TOP1 inhibitor may have been administered in the form of an antigen-binding molecule comprising a payload moiety comprising or consisting of the TOP1 inhibitor, or may have been administered in unconjugated form.

DNA topoisomerase I inhibitors and their use for the treatment of cancers is described *e.g.* in Pommier, Chem Rev. (2009) 109(7): 2894-2902, Li *et al.*, Am J Cancer Res. (2017) 7(12): 2350-2394 and Thomas and Pommier, Clin Cancer Res. (2019) 25(22): 6581-6589, all of which are hereby incorporated by reference in their entirety. In some embodiments, a TOP1 inhibitor according to the present disclosure is selected from: camptothecin, irinotecan, etirinotecan, SN-38, DX-8951f (extatecan mesylate), DXd(1), DXd(2), exatecan, FL118, topotecan, gimatecan, belotecan, deruxtecan, belotecan, rubitecan, lurtotecan, diflomotecan, karenitecan, silatecan, namitecan, elomotecan, DRF-1042, delimotecan, NSC606985, chimmitecan, ZBH-1205, Genz-644282, non-CPT1, indotecan (LMP-400), indimitecan (LMP-776) and LMP744.

In some embodiments, the cancer to be treated/prevented in accordance with the present disclosure is a cancer that is: relapsed or refractory with respect to treatment with a DDR inhibitor (*e.g.* a DDR inhibitor as described herein), and relapsed or refractory with respect to treatment with a TOP1 inhibitor (*e.g.* a TOP1 inhibitor as described herein). In some embodiments, the cancer is: refractory with respect to treatment with a DDR inhibitor (*e.g.* a DDR inhibitor as described herein), and refractory with respect to treatment with a TOP1 inhibitor (*e.g.* a TOP1 inhibitor as described herein). In some embodiments, the cancer is: relapsed with respect to treatment with a DDR inhibitor (*e.g.* a DDR inhibitor as described herein), and relapsed with respect to treatment with a TOP1 inhibitor (*e.g.* a TOP1 inhibitor as described herein). In some embodiments, the cancer is: refractory with respect to treatment with a DDR inhibitor

(*e.g.* a DDR inhibitor as described herein), and relapsed with respect to treatment with a TOP1 inhibitor (*e.g.* a TOP1 inhibitor as described herein). In some embodiments, the cancer is: relapsed with respect to treatment with a DDR inhibitor (*e.g.* a DDR inhibitor as described herein), and refractory with respect to treatment with a TOP1 inhibitor (*e.g.* a TOP1 inhibitor as described herein). In accordance with such embodiments, the TOP1 inhibitor and/or DDR inhibitor may have been administered in the form of an antigen-binding molecule comprising a payload moiety comprising or consisting of the TOP1 inhibitor/DDR inhibitor, or may have been administered in unconjugated form.

Treatment of a cancer in accordance with the methods of the present disclosure achieves one or more of the following treatment effects: reduces the number of cancer cells in the subject, reduces the size of a cancerous tumor/lesion in the subject, inhibits (*e.g.* prevents or slows) growth of cancer cells in the subject, inhibits (*e.g.* prevents or slows) growth of a cancerous tumor/lesion in the subject, inhibits (*e.g.* prevents or slows) the development/progression of a cancer (*e.g.* to a later stage, or metastasis), reduces the severity of symptoms of a cancer in the subject, increases survival of the subject (*e.g.* progression free survival or overall survival), reduces a correlate of the number or activity of cancer cells in the subject, and/or reduces cancer burden in the subject.

Subjects may be evaluated in accordance with the Revised Criteria for Response Assessment: The Lugano Classification (described *e.g.* in Cheson *et al.*, J Clin Oncol (2014) 32: 3059-3068, incorporated by reference hereinabove) in order to determine their response to treatment. In some embodiments, treatment of a subject in accordance with the methods of the present disclosure achieves one of the following: complete response, partial response, or stable disease.

Prevention may refer to prevention of development of a cancer, and/or prevention of worsening of a cancer, *e.g.* prevention of progression of a cancer, *e.g.* to a later stage (*e.g.* metastasis).

In some embodiments, administration of an antigen-binding molecule/composition according to the present disclosure may be associated with one or more of: inhibition of the development/progression of the cancer, a delay to/prevention of onset of the cancer, a reduction in/delay to/prevention of tumor growth, a reduction in/delay to/prevention of tissue invasion, a reduction in/delay to/prevention of metastasis, a reduction in the severity of one or more symptoms of the cancer, a reduction in the number of cancer cells, a reduction in the cancer burden, a reduction in tumor size/volume, and/or an increase in survival of subjects having the cancer (*e.g.* progression free survival or overall survival).

In accordance with various aspects of the present disclosure, a method of treating and/or preventing a cancer according to the present disclosure may comprise inhibiting the growth of a tumor, reducing the size/volume of a tumor and/or increasing the survival of a subject having the cancer.

In accordance with various aspects of the present disclosure, methods are provided which are for, or which comprise (*e.g.* in the context of treatment/prevention of a cancer, *e.g.* a cancer described herein), one or more of the following:

binding to cells expressing HER2;
inhibiting the proliferation of HER2-expressing cells;
killing cells expressing HER2;
inhibiting tumor growth and/or reducing tumor size/volume, *e.g.* of a HER2-expressing cancer; and/or
increasing the survival of subjects having a cancer, *e.g.* a HER2-expressing cancer.

Also provided are antigen-binding molecules and compositions according to the present disclosure for use in such methods, and the use of antigen-binding molecules and compositions according to the present disclosure in manufacture of compositions (*e.g.* medicaments) for use in such methods. It will be appreciated that the methods typically comprise administering an antigen-binding molecule according to the present disclosure to a subject.

Similarly, one or more of the following may be observed in a subject following therapeutic or prophylactic intervention in accordance with the present disclosure (*e.g.* compared to the level/number/proportion *etc.* prior to intervention):

inhibition of proliferation of HER2-expressing cells;
killing of cells expressing HER2;
inhibition of tumor growth and/or reduction of tumor size/volume, *e.g.* of a HER2-expressing cancer; and/or
increased survival of a subject having a cancer, *e.g.* a HER2-expressing cancer.

In some embodiments, therapeutic/prophylactic intervention in accordance with the present disclosure may be described as being 'associated with' one or more of the effects described in the preceding paragraph. The skilled person is readily able to evaluate such properties using techniques that are routinely practiced in the art.

Administration of the antigen-binding molecules and compositions of the present disclosure is preferably in a 'therapeutically-effective' or 'prophylactically-effective' amount, this being sufficient to show therapeutic or prophylactic benefit to the subject. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the disease/condition and the particular article administered. Prescription of treatment, *e.g.* decisions on dosage *etc.*, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disease/disorder to be treated, the condition of the individual subject, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's 'The Science and Practice of Pharmacy' (Ed. A. Adejare), 23rd Edition (2020), Academic Press.

Administration of the antigen-binding molecules and compositions of the present disclosure may be *e.g.* parenteral, systemic, topical, intracavitary, intravascular, intravenous, intra-arterial, intramuscular,

intrathecal, intraocular, intraconjunctival, intratumoral, subcutaneous, intradermal, oral or transdermal. Administration may be by injection, infusion or ingestion.

In some aspects and embodiments, articles of the present disclosure may be administered to a tissue/organ of interest (*e.g.* a tissue/organ affected by the disease/condition, *e.g.* a tissue/organ in which symptoms of the disease/condition manifest). In some aspects and embodiments, articles of the present disclosure may be administered to the blood (*i.e.* intravenous/intra-arterial administration) by injection or infusion (*e.g.* via cannula), or may be administered subcutaneously or orally. In some aspects and embodiments, articles of the present disclosure may be administered to a tumor.

In some embodiments, therapeutic or prophylactic intervention according to the present disclosure may further comprise administering another agent for the treatment/prevention of the relevant disease/condition. Administration of antigen-binding molecules and compositions described herein may be alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. Simultaneous administration refers to administration with another therapeutic agent together, for example as a pharmaceutical composition containing both agents (combined preparation), or immediately after each other (*e.g.* within 1, 4, 6, 8 or 12 hours) and optionally via the same route of administration (*e.g.* to the same tissue, artery, vein or other blood vessel). Sequential administration refers to administration of one agent followed after a given time interval by separate administration of another agent. It is not required that the two agents are administered by the same route, although this is the case in some embodiments. The time interval may be any time interval.

Multiple doses of the antigen-binding molecules and compositions may be provided. Multiple doses may be separated by a predetermined time interval, which may be selected to be one of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 days, or 1, 2, 3, 4, 5, or 6 months. By way of example, doses may be given once every 7, 14, 21 or 28 days (plus or minus 3, 2, or 1 days).

Methods of detection

The present disclosure also provides the articles of the present disclosure for use in methods for detecting, localising or imaging HER2, or cells expressing HER2.

The antigen-binding molecules described herein may be used in methods that involve detecting binding of the antigen-binding molecule to HER2. Such methods may involve detection of the bound complex of the antigen-binding molecule and HER2. It will be appreciated that the HER2 may be HER2 expressed by a cell, *e.g.* in or at the cell surface of a cell expressing HER2.

As such, a method is provided, comprising contacting a sample containing, or suspected to contain, HER2, and detecting the formation of a complex of the antigen-binding molecule and HER2. Also provided is a method comprising contacting a sample containing, or suspected to contain, a cell

expressing HER2, and detecting the formation of a complex of the antigen-binding molecule and a cell expressing HER2.

Suitable method formats are well known in the art, including immunoassays such as sandwich assays, *e.g.* ELISA. The methods may involve labelling the antigen-binding molecule, or target(s), or both, with a detectable moiety, *e.g.* a fluorescent label, phosphorescent label, luminescent label, immuno-detectable label, radiolabel, chemical, nucleic acid or enzymatic label as described herein. Detection techniques are well known to those of skill in the art and can be selected to correspond with the labelling agent.

Methods comprising detecting HER2, or cells expressing HER2, include methods for diagnosing/prognosing a disease/condition described herein.

Methods of this kind may be performed *in vitro* on a patient sample, or following processing of a patient sample. Once the sample is collected, the patient is not required to be present for the *in vitro* method to be performed, and therefore the method may be one which is not practised on the human or animal body. In some embodiments, the method is performed *in vivo*.

Such methods may involve detecting or quantifying HER2 and/or cells expressing HER2, *e.g.* in a patient sample. Where the method comprises quantifying the relevant factor, the method may further comprise comparing the determined amount against a standard or reference value as part of the diagnostic or prognostic evaluation. Other diagnostic/prognostic tests may be used in conjunction with those described herein to enhance the accuracy of the diagnosis or prognosis or to confirm a result obtained by using the tests described herein.

Detection in a sample may be used for the purpose of diagnosis of a disease/condition (*e.g.* a cancer), predisposition to a disease/condition, or for providing a prognosis (prognosticating) for a disease/condition, *e.g.* a disease/condition described herein. The diagnosis or prognosis may relate to an existing (previously diagnosed) disease/condition.

A sample may be taken from any tissue or bodily fluid. The sample may comprise or may be derived from: a quantity of blood; a quantity of serum derived from the individual's blood which may comprise the fluid portion of the blood obtained after removal of the fibrin clot and blood cells; a tissue sample or biopsy; pleural fluid; cerebrospinal fluid (CSF); or cells isolated from said individual. In some embodiments, the sample may be obtained or derived from a tissue or tissues which are affected by the disease/condition (*e.g.* tissue or tissues in which symptoms of the disease manifest, or which are involved in the pathogenesis of the disease/condition).

A subject may be selected for diagnostic/prognostic evaluation based on the presence of symptoms indicative of a disease/condition described herein, or based on the subject being considered to be at risk of developing a disease/condition described herein.

The present disclosure also provides methods for selecting/stratifying a subject for treatment with a HER2-targeted agent. In some embodiments a subject is selected for treatment/prevention in accordance with the methods of the present disclosure, or is identified as a subject which would benefit from such treatment/prevention, based on detection/quantification of HER2, or cells expressing HER2, *e.g.* in a sample obtained from the individual.

Subjects

The subject in accordance with aspects described herein may be any animal or human. The subject is preferably mammalian, more preferably human. The subject may be a non-human mammal, but is more preferably human. The subject may be male or female. The subject may be a patient. A subject may have been diagnosed with a disease or condition requiring treatment (*e.g.* a cancer, *e.g.* a cancer described herein), may be suspected of having such a disease/condition, or may be at risk of developing/contracting such a disease/condition.

In some embodiments, the subject to be treated according to a therapeutic or prophylactic method of the present disclosure herein is a subject having, or at risk of developing, a cancer, *e.g.* a cancer described herein. In embodiments according to the present disclosure, a subject may be selected for treatment according to the methods based on characterisation for certain markers of such disease/condition.

In some embodiments, a patient may be selected for treatment described herein based on the detection of a cancer expressing/overexpressing HER2, *e.g.* in a sample obtained from the subject (*e.g.* a biopsy, *e.g.* of a tumor).

Kits

The present disclosure also provides kits of parts. A kit according to the present disclosure may comprise components for performing a method described herein, in whole or in part.

The kit may have at least one container having a predetermined quantity of an antigen-binding molecule or composition described herein.

In some aspects of the present disclosure a kit of parts is provided. In some embodiments, the kit may comprise an antigen-binding molecule or composition described herein, and which may be provided in a predetermined quantity.

The kit may provide an antigen-binding molecule or composition described herein together with instructions for administration to a patient in order to treat a specified disease/condition (*e.g.* a disease/condition described herein, *e.g.* a cancer).

The kit may provide an antigen-binding moiety according to the disclosure, and a linker-payload moiety according to the present disclosure. The kit may further comprise reagents for conjugating the antigen-binding moiety and the linker-payload moiety.

The kit may further comprise reagents, buffers and/or standards required for execution of a method according to the present disclosure. Kits according to the present disclosure may include instructions for use, *e.g.* in the form of an instruction booklet or leaflet. The instructions may include a protocol for performing any one or more of the methods described herein.

Sequence identity

As used herein, 'sequence identity' refers to the percent of nucleotides/amino acid residues in a subject sequence that are identical to nucleotides/amino acid residues in a reference sequence, after aligning the sequences and, if necessary, introducing gaps, to achieve the maximum percent sequence identity between the sequences. Pairwise and multiple sequence alignment for the purposes of determining percent sequence identity between two or more amino acid or nucleic acid sequences can be achieved in various ways known to a person of skill in the art, for instance, using publicly available computer software such as ClustalOmega (Söding, J. 2005, Bioinformatics 21, 951-960), T-coffee (Notredame *et al.* 2000, J. Mol. Biol. (2000) 302, 205-217), Kalign (Lassmann and Sonnhammer 2005, BMC Bioinformatics, 6(298)) and MAFFT (Katoh and Standley 2013, Molecular Biology and Evolution, 30(4) 772–780) software. When using such software, the default parameters, *e.g.* for gap penalty and extension penalty, are preferably used.

Sequences

SEQ ID NO:	DESCRIPTION	SEQUENCE
1	Human HER2 isoform 1 (P04626-1 v1, 1987-08-13)	MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQ VVQGNLELTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFED NYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQD TILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVC AGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTY NTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGT QRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDG DPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNG AYSLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALL HTANRPEDECVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRV LQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR CPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSI ISAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQ MRILKETELRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEIL DEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDL LNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEY HADGGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREI PDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFRELVSEFSRMARDPQRF VVIQNEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAG GMVHHRHRSSSTRSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMG AAKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRP QPPSPREGPLPAARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQG GAAPQPHPPPAFSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
2	Human HER2 isoform 2 (P04626-2)	MPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVV LGVVFGILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRK VKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVG SPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKG MSYLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIK WMALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLP QPPICTIDVYMIMVKCWMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPA SPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSS STRSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTH DPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLP

		AARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPA FSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
3	Human HER2 isoform 3 (P04626-3)	MRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIP DGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVT QLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARNV LVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWS YGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDS ECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDL VDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEEEA PRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSET DGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERPKTLSPGKN GVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPAFSPAFDNLYYWDQDPPERG APPSTFKGTPTAENPEYLGLDVPV
4	Human HER2 isoform 4 (P04626-4)	MPRGSWKPQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTN ASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPL NNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQL ALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLP TDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPE GRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCAR VCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPE QLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISW LGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGE GLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARH CLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMP IWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLG VVFGILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVK VLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSP YVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMS YLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWM ALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQP PICTIDVYMIMVKCWMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASP LDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSST RSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHD PSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPA ARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPAF SPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
5	Human HER2 isoform 5 (P04626-5)	MKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQGYVL IAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLREL QLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSP MCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKH SDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYL STDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVT SANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISA WPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLALIHH NTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLCARGHCWGP GPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTC FGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINC THSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVVFGILIKRRQQKIRKYTM RRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPD GENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQ LMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARNVL VKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWS YGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDS ECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDL VDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEEEA PRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSET DGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERPKTLSPGKN GVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPAFSPAFDNLYYWDQDPPERG APPSTFKGTPTAENPEYLGLDVPV
6	Human HER2 isoform 6 (P04626-6)	MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQ VVQGNLELTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFED NYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQD TILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVC AGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTY NTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGT QRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDG DPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNG AYSLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALL

		HTANRPEDECVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRV LQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR CPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSPLTSIISAVVGILLVVVLGVVF GILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLG SGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDETISNLFSNFAPRGP SACCEPTCWCHSGKGQDSLPREEWGRQRRFCLWGCRGEPRVLDTPGRSCPS APPSSCLQPSLRQPLLLGPGPTRAGGSTQHLQRDTYGREPRVPGSGRASVNQK AKSAEALMCPQGAGKA
7	Mature human HER2 isoform 1	TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDI QEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGA SPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRS RACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCA AGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASC VTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGME HLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLE EITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLREL GSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLC ARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPEC QPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEE GACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVVFGILIKR RQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFG TVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGIC LTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVH RDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRF THQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMI MVKCWMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSL LEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTL GLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQRYSE DPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLE RPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPAFSPAFDNLYY WDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
8	Human HER2 isoform 1 signal peptide	MELAALCRWGLLLALLPPGAAS
9	Human HER2 isoform 1 extracellular domain	TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDI QEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGA SPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRS RACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCA AGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASC VTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGME HLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLE EITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLREL GSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLC ARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPEC QPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEE GACQPCPINCTHSCVDLDDKGCPAEQRASPLT
10	Human HER2 isoform 1 transmembrane domain	SIISAVVGILLVVVLGVVFGILI
11	Human HER2 isoform 1 cytoplasmic domain	KRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGA FGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLL GICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVR LVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILR RRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDV YMIMVKCWMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFY RSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGG DLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQR YSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGA TLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPAFSPAFDN LYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
12	Trastuzumab HC (hIgG1, N297A, D356E and L358M (EU numbering) relative to P01857-1)	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPT NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAM DYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK KVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKC KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDI

		AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGK
13	Trastuzumab κ LC	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLY SGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTV AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT EQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
14	Trastuzumab VH	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPT NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAM DYWGQGTLVTVSS
15	Trastuzumab HC-CDR1	GFNIKDTY
16	Trastuzumab HC-CDR2	IYPTNGYT
17	Trastuzumab HC-CDR3	SRWGGDGFYAMDY
18	Trastuzumab HC-FR1	EVQLVESGGGLVQPGGSLRLSCAAS
19	Trastuzumab HC-FR2	IHWVRQAPGKGLEWVAR
20	Trastuzumab HC-FR3	RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
21	Trastuzumab HC-FR4	WGQGTLVTVSS
22	Trastuzumab VL	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLY SGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK
23	Trastuzumab LC-CDR1	QDVNTA
24	Trastuzumab LC-CDR2	SAS
25	Trastuzumab LC-CDR3	QQHYTTPPT
26	Trastuzumab LC-FR1	DIQMTQSPSSLSASVGDRVTITCRAS
27	Trastuzumab LC-FR2	VAWYQQKPGKAPKLLIY
28	Trastuzumab LC-FR3	FLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYC
29	Trastuzumab LC-FR4	FGQGTKVEIK
30	Human IgG1 constant region (IGHG1; UniProt:P01857-1, v1)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
31	CH1 IgG1 (positions 1-98 of P01857-1, v1)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
32	Hinge IgG1 (positions 99-110 of P01857-1, v1)	EPKSCDKTHTCP
33	CH2 IgG1 (positions 111-223 of P01857-1, v1)	PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAK
34	CH3 IgG1 (positions 224-330 of P01857-1, v1)	GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
35	Human IgG1 constant region G1m3 allotype (K214R, D356E and L358M (EU numbering)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS

	relative to P01857-1)	PGK
36	CH1 IgG1 G1m3 allotype	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRV
37	CH3 IgG1 G1m3 allotype	GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
38	Human IgG1 CH2-CH3 region	PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
39	Human IgG1 G1m3 allotype CH2-CH3 region	PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP G
40	Cκ CL (IGKC; UniProt: P01834-1, v2))	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
41	Cλ CL1 (IGLC1; UniProt: P0CG04-1, v1)	GQPKANPTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVET TKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS
42	Cλ CL2 (IGLC2; UniProt: P0DOY2-1, v1)	GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVET TTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS
43	Cλ CL3 (IGLC3; UniProt: P0DOY3-1, v1)	GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVET TTPSKQSNNKYAASSYLSLTPEQWKSHKSYSCQVTHEGSTVEKTVAPTECS
44	Cλ CL6 (IGLC6; UniProt: P0CF74-1, v1)	GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVKVAWKADGSPVNTGVET TTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPAECS
45	Cλ CL7 (IGLC7; UniProt: A0M8Q6-1, v3)	GQPKAAPSVTLFPPSSEELQANKATLVCLVSDFNPGAVTVAWKADGSPVKVGVE TTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCRVTHEGSTVEKTVAPAECS
46	Human IgG1 constant region (IGHG1; UniProt:P01857-1, v1) comprising N297A	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
47	Human IgG1 constant region G1m3 allotype (K214R, D356E and L358M (EU numbering) relative to P01857-1) comprising N297A	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK
48	CH2 IgG1 comprising N297A	PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAK
49	Human IgG1 CH2-CH3 region comprising N297A	PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
50	Human IgG1 G1m3 allotype CH2-CH3 region comprising N297A	PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP G

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The present disclosure includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Aspects and embodiments of the present disclosure will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Throughout this specification, including the claims which follow, unless the context requires otherwise, the word 'comprise,' and variations such as 'comprises' and 'comprising,' will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in the specification and the appended claims, the singular forms 'a', 'an', and 'the' include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from 'about' one particular value, and/or to 'about' another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent 'about', it will be understood that the particular value forms another embodiment.

Where a nucleic acid sequence is disclosed or referred to herein, the reverse complement thereof is also expressly contemplated.

Methods described herein may preferably be performed *in vitro*. The term '*in vitro*' is intended to encompass procedures performed with cells in culture whereas the term '*in vivo*' is intended to encompass procedures with/on intact multi-cellular organisms.

Values may be expressed herein as 'about' a particular value. Similarly, ranges may be expressed herein as from 'about' a particular value, and/or to 'about' another particular value. The term 'about' in relation to a numerical value is optional, and means for example +/- 10 %. By way of illustration, reference *e.g.* to 'about 10 %' is to be construed as 9 % to 11 %. In instances herein where 'about' is recited, the value it precedes is also specifically contemplated. By way of illustration, reference *e.g.* to 'about 10 %' also specifically contemplates 10 %.

Brief Description of the Figures

Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying figures.

Figure 1A shows % inhibition for a TOP1 inhibitor and an ATR inhibitor alone and in combination in HCT-116 cells.

Figure 1B shows % inhibition for a TOP1 inhibitor and a CHK1 inhibitor alone and in combination in HCT-116 cells.

Figure 1C shows % inhibition for a TOP1 inhibitor and an ATR inhibitor alone and in combination in HEC-1B cells.

Figure 1D shows % inhibition for a TOP1 inhibitor and a CHK1 inhibitor alone and in combination in HEC-1B cells.

Figure 2 shows % cell death following treatment *in vitro* of cells of the indicated cancer cell lines for 7 days with trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), Trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control).

Figure 3 shows tumor volume over time, for mice having a JIMT-1 cell line-derived xenograft model of breast ductal carcinoma, and treated with PBS (vehicle), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)) or trastuzumab deruxtecan (T-DXd)).

Figure 4 shows bodyweight in grams (g) over time, for mice having a JIMT-1 cell line-derived xenograft model of breast ductal carcinoma, and treated with PBS (vehicle), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)) or trastuzumab deruxtecan (T-DXd)

Figure 5A shows binding of trastuzumab (T (naked), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), Trastuzumab deruxtecan (T-DXd), isotype-matched control antibody (Isotype (Naked)) or isotype-matched control antibody conjugated to exatecan (Isotype (Exa)) to live BT-474, NCI-N87, JIMT-1, HEC-1-B or HCT116 cells, as determined by flow cytometry.

Figure 5B shows the mean fluorescence intensity (MFI) for trastuzumab (T (naked), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), F trastuzumab deruxtecan (T-DXd), isotype-matched control antibody (Isotype (Naked)) or isotype-matched control antibody conjugated to exatecan (Isotype (Exa)) bound to live BT-474, NCI-N87, JIMT-1, HEC-1-B or HCT116 cells, as determined by flow cytometry.

Figure 6A shows subcellular localization of trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)) or trastuzumab deruxtecan (T-DXd) within HEC-1-B cells after incubation for 0h, 0.5h or 2h, as determined by immunofluorescence microscopy.

Figure 6B shows subcellular localization of trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)) or trastuzumab deruxtecan (T-DXd) within NCI-N87 cells after incubation for 0h, 0.5h or 2h, as determined by immunofluorescence microscopy.

Figure 7A shows % cell death following treatment *in vitro* of HEC1-B cells for 3 days with trastuzumab (T (naked)), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at a concentration of 333 nM.

Figure 7B shows % cell death following treatment *in vitro* of NCI-N87 cells for 3 days with trastuzumab (T (naked)), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at a concentration of 333 nM.

Figure 7C shows % cell death following treatment *in vitro* of HCT-116 cells for 3 days with trastuzumab (T (naked)), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at a concentration of 333 nM.

Figure 7D shows % cell death following treatment *in vitro* of BT474 cells for 3 days with trastuzumab (T (naked)), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at a concentration of 333 nM.

Figure 7E shows % cell death following treatment *in vitro* of JIMT-1 cells for 3 days with trastuzumab (T (naked)), trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at a concentration of 333 nM.

Figure 7F shows % cell death following treatment *in vitro* of HEC1-B cells for 3 days with trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at concentrations of the antigen-binding molecules providing equivalent 0.857 nM payload concentration.

Figure 7G shows % cell death following treatment *in vitro* of NCI-N87 cells for 3 days with trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at concentrations of the antigen-binding molecules providing equivalent 0.857 nM payload concentration.

Figure 7H shows % cell death following treatment *in vitro* of HCT-116 cells for 3 days with trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at concentrations of the antigen-binding molecules providing equivalent 0.857 nM payload concentration.

Figure 7I shows % cell death following treatment *in vitro* of BT474 cells for 3 days with trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at concentrations of the antigen-binding molecules providing equivalent 0.857 nM payload concentration.

Figure 7J shows % cell death following treatment *in vitro* of JIMT-1 cells for 3 days with trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)), trastuzumab deruxtecan (T-DXd) or isotype-matched control antibody conjugated to exatecan (Isotype Control), at concentrations of the antigen-binding molecules providing equivalent 0.857 nM payload concentration.

Figure 8 shows the Loewe synergy score for exatecan and berzosertib (upper panel) and the % inhibition for exatecan and berzosertib alone and in combination (lower panel) in HEC1-B cells.

Figure 9 shows the Loewe synergy score for exatecan and berzosertib (upper panel) and the % inhibition for exatecan and berzosertib alone and in combination (lower panel) in HCT-116 cells.

Figure 10 shows the level of pATR, pCHK1, pH2AX and CHK1 in JIMT-1 cells untreated or treated with 100 nM exatecan, 100 nM berzosertib, or 100 nM exatecan and 100 nM berzosertib in combination.

Figure 11 shows the level of pATR, pCHK1, pH2AX and CHK1 in HCT-116 cells untreated or treated with 75 nM exatecan, 75 nM berzosertib, or 75 nM exatecan and 75 nM berzosertib in combination.

Figure 12 shows survival of Sprague-Dawley rats following administration of vehicle, exatecan (1, 3, 10 or 30 mg/kg), berzosertib (35 mg/kg) or exatecan and berzosertib in combination (1, 3, 10 or 30 mg/kg exatecan + 35 mg/kg berzosertib).

Figure 13 shows body weight change in Sprague-Dawley rats following administration of vehicle, exatecan (1, 3, 10 or 30 mg/kg), berzosertib (35 mg/kg) or exatecan and berzosertib in combination (1, 3, 10 or 30 mg/kg exatecan + 35 mg/kg berzosertib).

Figure 14 shows results of hematology assessments in Sprague-Dawley rats following administration of vehicle, exatecan (1, 3, 10 or 30 mg/kg), berzosertib (35 mg/kg) or exatecan and berzosertib in combination (1, 3, 10 or 30 mg/kg exatecan + 35 mg/kg berzosertib).

Figure 15 shows results of clinical chemistry assessments in Sprague-Dawley rats following administration of vehicle, exatecan (1, 3, 10 or 30 mg/kg), berzosertib (35 mg/kg) or exatecan and berzosertib in combination (1, 3, 10 or 30 mg/kg exatecan + 35 mg/kg berzosertib).

Figure 16 shows tumour volume in a T-DXd resistant NCI-N87 CDX model following administration of vehicle, Trastuzumab deruxtecan (T-DXd) (3 mg/kg) or trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber) (3 mg/kg, 9 mg/kg).

Figure 17 shows % inhibition of HER2-negative MDA-MB-231 cells alone or co-cultured with HER2-positive NCI-N87 cells and exposed to varying concentrations of trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)) or Trastuzumab deruxtecan (T-DXd).

Figure 18 shows megakaryocyte uptake via macropinocytosis of trastuzumab conjugated to both exatecan and berzosertib (T-(Exa+Ber)) (100 ng/ml) or trastuzumab deruxtecan (T-DXd) (100 ng/ml).

Figure 19 shows % increase in hydrophobic interaction chromatography retention time (RT) for a single payload ADC and T-(Exa+Ber).

Figure 20 shows the stability of ADCs 5 and 6 in plasma over 7 days.

Figure 21 shows the efficacy of dual payload ADCs on NCI-N87 gastric cell line cells.

Figure 22 shows the efficacy of dual and single payload ADCs in killing NCI-N87 gastric cell line cells.

Figure 23 shows the efficacy of dual and single payload ADCs in killing NCI-N87 gastric cell line cells.

Figure 24 shows the in vivo anti-tumor efficacy of dual and single payload ADCs in a xenograft mouse model.

Figure 25 shows the in vivo efficacy of dual payload ADCs with berzosertib (ATRi) or prexasertib (CHK1i) in a mouse model.

Figure 26 shows the in vivo efficacy of dual payload ADCs with different target DAR ratios.

Figure 27 shows the weight change results in a primate study following treatment with a dual payload ADC.

Figure 28 shows the biochemistry and haemotology results in a primate study following treatment with a dual payload ADC.

Figure 29 shows the efficacy of dual payload ADCs with TMTHSI or DBCO moieties on NCI-N87 gastric cell lines.

Figure 30 shows the clearance of dual payload ADCs with TMTHSI or DBCO moieties compared to the unconjugated control antibody in immunocompetent mice.

Figure 31 shows efficacy of dual payload ADCs with different conjugations.

Examples

Example 1 – *in vitro* combination of DDR inhibitors and TOP1 inhibitors

In order to further demonstrate the benefits of combining DDR inhibitors and TOP1 inhibitors, certain inhibitors were combined in a two-dimensional proliferation assay as follows.

Cell Lines

HCT-116

HEC-1B

Test Compounds

Exatecan (MedChemExpress,#HY-13631)

Ceralasertib (MedChemExpress,#HY-19323)

Prexasertib (MedChemExpress,#HY-18174)

Controls

Vehicle control (0.25% DMSO)

Only Cells

Detecting Reagent

Cell Titre Glo 2.0, Promega #G9243

The cells were seeded in wells of a white 96-well plate at a density of 7,000 cells per well for HEC-1B cells, and 3,000 cells per well for HCT-116 cells, with 150 µl of media in each well. For the single compound experiment, 25 µl of the test compound and 25 µl of media were added to the respective wells at varying concentrations. For the synergy experiment, 25 µl of compound 1 and 25 µl of another compound were added to the respective wells at different concentrations. The plates were then incubated for 3 days at 37°C with 5% CO_2. Following incubation, 50µl of the detection reagent was added per well and shaken at 600 rpm for 20 minutes. The resulting luminescence was measured using Perkin Elmer Victor Nivo, and percent inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = 100-((Lum_{Treatment}/Lum_{Vehicle})*100)$$

Synergy between the test compounds (Loewe synergy score) was then determined using the Synergy Finder tool at synergyfinder.fimm.fi. A Loewe synergy score of above 10 indicates synergy, between 10 and -10 indicates an additive effect, and below -10 indicates antagonism.

A: Exatecan and ceralasertib (an ATR inhibitor) in HCT-116 cells

Fig 1A shows the % inhibition for exatecan and ceralasertib alone and in combination. The Loewe synergy score is shown in table 1A below. The IC_{50} for exatecan alone was 0.626 nM, when combined with ceralasertib at 375 nM was 0.080 nM and when combined with ceralasertib at 750 nM was 0.044 nM.

Table 1A

Ceralasertib (nM)	Exatecan (nM)									
	0.009	0.019	0.039	0.078	0.156	0.313	0.63	1.25	2.51	5.0
3000	14.47	7.75	14.24	22.99	29.46	28.14	19.70	14.41	18.67	26.21
1500	16.96	26.24	1.73	14.76	26.42	25.94	16.68	6.20	9.53	20.48
750	6.37	12.63	25.21	42.94	17.98	22.09	9.32	0.50	-1.53	9.67
375	-5.16	-1.60	4.08	19.51	1.90	15.52	1.24	-5.98	-10.88	0.65
188	-7.41	-4.77	-3.19	-2.33	20.61	1.32	11.36	-6.17	-12.77	-3.84
94	-8.44	-6.34	-3.72	-6.87	3.28	20.33	9.72	-4.78	-10.01	-5.03
47	-3.10	-2.85	-1.45	-5.41	-2.03	10.27	6.29	-4.23	-7.76	-2.24
23	-3.04	0.99	3.88	-0.49	-0.78	5.97	4.24	-4.06	-6.20	-0.96
12	1.29	5.83	4.58	2.40	3.49	5.59	5.02	-1.15	-3.59	0.63
6	5.29	5.46	6.95	4.14	9.33	10.49	9.10	1.97	-1.21	4.21

B: Exatecan and prexasertib (a CHK1 inhibitor) in HCT-116 cells

Fig 1B shows the % inhibition for exatecan and prexasertib alone and in combination. The Loewe synergy score is shown in table 1B below. The IC_{50} for exatecan alone was 0.626 nM, when combined with prexasertib at 50 nM was 0.166 nM and when combined with prexasertib at 100 nM was 0.158 nM. The IC_{50} for prexasertib alone was 61.14 nM.

Table 1B

Prexasertib (nM)	Exatecan (nM)									
	0.009	0.019	0.039	0.078	0.156	0.313	0.63	1.25	2.51	5.0
200	1.44	0.54	4.30	4.73	0.55	10.69	13.40	10.59	12.37	16.76
100	-0.70	-0.97	1.99	3.22	11.68	4.53	10.68	7.28	8.16	14.77
50	-0.93	-3.65	1.15	-0.38	11.15	18.31	5.15	1.93	4.48	10.30
25.0	-4.31	-5.15	-3.38	-4.93	6.01	14.06	1.73	4.34	-0.95	6.29
12.5	-5.23	-5.36	1.16	-8.54	2.10	10.25	18.44	3.76	-3.49	3.33
6.3	-4.45	-5.70	-0.48	-6.58	-4.00	0.64	10.86	2.45	-6.72	-0.59
3.13	-4.52	-3.53	-2.03	-5.26	-4.81	-5.45	5.90	2.56	-6.85	-2.46
1.56	-3.41	-2.52	0.97	-3.23	-2.10	1.45	4.34	1.67	-6.14	-1.63
0.78	0.04	1.41	5.32	-2.11	-0.21	2.92	5.53	0.01	-4.94	-2.74
0.39	-0.86	2.10	4.13	0.29	5.42	6.59	6.01	-2.13	-1.71	2.17

C: Exatecan and ceralasertib (an ATR inhibitor) in HEC-1B cells

Fig 1C shows the % inhibition for exatecan and ceralasertib alone and in combination. The Loewe synergy score is shown in table 1C below. The IC_{50} for exatecan alone was 113.9 nM, when combined with ceralasertib at 0.37 µM was 19.01 nM and when combined with ceralasertib at 1.1 µM was 3.719 nM.

The IC_{50} for ceralasertib alone was 2.118 μM.

Table 1C

Ceralasertib (nM)	Exatecan (nM)									
	0.12	0.38	1.1	3.4	10	30	92	278	833	2500
10000	-3.21	-4.57	-0.59	-0.59	-2.38	0.19	-1.02	-2.42	-6.30	-2.55
3333	-0.77	1.10	9.54	12.30	14.87	17.21	16.73	14.82	11.81	11.81
1111	13.74	13.98	23.52	30.96	41.65	42.89	35.75	28.82	21.90	16.60
370	3.36	2.76	7.85	0.83	13.33	29.05	29.00	26.53	20.11	16.47
123	0.07	-0.28	2.87	-4.88	-1.40	8.40	12.10	11.47	10.53	8.84
41	1.41	-1.48	3.94	-2.08	0.04	0.57	1.91	1.86	2.09	1.87
13.7	-0.91	2.38	6.41	1.37	3.06	1.41	0.37	-0.97	0.36	-0.21
4.6	2.34	1.73	6.53	2.58	5.53	4.57	1.48	3.16	1.14	0.56
1.5	6.29	4.36	7.14	7.64	6.22	5.52	2.93	3.25	2.36	0.23
0.5	4.68	6.42	8.44	7.46	3.80	5.70	5.00	6.34	3.45	0.76

D: Exatecan and prexasertib (a CHK1 inhibitor) in HEC-1B cells

Fig 1D shows the % inhibition for exatecan and prexasertib alone and in combination. The Loewe synergy score is shown in table 1D below. The IC_{50} for exatecan alone was 113.9 nM, when combined with prexasertib at 1.9 nM was 8.988 nM and when combined with prexasertib at 3.8 nM was 2.013 nM. The IC_{50} for prexasertib alone was 4.466 nM.

Table 1D

Prexasertib (nM)	Exatecan (nM)									
	0.12	0.38	1.1	3.4	10	30	92	278	833	2500
30	-6.77	-4.80	-3.82	-3.12	-3.26	-3.16	-1.74	-1.53	-2.55	-2.76
15	-6.37	-6.95	-4.81	-5.04	-4.40	-3.88	-2.38	-1.03	-2.55	-1.84
7.5	-6.19	-4.10	-0.89	0.98	3.68	4.20	4.84	7.43	6.02	5.36
3.8	-7.79	-1.21	8.67	20.88	31.37	30.83	26.49	23.84	18.35	15.01
1.9	-8.06	-7.30	-1.57	9.34	24.03	24.85	26.19	27.04	21.05	16.08
0.9	-7.29	-6.70	1.87	1.71	9.99	8.60	15.58	18.82	18.26	14.51
0.47	-4.93	-6.53	0.96	0.09	4.58	2.46	5.75	12.52	11.43	10.24
0.23	-2.37	-4.55	3.79	1.13	5.15	1.37	3.30	6.28	6.12	5.68
0.12	-2.99	-3.35	1.01	4.08	4.30	2.92	0.52	5.06	4.29	3.57
0.06	-0.84	-2.67	4.13	6.75	6.19	3.18	-0.08	4.14	1.20	0.78

In further experiments an additional DDR inhibitor, berzosertib (an ATR inhibitor), was combined with TOP1 inhibitor exatecan, in a two-dimensional proliferation assay as follows.

Cell Lines

HEC-1-B (HTB-113)

HCT-116 (CCL-247)

Test Compounds

Exatecan (MedChemExpress,#HY-13631)

Berzosertib (Selleckchem, #S7102)

Detection

CellTiter-Glo® 2.0D Cell Viability Assay (Promega, #G9243)

The cells were seeded in 96-well white opaque plates in 150 µl of media, and incubated at 37°C with 5% CO_2 for 24 hours. HEC-1B was seeded at 7000 cells/well and HCT-116 was seeded at 3000 cells/well The next day, 25 µl of exatecan, berzosertib, or exatecan and berzosertib were added to cells at varying concentrations and incubated for 3 days at 37°C with 5% CO_2. Following incubation, 50 µl of CellTiter-Glo reagent was added to the plates and incubated for 25 mins with gentle shaking at 600 rpm. Cell viability was measured via Luminescence using Victor Nivo, PerkinElmer.

Background was subtracted using Luminescence values from wells having only media (without any cells). Percent inhibition was calculated using the formula 100- {(Lum of cells treated with drug / Lum of cells treated with buffer control)*100} (Lum= Luminescence). The LOEWE synergy score was calculated using the Synergy Finder tool at synergyfinder.fimm.fi.

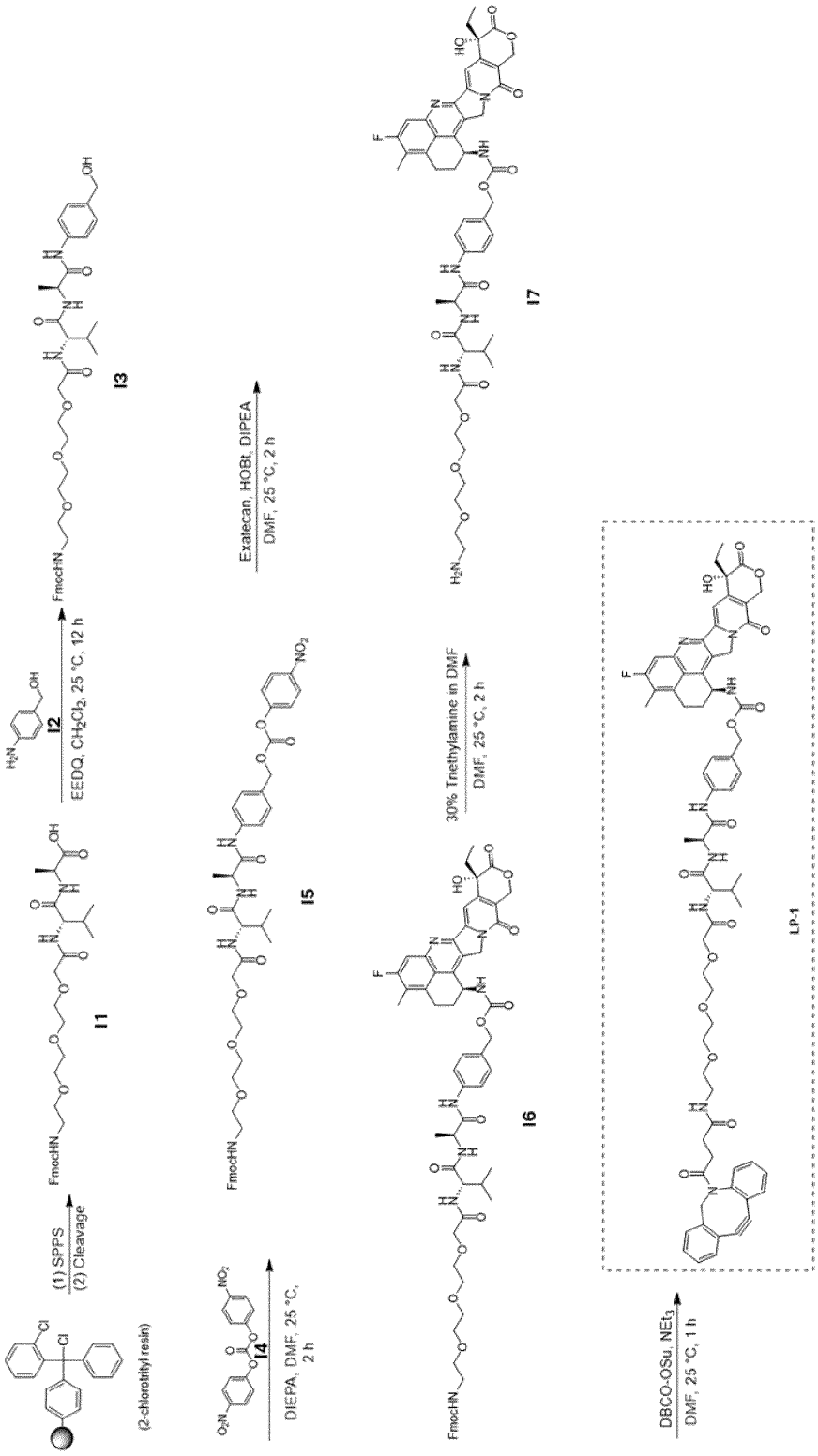
The results are shown in Figure 8 and Figure 9. A synergistic effect is observed across a range of exatecan and berzosertib concentrations.

To summarise, exatecan was tested with two clinical stage ATR inhibitors (berzosertib and ceralasertib) for *in vitro* synergy in TOP1 inhibitor-low sensitivity cell line HEC-1B and TOP1 inhibitor--high sensitivity cell line HCT-116. Both berzosertib and ceralasertib show synergy with exatecan across a wide range of concentrations tested in the cell lines. The effects in HEC-1B demonstrate the TOP1 inhibitor and DDR inhibitor combination can sensitize inherently -less sensitive cells to TOP1 inhibitor therapy.

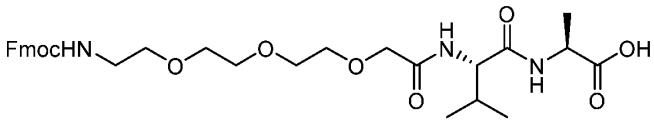
General conditions

All chemicals, raw materials and solvents were purchased from commercial sources, unless indicated otherwise. All chemical reactions were run under ambient conditions, unless otherwise indicated. Flash column chromatography was performed with CombiFlash® NEXTGEN 100, and the column was purchased from Agela Technologies. Prep-HPLC purifications were carried out using AUNO LC-2000, and the column is of Phenomenex Luna C18, 250 × 100 mm, 10µm, 10nm. ^{1}H NMR spectra were recorded on a Bruker spectrometer (400 MHz). ^{1}H NMR chemical shifts are expressed in parts per million (δ) downfield from tetramethylsilane (with the $CDCl_3$ peak at 7.26 ppm used as a standard). Mass Spectrometric data were recorded on SHIMADZU LCMS-2020 (ESI-MS) and Agilent 1260\G6125B (ESI-MS), and the column is of Kinetex® EVO C18 4.6×50mm, 5µm, Kinetex® EVO C18 2.1*30mm, 5µm, Shim-pack Scepter C18-120 3.0×33mm 3 µm and Poroshell 120 EC C18 2.7µm 3.0*30mm.

Example 2 – Synthesis of Linker-Payload molecule-1 (LP-1)



i) (1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-oyl)-L-valyl-L-alanine (compound I1)



I1

Compound **I1** was synthesized using standard solid-phase Fmoc chemistry.

a) Resin loading: CH_2Cl_2 (200 mL) was added to 2-chlorotrityl chloride resin (6.0 mmol, 1.00 equiv.), followed by the addition of Fmoc-Ala-OH (1.0 equiv.) and DIPEA (6.0 equiv.), the mixture was agitated under N_2 atmosphere at 25 °C for 2 h. Thereafter methanol (9.5 mL) was added to the resin and the agitation continued for 30 min. The resin was then filtered and washed with DMF (300 mL x 3).

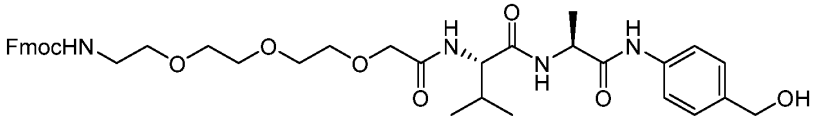
b) Deprotection: 20% piperidine in DMF (200 mL) was added to the resin and agitated under N_2 atmosphere at 25 °C for 30 min. The resin was washed with DMF (200 mL x 5) and filtered to get the resin with reactive amine group.

c) Coupling: A solution of HBTU (2.85 equiv.), and Fmoc-Val-OH (3.0 equiv.) in DMF (200 mL) was added to the resin followed by the addition of DIEPA (6.0 equiv.). The mixture was agitated under N_2 atmosphere at 25 ℃ for 30 min. The resin was then washed with DMF (200 mL x 3).

d) Repeat step b to deprotect Fmoc group. Treat the resulting resin with Fmoc-N-amido-PEG3-acid (2.0 equiv.), HATU (1.9 equiv.) and DIPEA (4.0 equiv.) in DMF. The mixture was agitated under N_2 atmosphere at 25 ℃ for 30 min. The resulting resin was washed with DMF (200 mL x 3).

e) Peptide cleavage and purification: The resin was washed with methanol (200 mL x 3) and dried under vacuum. The dried resin was treated with the cleavage buffer consisting of 20% HFIP in CH_2Cl_2 and stirred for 30 min and filtered. Concentration of the filtrate under reduced pressure furnished the crude compound **I1** (1.71 g) which was taken forward without further purification.

ii) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-(hydroxymethyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (Compound I3)

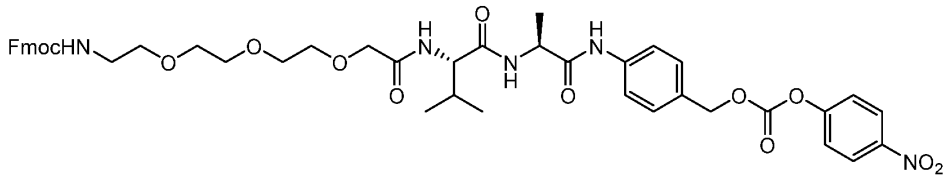


I3

To a solution of compound **I1** (1.60 g, 2.67 mmol, 1.0 equiv.) in CH_2Cl_2 (16.0 mL) was added compound **I2** (657 mg, 5.34 mmol, 2.0 equiv.), and EEDQ (1.32 g, 5.34 mmol, 2.0 equiv.). The reaction mixture was stirred at 25 °C for 12 h. LCMS analysis showed the starting material **I1** was consumed completely, and desired product mass was detected. The reaction mixture was added to 160 mL isopropyl ether, then

centrifuged to afford the crude compound **I3** (2.00 g) as a yellow oil. The crude product was used for the subsequent reaction without further purification. **MS (ESI)**: $[M+Na]^+$: 727.4.

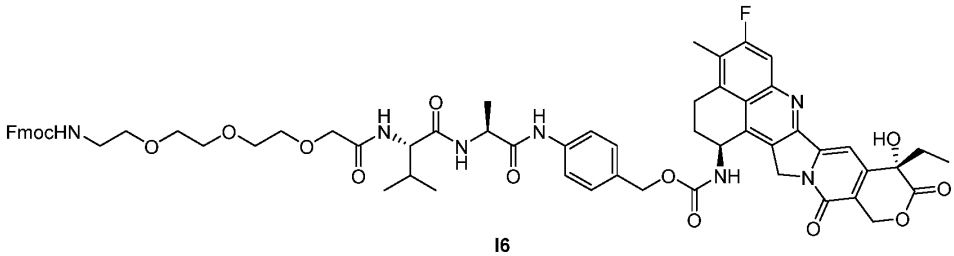
iii) (9H-fluoren-9-yl)methyl ((2S,5S)-5-isopropyl-2-methyl-1-((4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (Compound I5)



I5

To a solution of compound **I3** (2.00 g, 2.6 mmol, 1.0 equiv.), and compound **I4** (1.58 g, 5.2 mmol, 2.0 equiv.) in DMF (20 mL) was added DIEPA (671 mg, 5.20 mmol, 905 µL, 2.0 equiv.). The mixture was stirred at 25 °C for 2 h. LCMS analysis showed 31% of desired product formation and 2% of the starting alcohol **I3**. The mixture was purified directly by purified by prep-HPLC (TFA condition) and the solvent was removed to furnish compound **I5** (680 mg, 765 µmol, 29.4% yield, 97.9% purity) as a yellow solid. **MS (ESI)**: $[M+Na]^+$: 870.4.

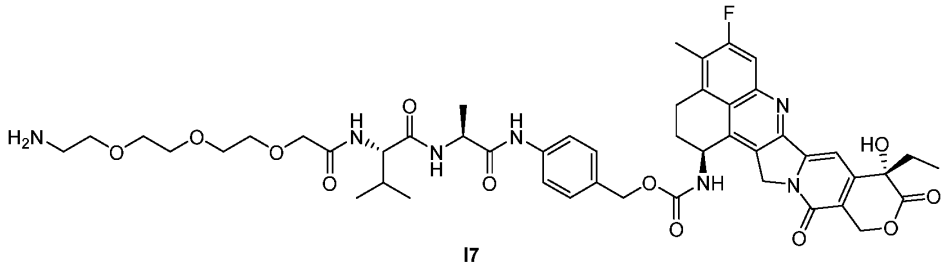
iv) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-((((((1R,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (compound I6)



I6

To a solution of compound **I5** (200 mg, 225 µmol, 1.0 equiv.), and Exatecan (119 mg, 225 µmol, 1.00 equiv.) in DMF (3.40 mL) was added HOBt (33.4 mg, 247 µmol, 1.1 equiv.) and DIPEA (58 mg, 450 µmol, 78.4 µL, 2.0 equiv.). The mixture was stirred at 25 °C for 2 h. LCMS analysis showed compound **I5** was completely consumed and the desired product mass was identified. The mixture was purified directly by prep-HPLC (TFA condition) to afford compound **I6** (180 mg, 148 µmol, 66% yield, 96.3% purity) as a yellow solid. **MS (ESI)**: $[M+H]^+$: 1167.7

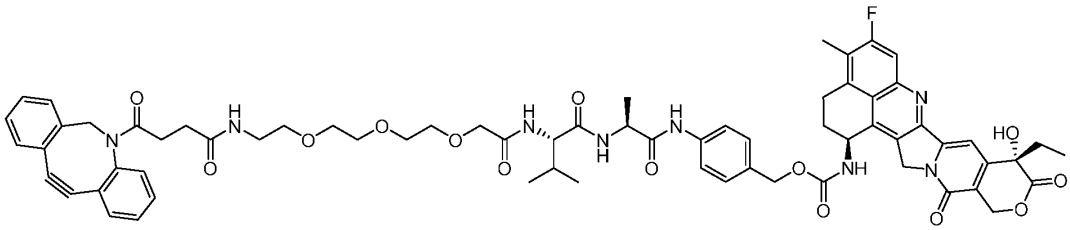
v) 4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl ((1R,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamate (Compound I7)



I7

To a solution of compound **I6** (180 mg, 148 μmol, 1.0 equiv.) in DMF (1.26 mL) was added triethylamine (392 mg, 3.88 mmol, 0.54 mL, 26 equiv.). The mixture was stirred at 25 °C for 2 h. LCMS analysis showed compound **I6** was consumed completely, and desired product mass was detected. Compound **I7** (140 mg, crude) was obtained as a brown liquid and used directly for the next step. **MS (ESI)**: $[M+H]^+$: 945.4

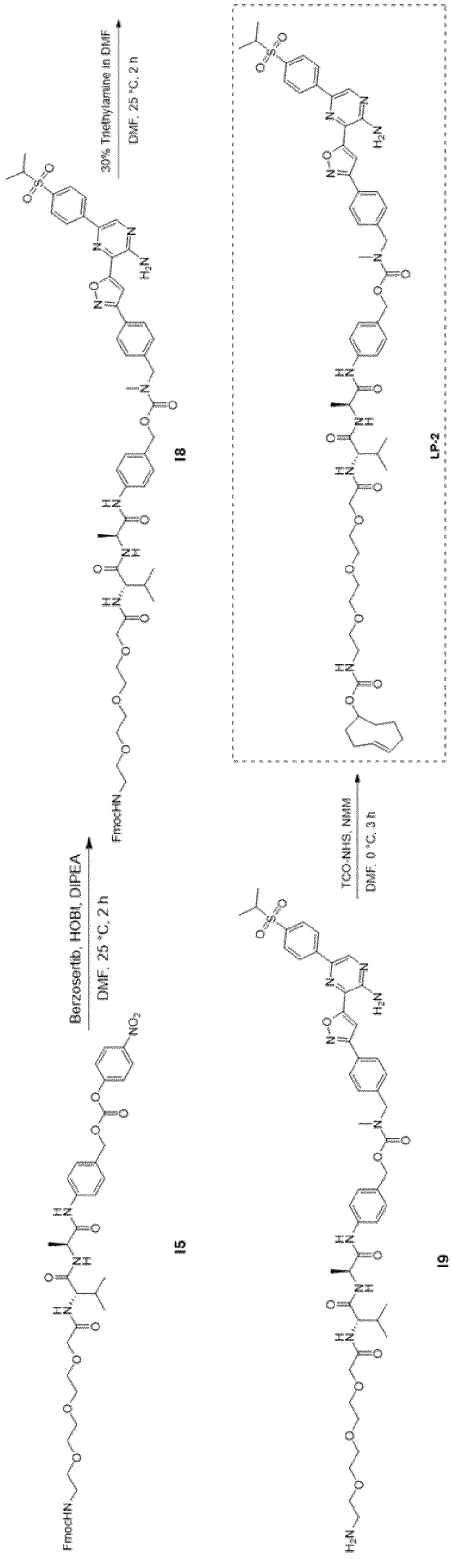
vi) Synthesis of LP-1



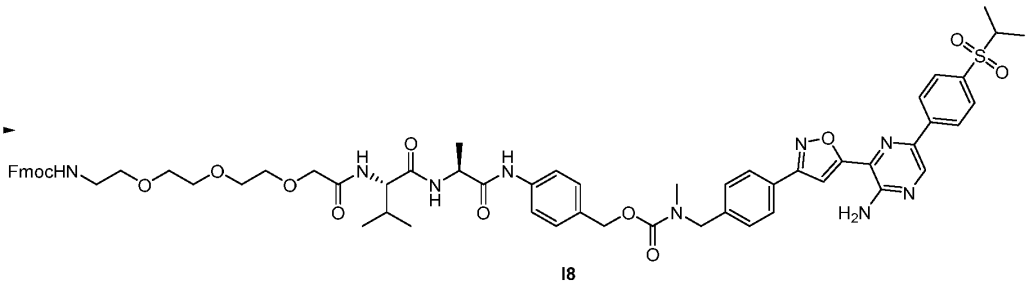
LP-1

To a solution of compound **I7** (140 mg, 148 μmol, 1.0 equiv.) in DMF (1.26 mL) was added triethylamine (392 mg, 3.9 mmol, 0.54 mL, 26.1 equiv.), and DBCO-OSu (59.6 mg, 148 μmol, 1.0 equiv.). The mixture was stirred at 25 °C for 1 h. LC-MS showed compound **I7** was consumed completely, and desired mass was detected. The reaction mixture was added to 18.0 mL of isopropyl ether, and the crude product was slowly precipitated out. Centrifuged to get the crude product and discarded the liquid supernatant. The residue was purified by prep-HPLC (TFA condition) to obtain **LP-1** (61.0 mg, 47.5 μmol, 32.5% yield, 97.5% purity) as a yellow solid. **MS (ESI)**: $[M+Na]^+$: 1253.6; **^{1}H NMR (400 MHz, DMSO-*d*$_6$)** δ ppm 9.98 (s, 1 H), 8.36 (d, *J* = 6.80 Hz, 1 H), 8.04 - 8.06 (m, 1 H), 7.71 - 7.78 (m, 2 H), 7.64 - 7.66 (m, 1 H), 7.58 (br d, *J* =8.8 Hz, 3 H), 7.41 - 7.47 (m, 4 H), 7.25 - 7.37 (m, 6 H), 5.44 (s, 2 H), 5.28 (br s, 2 H), 5.07 (s, 2 H), 5.00 (br d, *J* =13.88 Hz, 1 H), 4.38 (br t, *J* =6.94 Hz, 1 H), 4.28 (dd, *J* =9.13, 6.63 Hz, 1 H), 3.93 (s, 2 H), 3.43 - 3.61 (m, 12 H), 3.27 (br t, *J* =6.00 Hz, 4 H), 3.03 - 3.11 (m, 2 H), 2.37 (s, 3 H), 2.13 - 2.26 (m, 3 H), 1.93 - 2.03 (m, 2 H), 1.81 - 1.92 (m, 2 H), 1.70 - 1.80 (m, 1 H), 1.30 (d, *J* =7.00 Hz, 3 H), 0.84 - 0.91 (m, 6 H), 0.81 (br d, *J* =6.75 Hz, 3 H).

Example 3 - Synthesis of linker-payload molecule-2 (LP-2)



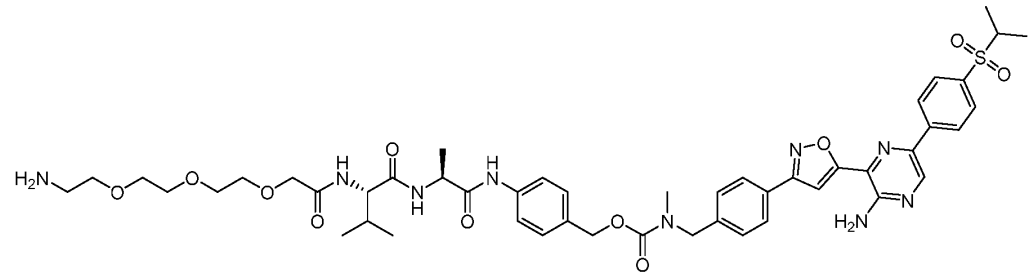
i) 4-((17S,20S)-1-(9H-fluoren-9-yl)-17-isopropyl-20-methyl-3,15,18-trioxo-2,7,10,13-tetraoxa-4,16,19-triazahenicosan-21-amido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (compound I8)



I8

To a solution of compound **I5** (150 mg, 168 µmol, 1.0 equiv.), and berzosertib (86 mg, 185 µmol, 1.1 equiv.) in DMF (1.5 mL) was added HOBt (25.0 mg, 185 µmol, 1.10 eq) and DIPEA (43.6 mg, 337 µmol, 58.8 µL, 2.00 eq). The mixture was stirred at 25 °C for 2 h. LCMS analysis showed compound **I5** was consumed completely and the desired mass was detected. The mixture was purified directly by prep-HPLC (TFA condition) to afford compound **I8** (150 mg, 121 µmol, 72.2% yield, 97.1% purity) as a yellow solid. **MS (ESI)**: $[M+H]^+$: 1194.6

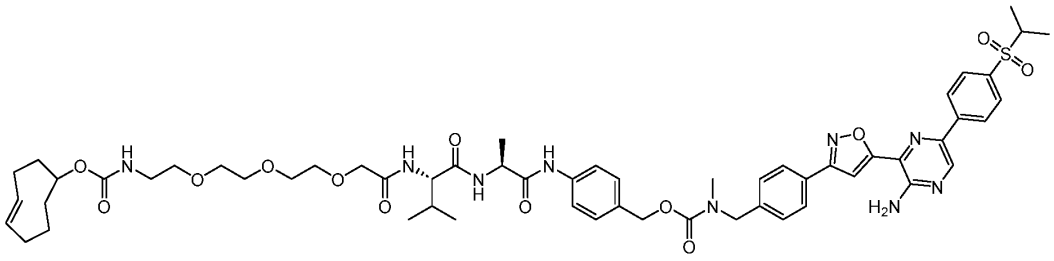
ii) 4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (Compound I9)



I9

To a solution of compound **I8** (150 mg, 121 µmol, 1.0 equiv.) in DMF (1.0 mL) was added triethylamine (327 mg, 3.23 mmol, 450 µL, 26.5 equiv.). The mixture was stirred at 25 °C for 2 h. LCMS analysis showed compound **I8** was consumed completely and the desired product mass was observed. The reaction mixture was added to 15.0 mL isopropyl ether and centrifuged to get the crude compound **I9** (200 mg, crude) as a yellow oil. **MS (ESI)**: $[M+H]^+$: 972.5

iii) 4-((15S,18S)-1-(((E)-cyclooct-4-en-1-yl)oxy)-15-isopropyl-18-methyl-1,13,16-trioxo-5,8,11-trioxa-2,14,17-triazanonadecan-19-amido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (LP-2)

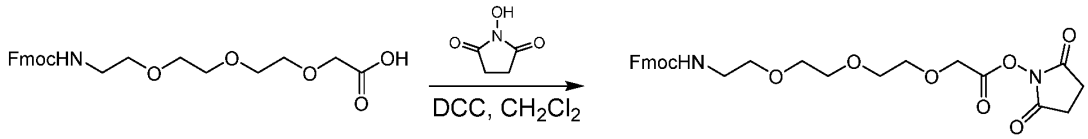


LP-2

To a solution of compound **I9** (190 mg, 169 µmol, 1.0 equiv.), and TCO-NHS (45.2 mg, 169 µmol, 1.0 equiv.) in DMF (1.5 mL) was added NMM (17.1 mg, 169 µmol, 18.6 µL, 1.00 equiv.). The mixture was stirred at 0 °C for 3 h. LCMS showed compound **I9** was consumed completely. The resultant reaction mixture was purified directly by prep-HPLC (neutral condition) to afford compound **LP-2** (55.0 mg, 45.0 µmol, 27.6% yield, 95.6% purity) as a light-yellow solid. **MS (ESI)**: $[M+Na]^+$:1146.6; **^{1}H NMR (400 MHz, DMSO-*d*$_6$)**: δ ppm 9.99 (s, 1 H), 8.94 (s, 1 H), 8.38 (d, *J* =8.50 Hz, 3 H), 7.96 - 8.03 (m, 2 H), 7.93 (d, *J* =8.63 Hz, 2 H), 7.77 (s, 1 H), 7.53 - 7.63 (m, 2 H), 7.25 - 7.46 (m, 5 H), 7.19 (br s, 2 H), 6.86 - 6.93 (m, 1 H), 5.49 - 5.60 (m, 1 H), 5.36 - 5.46 (m, 1 H), 5.07 (br s, 2 H), 4.53 (s, 2 H), 4.35 - 4.43 (m, 1 H), 4.26 - 4.32 (m, 1 H), 4.14 - 4.23 (m, 1 H), 3.93 (s, 2 H), 3.43 - 3.63 (m, 10 H), 2.98 - 3.11 (m, 2 H), 2.88 (s, 3 H), 2.17 - 2.30 (m, 3 H), 1.93 - 2.05 (m, 1 H), 1.75 - 1.92 (m, 4 H), 1.44 - 1.68 (m, 3 H), 1.26 - 1.35 (m, 3 H), 1.19 (d, *J* =6.75 Hz, 6 H), 0.77 - 0.93 (m, 6 H).

Example 4 – Synthesis of Linker-payload payload molecule-3 (LP-3)

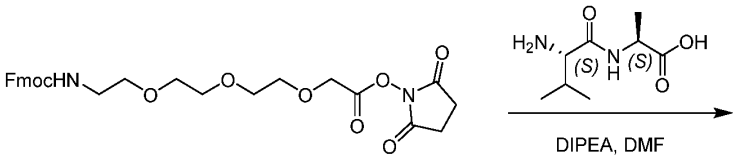
i) 2,5-dioxopyrrolidin-1-yl 1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-oate (Compound I11)



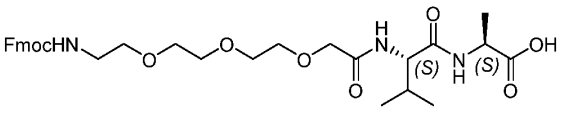
I10 **I11**

To a solution of compound **I10** (25.5 g, 59.4 mmol, 1.0 eq.) in CH_2Cl_2 (250 mL) was added HOSu (6.84 g, 59.4 mmol, 1.0 eq.) and DCC (12.2 g, 59.4 mmol, 12.0 mL, 1.0 eq.). The mixture was stirred at 25 °C for 12 h. Upon completion, the reaction mixture was filtered and concentrated under reduced pressure to give compound **I11** as a yellow oil (31.7 g, crude), which was used without any further purification. **MS (ESI)**: MS calculated: 526.53, MS observed: $[M+H]^+$ = 527.1.

ii) (1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-oyl)-L-valyl-L-alanine (Compound I12)



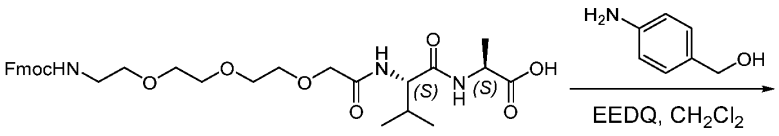
I11



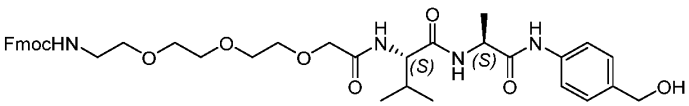
I12

To a solution of compound **I11** (31.3 g, 59.4 mmol, 1.0 eq.) and dipeptide Val-Ala-OH (11.1 g, 59.4 mmol, 1.0 eq.) in DMF (300 mL) was added DIPEA (7.68 g, 59.4 mmol, 10.3 mL, 1.0 eq.). The mixture was stirred at 25 °C for 4 h. LC-MS showed compound **I11** was consumed completely. The reaction mixture was concentrated and the residue was purified by preparative HPLC to obtain compound **I12** (23.5 g, 37.5 mmol, 63% yield for 2 steps) as a white solid. **MS (ESI)**: MS calculated: 599.67, MS observed: $[M+H]^+$ = 600.3

iii) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-(hydroxymethyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (Compound I13)



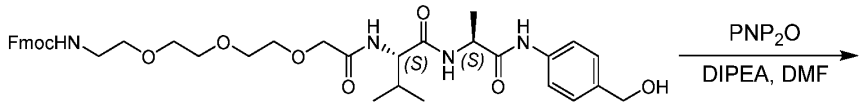
I12



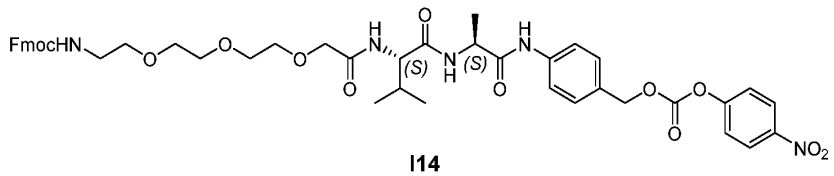
I13

To a solution of compound **I12** (23.5 g, 37.5 mmol, 1.0 eq.) in CH_2Cl_2 (235 mL) was added 4-aminobenzyl alcohol (9.25 g, 75 mmol, 2.0 eq.) and EEDQ (18.5 g, 75.0 mmol, 2.0 eq.). The mixture was stirred at 25 °C for 12 h under dark conditions. After completion, the mixture was concentrated and washed with isopropyl ether (2.35 L) to obtain compound **I13** (34 g, crude) as a yellow gelatinous solid and used without further purifications. **MS (ESI)**: MS calculated.: 704.8, MS observed: $[M+H]^+$ = 705.3

iv) (9H-fluoren-9-yl)methyl ((2S,5S)-5-isopropyl-2-methyl-1-((4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (Compound I14)



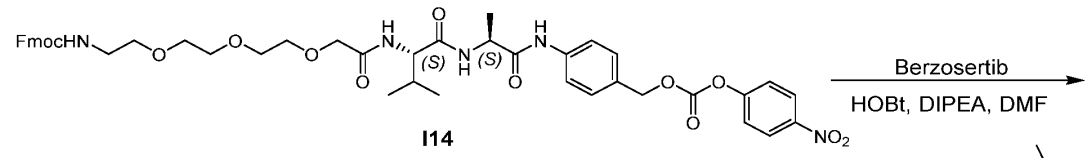
I13



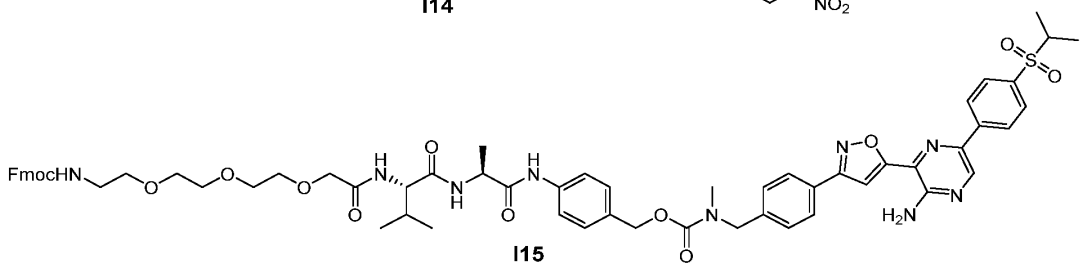
I14

To a solution of compound **I13** (34 g, 45.4 mmol, 1.0 eq.) in DMF (320 mL) was added PNP_2O (34.5 g, 113 mmol, 2.5 eq.) and DIPEA (15 mL, 90.8 mmol, 2.0 eq.). The mixture was stirred at 25 °C for 4 h. Upon completion, the reaction mixture was purified by preparative HPLC to obtain compound **I14** (22 g, 24.6 mmol, 63% yield for 2 steps) as a yellow solid. **MS (ESI)**: MS calculated: 869.35, MS observed: $[M+H]^+$ = 870.4

v) 4-((17S,20S)-1-(9H-fluoren-9-yl)-17-isopropyl-20-methyl-3,15,18-trioxo-2,7,10,13-etraoxa-4,16,19-triazahenicosan-21-amido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (Compound I15)



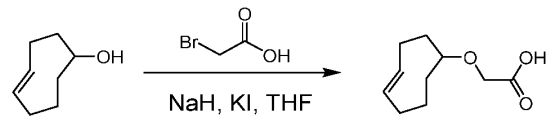
I14



I15

To a solution of compound **I14** (2.1 g, 2.35 mmol, 1.0 eq.) and berzosertib (1.14 g, 2.47 mmol, 1.05 eq.) in DMF (11 mL) was added HOBt (350 mg, 2.59 mmol, 1.1 eq.) and DIPEA (778 µL, 4.71 mmol, 2.0 eq.). The mixture was stirred at 28 °C for 2 h. LC-MS analysis showed compound **I14** was consumed completely. Then the reaction was concentrated and triturated with isopropyl ether (3 x 110 mL) and THE residue obtained was further purified by preparative HPLC to obtain compound **I15** (2.4 g, 1.97 mmol, 86% yield) as a yellow solid. **MS (ESI):** MS calculated.: 1193.49, MS observed: [M+H]+ = 1194.5

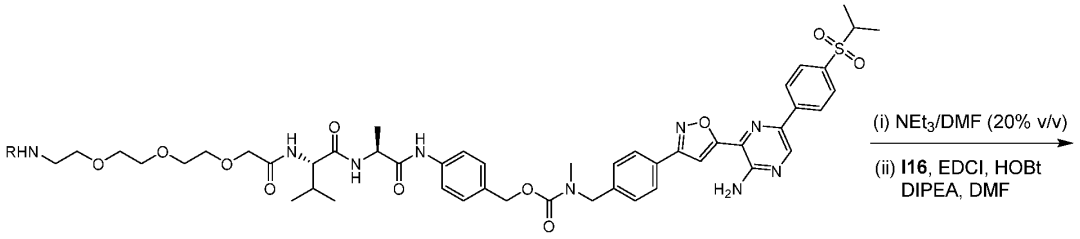
vi) (E)-2-(cyclooct-4-en-1-yloxy)acetic acid (Compound I16)



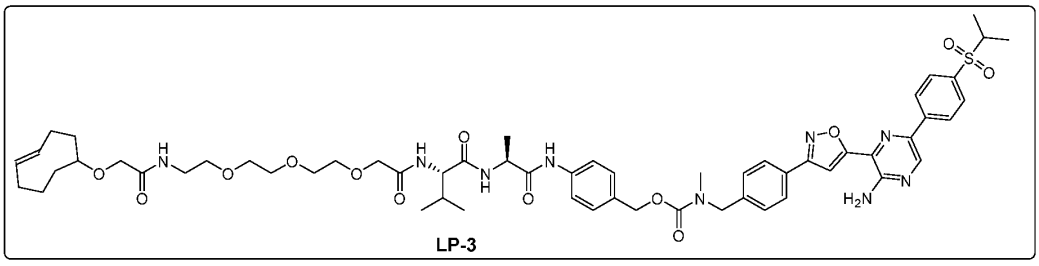
TCO-OH **I16**

To a solution of compound TCO-OH (200 mg, 1.58 mmol, 1.0 eq.) in dry THF (2.0 mL) was added NaH (60% dispersion in mineral oil, 190 mg, 4.75 mmol, 3.0 eq.). The mixture was stirred at 25 °C for 1 h under N_2 atmosphere. Then 2-bromoacetic acid (264 mg, 1.9 mmol, 1.2 eq.) and KI (26.3 mg, 158 µmol, 0.10 eq.) were added. The mixture was stirred at 70 °C for 12 h. Upon completion, the reaction mixture was quenched with H_2O (10 mL), and adjusted to pH 2 using 1N HCl. The product was extracted with CH_2Cl_2 (6 x 5 mL). The combined organic layers were washed with brine (3 x 5 mL) and dried over anhydrous Na_2SO_4, filtered, concentrated and purified by preparative HPLC to give compound **I16** (82 mg, 445 µmol, 28% yield) as a white solid. **MS (ESI):** MS calculated.: 184.11, MS observed: $[M-H]^-$ = 183.1. **^{1}H NMR (400 MHz, $CDCl_3$)** *δ* 5.77 - 5.48 (m, 1H), 5.47 - 5.25 (m, 1H), 3.87 - 3.69 (m, 1H), 3.69 - 3.53 (m, 1H), 2.99 (br d, *J* = 8.5 Hz, 1H), 2.52 - 1.63 (m, 8H), 1.61 - 1.40 (m, 2H).

vii) 4-((2S,5S)-20-(((E)-cyclooct-4-en-1-yl)oxy)-5-isopropyl-2-methyl-4,7,19-trioxo-9,12,15-trioxa-3,6,18-triazaicosanamido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (LP-3)



R= Fmoc: **I15**



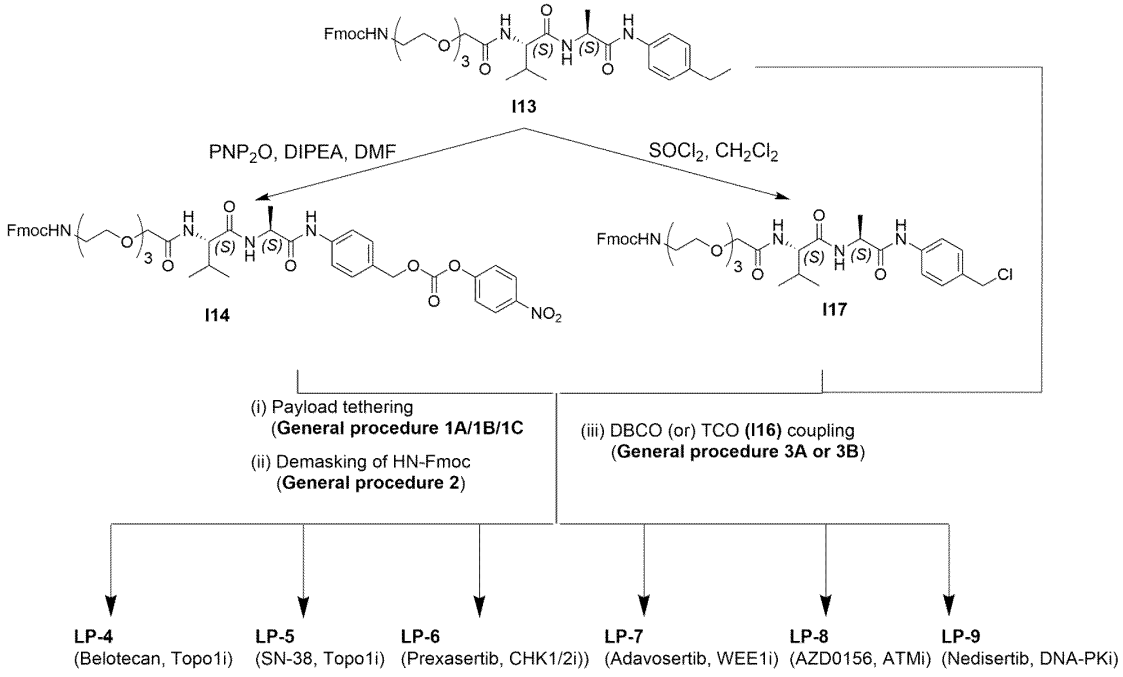
LP-3

To a solution of compound **I15** (2.0 g) in DMF (14 mL) was added triethylamine (6 mL). The mixture was stirred at 25 °C for 12 h. Upon completion, isopropyl ether (200 mL) was added to the reaction mixture and compound **I15*** (2.0 g, crude) was collected upon centrifugation. The product was used directly for the next step without further purification. [Note I15* has the same chemical formula as I15, where R=H].

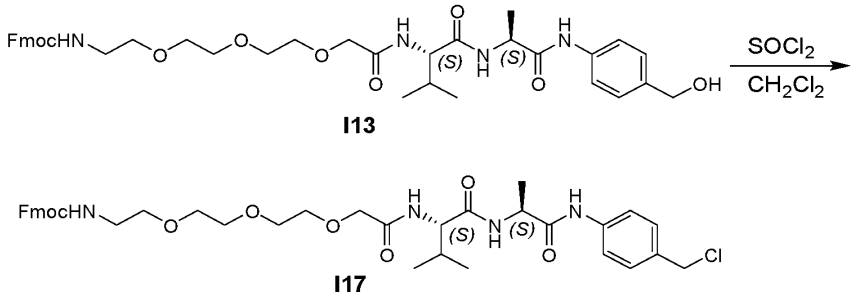
To a solution of crude **I15*** (265 mg, 272 µmol, 1.0 eq.) and compound **I16** (50 mg, 271 µmol, 1.0 eq.) in DMF (2.5 mL) was added HATU (154 mg, 407 µmol, 1.5 eq.) and DIPEA (89.7 µL, 542 µmol, 2.0 eq.). The mixture was stirred at 25 °C for 2 h. LC-MS analysis indicated the completion of the reaction. The reaction mixture was purified by preparative HPLC to give **LP-3** (205 mg, 49% yield) as a pale-yellow solid. **^{1}H NMR (400 MHz, DMSO-d6)** δ 10.05 - 9.97 (m, 1H), 8.95 (s, 1H), 8.46 - 8.20 (m, 3H), 8.04 - 7.88 (m, 4H), 7.78 (s, 1H), 7.65 - 7.52 (m, 2H), 7.52 - 7.10 (m, 8H), 5.50 (br dd, J = 3.6, 11.6 Hz, 1H), 5.34 (br dd, J = 4.0, 11.4 Hz, 1H), 5.07 (br s, 2H), 4.53 (s, 2H), 4.45 - 4.20 (m, 2H), 3.97 - 3.90 (m, 2H), 3.78 - 3.64 (m, 2H), 3.63 - 3.40 (m, 9H), 3.23 (q, J = 6.0 Hz, 2H), 2.93 - 2.82 (m, 3H), 2.31 - 1.67 (m, 11H), 1.49

- 1.38 (m, 2H), 1.38 - 1.23 (m, 4H), 1.19 (d, J = 6.8 Hz, 6H), 0.94 - 0.76 (m, 6H). **MS (ESI)**: MS calculated.: 1137.52, MS observed: [M+H]+ = 1138.0. HPLC purity (254 nm) = 96.8%

Example 5 – Synthesis of Linker-payload molecules LP-4, LP-5, LP-6, LP-7, LP-8 and LP-9



i) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-(chloromethyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (Compound I17)



To a solution of compound **I13** (180 mg, 250 µmol, 1.0 eq.) in CH_2Cl_2 (2.0 mL), $SOCl_2$ (15.2 µL) was added slowly. The mixture was stirred for 2 h at 25 °C. LC-MS analysis indicated the completion of the reaction. The reaction mixture was added dropwise to the ice-cold isopropyl ether (20 ml), centrifuged, and the desired product **I17** was isolated as a yellow oily residue (160 mg, crude) and used directly for the next reaction.

ii) General Procedure for payload tethering (General Procedure 1A):

To a solution of compound **I14** (140 µmol, 1.2 eq) in DMF (~1.0 mL) was added DIPEA (3.0 eq.), and **payload molecule** (1.0 eq.) at 25 °C. The mixture was stirred at 25 °C for 2 h. The reaction progress was

monitored by LC-MS analysis. Upon completion, the reaction mixture was concentrated, and the residue was either used directly or purified by preparative HPLC.

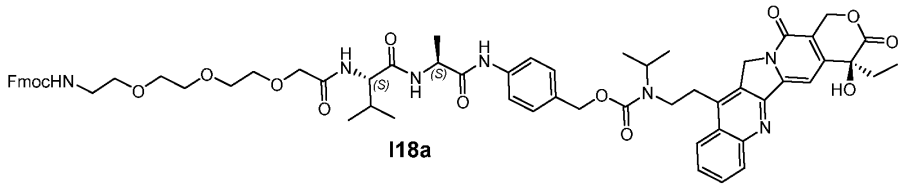
iii) General Procedure for payload tethering (General Procedure 1B):

To a solution of **payload molecule** (140 µmol, 1.0 eq.) in CH_2Cl_2 (1.0 mL), DMAP (3.0 eq.) and triphosgene (0.8 eq.) were added. The mixture was stirred for 2 min at 25 °C. DIPEA (2.0 eq.) was added followed by compound **I13** (1.0 eq.). The mixture was stirred for 2 h at 25 °C. The reaction was monitored by LC-MS analysis. Upon completion the reaction mixture was poured into ice-cold isopropyl ether. The precipitate was collected and further purified by preparative HPLC.

iv) General Procedure for payload tethering (General Procedure 1C):

To a solution of compound **I17** (~110 µmol, 1.0 eq.) in DMF (800 µL), **payload molecule** (0.7 eq.) and DIPEA (3.0 eq.) were added. The mixture was stirred for 12 h at 25 °C. The reaction was monitored by LC-MS analysis. Upon completion, the reaction mixture was poured into ice-cold isopropyl ether. The precipitated product was collected and used directly without further purification.

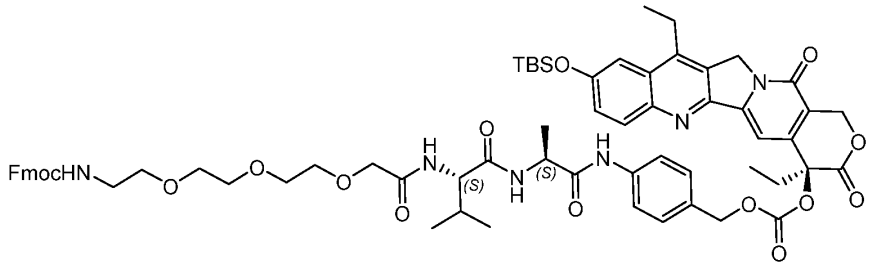
v) 4-((17S,20S)-1-(9H-fluoren-9-yl)-17-isopropyl-20-methyl-3,15,18-trioxo-2,7,10,13-tetraoxa-4,16,19-triazahenicosan-21-amido)benzyl (2-((S)-4-ethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-11-yl)ethyl)(isopropyl)carbamate (Compound I18a)



I18a

Prepared according to the General Procedure 1A: Payload: Belotecan. **I18a** obtained as a white solid (42.0 mg, 76% yield). **MS (ESI):** MS calculated: 1164.3, MS observed: $[M+H]^+$ = 1164.0

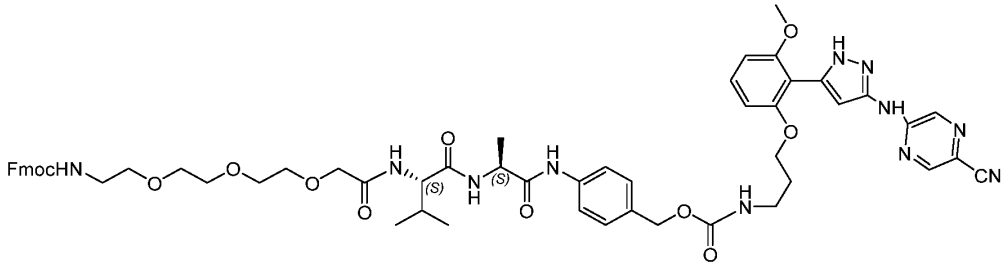
vi) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-((((((S)-9-((tert-butyldimethylsilyl)oxy)-4,11-diethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)carbonyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (Compound I18b)



I18b

Prepared according to the General Procedure 1B: Payload: SN38-(OTBS). **I18b** collected as a yellow solid (75 mg, 57%). **MS (ESI):** MS calculated: 1236.5, MS observed: $[M+H]^+$ = 1237.5

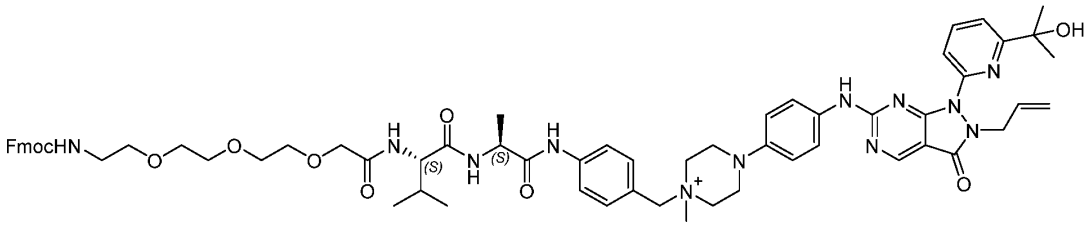
vii) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-((((3-(2-(3-((5-cyanopyrazin-2-yl)amino)-1H-pyrazol-5-yl)-3-methoxyphenoxy)propyl)carbamoyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (compound I18c)



I18c

Prepared according to the General Procedure 1A: Payload: Prexasertib. **I18c** was obtained as a colourless solid (150 mg, crude) and used directly without further purification. **MS (ESI):** MS calculated: 1095.48, MS observed: $[M+H]^+$ = 1096.1.

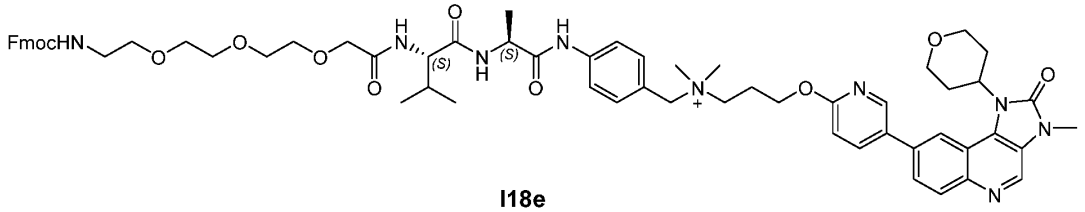
viii) 1-(4-((17S,20S)-1-(9H-fluoren-9-yl)-17-isopropyl-20-methyl-3,15,18-trioxo-2,7,10,13-tetraoxa-4,16,19-triazahenicosan-21-amido)benzyl)-4-(4-((2-allyl-1-(6-(2-hydroxypropan-2-yl)pyridin-2-yl)-3-oxo-2,3-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)amino)phenyl)-1-methylpiperazin-1-ium (Compound I18d)



I18d

Prepared according to the General Procedure 1C: Payload: Adavosertib; **I18d** was obtained as a yellow oil (70 mg, crude). **MS (ESI):** MS calculated: 1187.6, MS observed: $[M+H]^+$ = 1188.9

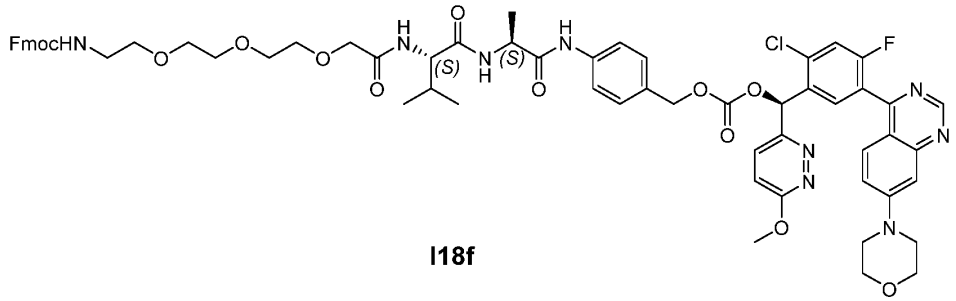
ix) N-(4-((17S,20S)-1-(9H-fluoren-9-yl)-17-isopropyl-20-methyl-3,15,18-trioxo-2,7,10,13-tetraoxa-4,16,19-triazahenicosan-21-amido)benzyl)-N,N-dimethyl-3-((5-(3-methyl-2-oxo-1-(tetrahydro-2H-pyran-4-yl)-2,3-dihydro-1H-imidazo[4,5-c]quinolin-8-yl)pyridin-2-yl)oxy)propan-1-aminium (Compound I18e)



I18e

Prepared according to the General Procedure 1C: Payload: AZD0156; **I18e** was obtained as a yellow oil (70 mg, crude). **MS (ESI):** MS calculated: MS calculated: 1148.5, MS observed: $[M+H]^+$ = 1149.5

x) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-(((((S)-(2-chloro-4-fluoro-5-(7-morpholinoquinazolin-4-yl)phenyl)(6-methoxypyridazin-3-yl)methoxy)carbonyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (Compound I18f)

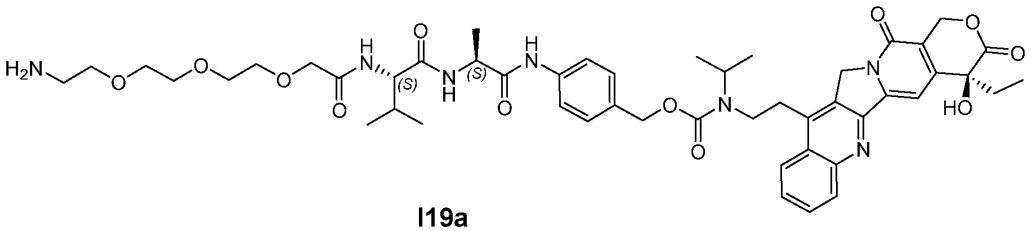


I18f

Prepared according to the General Procedure 1A: Payload: Nedisertib; **I18f** (50 mg, 40%, white solid).
MS (ESI): MS calculated: 1211.45, MS observed: $[M+H]^+$ = 1212.0.

xi) General Procedure for the -NHFmoc deprotection (General Procedure 2):
To a solution of Fmoc protected amine (~40-60 µmol, 1.0 eq.) in DMF (~0.60 mL) was added triethylamine (~0.25 mL). The reaction mixture was then stirred for 3 h at 25 °C. Upon completion, the solvents were removed and purified by preparative HPLC to get the desired product.

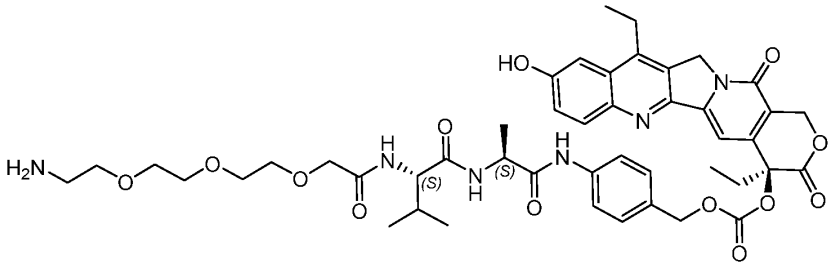
xii) 4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl (2-((S)-4-ethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-11-yl)ethyl)(isopropyl)carbamate (Compound I19a)



I19a

Prepared according to the General Procedure 2: **I19a** was obtained as a white solid (30 mg, 88% yield).
MS (ESI): MS calculated.: 942.0, MS observed: $[M]^+$ = 942.0.

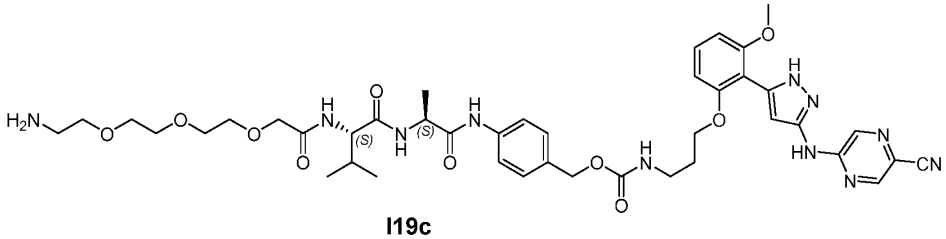
xiii) 4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl ((S)-4,11-diethyl-9-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl) carbonate (Compound I19b)



I19b

Prepared according to the General Procedure 2: **I19b** was obtained as a yellow solid (50 mg, crude). **LC (ESI):** MS calculated: 900.3, MS observed: $[M+H]^+$ =901.5

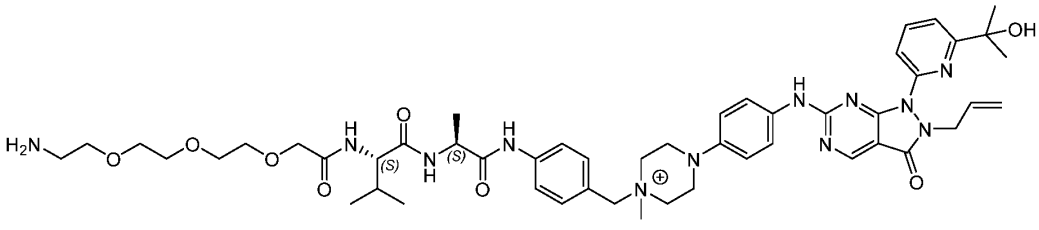
xiv) 4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl (3-(2-(3-((5-cyanopyrazin-2-yl)amino)-1H-pyrazol-5-yl)-3-methoxyphenoxy)propyl)carbamate (Compound I19c)



I19c

Prepared according to the General Procedure 2: **I19c** was obtained as a white solid (30 mg, 42% yield). **MS (ESI):** MS calculated: 873.41, MS observed: $[M+H]^+$ = 874.0.

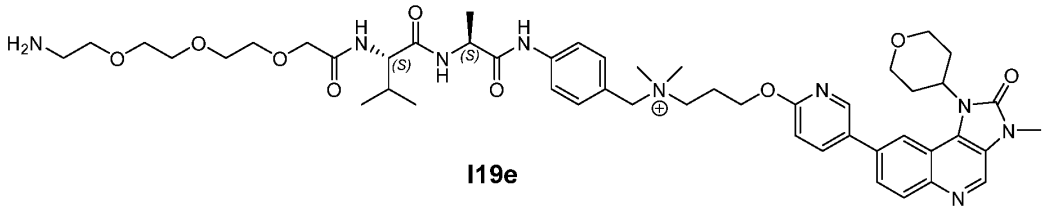
xv) 4-(4-((2-allyl-1-(6-(2-hydroxypropan-2-yl)pyridin-2-yl)-3-oxo-2,3-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)amino)phenyl)-1-(4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl)-1-methylpiperazin-1-ium (Compound I19d)



I19d

Prepared according to the General Procedure 2: **I19d** was obtained as a yellow solid (40 mg, 70% yield). **MS (ESI):** MS cal.: 965.5, MS observed: $[M+H]^+$ = 966.5

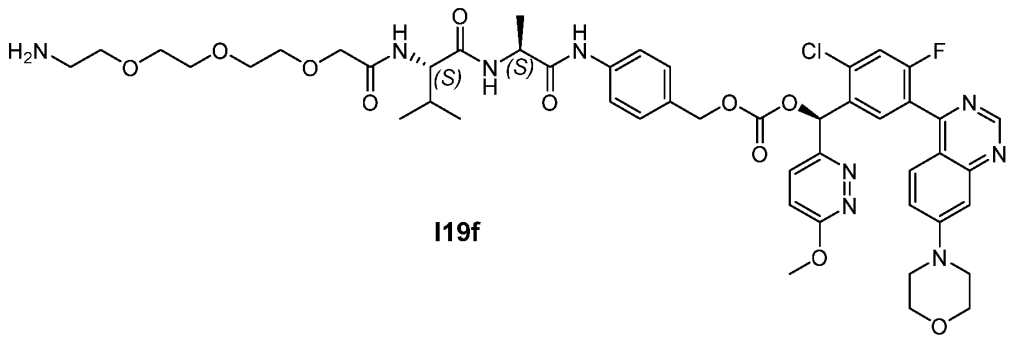
xvi) N-(4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl)-N,N-dimethyl-3-((5-(3-methyl-2-oxo-1-(tetrahydro-2H-pyran-4-yl)-2,3-dihydro-1H-imidazo[4,5-c]quinolin-8-yl)pyridin-2-yl)oxy)propan-1-aminium (Compound I19e)



I19e

Prepared according to the General Procedure 2: **I19e** was obtained as a yellow solid (40 mg, 31% yield). **MS (ESI):** MS calculated: 926.5, MS observed: $[M+H]^+$ = 927.5

xvii) 4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl ((S)-(2-chloro-4-fluoro-5-(7-morpholinoquinazolin-4-yl)phenyl)(6-methoxypyridazin-3-yl)methyl) carbonate (Compound I19f)



I19f

Prepared according to the General Procedure 2: **I19f** was isolated as a yellow solid (40 mg, 98% yield)**.** **MS (ESI):** MS calculated: 989.39, MS observed: $[M+H]^+$ = 990.4.

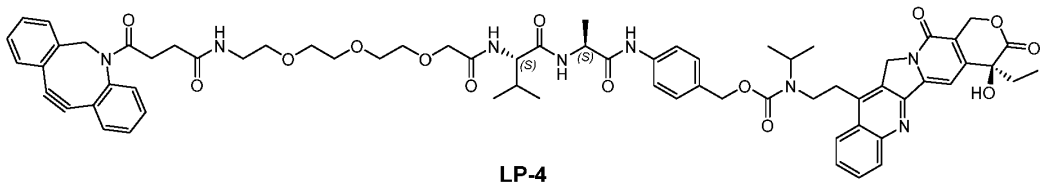
xviii) General Procedure for DBCO coupling (General Procedure 3A):

To a solution of amine (30 µmol, 1.0 eq.) in DMF (1.0 mL) was added DBCO-OSu (2.0 eq.) and DIPEA (3.0 eq.). The mixture was stirred for 2 h at 25 °C. LC-MS analysis indicated the complete conversion of the amine to the desired product. The reaction mixture was concentrated under reduced pressure and directly purified by preparative HPLC to afford the desired product.

xix) General Procedure for TCO coupling (General Procedure 3B):

To a solution of amine (35 µmol, 1.0 eq.) in DMF (0.3 mL) was added compound **I16** (1.0 eq.), HATU (2.0 eq.) and DIPEA (2.0 eq.). The reaction mixture was stirred for 2 h at 25 °C. LC-MS analysis indicated the complete conversion of the amine to the desired product. The reaction mixture was concentred under reduced pressure and the residue was directly purified by preparative HPLC to give the desired product.

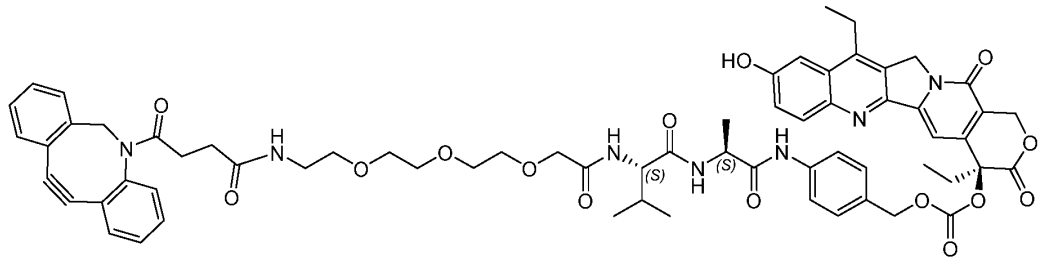
xx) [4-[[(2S)-2-[[(2S)-2-[[2-[2-[2-[2-[[4-(2-azatricyclo[10.4.0.04,9]hexadeca-1(12),4(9),5,7,13,15-hexaen-10-yn-2-yl)-4-oxo-butanoyl]amino]ethoxy]ethoxy]ethoxy]acetyl]amino]-3-methyl-butanoyl]amino]propanoyl]amino]phenyl]methyl N-[2-[(19S)-19-ethyl-19-hydroxy-14,18-dioxo-17-oxa-3,13-diazapentacyclo[11.8.0.02,11.04,9.015,20]henicosa-1(21),2,4,6,8,10,15(20)-heptaen-10-yl]ethyl]-N-isopropyl-carbamate (LP-4)



LP-4

LP-4 was synthesized according to General Procedure 3A from **I19a**. **Yield:** 45%. **^{1}H NMR (400 MHz, DMSO-*d*$_6$.)** δ ppm 10.13 - 9.88 (m, 1H), 8.43 - 8.29 (m, 1H), 8.25 - 8.09 (m, 1H), 8.01 - 7.82 (m, 1H), 7.79 - 7.54 (m, 6H), 7.53 - 7.29 (m, 10H), 6.60 - 6.40 (m, 1H), 5.51 - 5.28 (m, 4H), 5.18 - 5.05 (m, 2H), 4.49 - 4.11 (m, 3H), 4.06 - 3.85 (m, 2H), 3.67 - 3.39 (m, 18H), 3.11 - 3.03 (m, 4H), 2.71 - 2.65 (m, 2H), 2.02 - 1.97 (m, 2H), 1.32 - 1.23 (m, 6H), 1.14 - 1.11 (m, 3H), 0.91 - 0.80 (m, 9H). **MS (ESI):** MS calculated: 1229.3, MS observed: $[M+H]^+$ = 1229.4. **Purity (HPLC, 254 nm)** = 95.5%

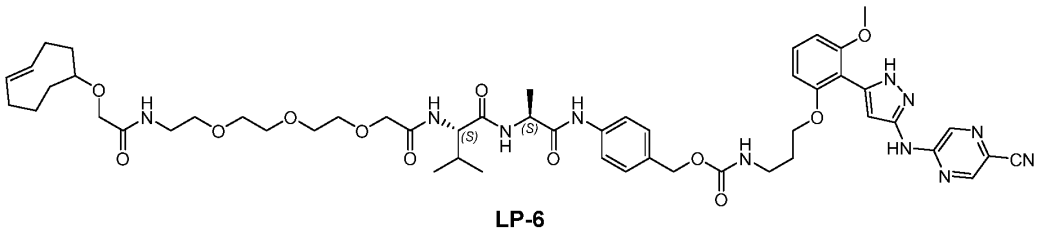
xxi) DBCO-PEG3-VA-PABC-SN38 (LP-5)



LP-5

LP-5 was synthesized according to General Procedure 3A from **I19b**. **Yield:** 38%. **^{1}H NMR (400 MHz, DMSO-*d6*)** δ ppm 10.16 - 10.72 (m, 1 H), 9.93 - 10.11 (m, 1 H), 8.35 - 8.46 (m, 1 H), 8.02 - 8.09 (m, 1 H), 7.73 - 7.78 (m, 1 H), 7.65 - 7.69 (m, 1 H), 7.57 - 7.60 (m, 2 H), 7.45 - 7.46 (m, 1 H), 7.43 - 7.44 (m, 1 H), 7.41 - 7.42 (m, 1 H), 7.35 - 7.37 (m, 1 H), 7.31 - 7.34 (m, 2 H), 7.29 - 7.30 (m, 1 H), 6.93 - 6.96 (m, 1 H), 5.48 - 5.55 (m, 2 H), 5.29 - 5.34 (m, 2 H), 5.27 - 5.35 (m, 2 H), 5.05 - 5.11 (m, 2 H), 4.36 - 4.43 (m, 1 H), 4.27 - 4.34 (m, 1 H), 3.92 - 3.98 (m, 2 H), 3.57 - 3.60 (m, 2 H), 3.53 - 3.55 (m, 2 H), 3.49 - 3.51 (m, 2 H), 3.44 - 3.47 (m, 3 H), 3.44 - 3.47 (m, 3 H), 3.07 - 3.12 (m, 3 H), 2.65 - 2.71 (m, 1 H), 2.31 - 2.36 (m, 1 H), 2.12 - 2.28 (m, 4 H), 2.06 - 2.10 (m, 1 H), 1.96 - 2.04 (m, 3 H), 1.86 - 1.94 (m, 2 H), 1.28 - 1.32 (m, 6 H), 1.23 - 1.26 (m, 3 H), 0.87 - 0.92 (m, 6 H), 0.80 - 0.83 (m, 3 H). **MS (ESI):** MS cal.: 1187.4, MS observed: $[M+H]^+$ = 1188.7. **Purity (HPLC, 254 nm)** = 96.2%

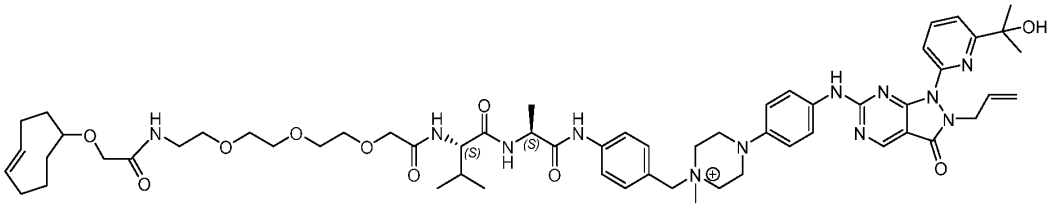
xxii) 4-((2S,5S)-20-(((E)-cyclooct-4-en-1-yl)oxy)-5-isopropyl-2-methyl-4,7,19-trioxo-9,12,15-trioxa-3,6,18-triazaicosanamido)benzyl (3-(2-(3-((5-cyanopyrazin-2-yl)amino)-1H-pyrazol-5-yl)-3-methoxyphenoxy)propyl)carbamate (LP-6)



LP-6

LP-6 was synthesized according to General Procedure 3B from **I18c**. **Yield:** 46%. **^{1}H NMR(400 MHz, DMSO-d_6)** *δ ppm* 12.31 - 12.25 (m, 1H), 10.68 (br s, 1H), 10.00 (s, 1H), 8.61 (s, 1H), 8.40 (d, *J* = 6.8 Hz, 1H), 7.56 (br d, *J* = 8.4 Hz, 2H), 7.49 - 7.43 (m, 2H), 7.34 - 7.21 (m, 4H), 6.91 (br d, *J* = 4.5 Hz, 1H), 6.74 (t, *J* = 8.6 Hz, 2H), 5.59 - 5.47 (m, 1H), 5.39 - 5.27 (m, 1H), 4.92 (s, 2H), 4.34 (s, 2H), 4.02 (br t, *J* = 5.8 Hz, 2H), 3.94 (s, 2H), 3.81 (s, 2H), 3.71 (d, *J* = 8.6 Hz, 2H), 3.59 - 3.50 (m, 8H), 3.42 - 3.39 (m, 2H), 3.26 - 3.21 (m, 2H), 3.19 - 3.15 (m, 2H), 3.05 - 2.99 (m, 1H), 2.33 - 2.15 (m, 4H), 2.05 - 1.97 (m, 2H), 1.90 - 1.83 (m, 4H), 1.78 - 1.70 (m, 3H), 1.48 - 1.40 (m, 2H), 1.30 (d, *J* = 7.1 Hz, 3H), 0.89 - 0.81 (m, 6H). **MS (ESI):** MS calculated: 1039.51, MS observed: $[M+H]^+$ = 1040.5. **Purity (HPLC, 254 nm)** = 96.4%

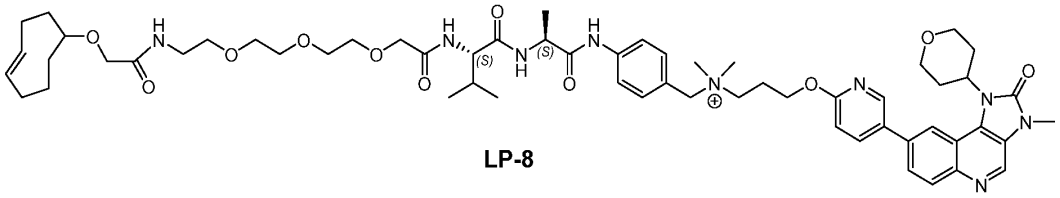
xxiii) 4-(4-((2-allyl-1-(6-(2-hydroxypropan-2-yl)pyridin-2-yl)-3-oxo-2,3-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)amino)phenyl)-1-(4-((2S,5S)-20-(((E)-cyclooct-4-en-1-yl)oxy)-5-isopropyl-2-methyl-4,7,19-trioxo-9,12,15-trioxa-3,6,18-triazaicosanamido)benzyl)-1-methylpiperazin-1-ium (LP-7)



LP-7

LP-7 was synthesized according to General Procedure 3B using **I19d**. **Yield:** 37%. **^{1}H NMR(400 MHz, DMSO-d_6)** δ ppm 10.23 - 10.57 (m, 1 H), 8.83 - 8.88 (m, 1 H), 8.69 - 8.81 (m, 1 H), 7.99 - 8.06 (m, 1 H), 7.73 - 7.80 (m, 3 H), 7.58 - 7.67 (m, 3 H), 7.44 - 7.54 (m, 4 H), 6.99 - 7.04 (m, 2 H), 5.61 - 5.72 (m, 1 H), 5.49 - 5.57 (m, 1 H), 5.28 - 5.37 (m, 2 H), 4.96 - 5.02 (m, 1 H), 4.78 - 4.86 (m, 1 H), 4.61 - 4.71 (m, 4 H), 4.26 - 4.43 (m, 2 H), 3.92 - 3.97 (m, 2 H), 3.64 - 3.73 (m, 4 H), 3.54 - 3.62 (m, 6 H), 3.50 - 3.53 (m, 4 H), 3.38 - 3.43 (m, 4 H), 3.21 - 3.25 (m, 3 H), 2.99 - 3.04 (m, 4 H), 2.31 - 2.34 (m, 1 H), 2.23 - 2.29 (m, 3 H), 2.15 - 2.20 (m, 1 H), 1.97 - 2.07 (m, 3 H), 1.83 - 1.92 (m, 3 H), 1.74 - 1.79 (m, 2 H), 1.46 (s, 6 H), 1.31 - 1.35 (m, 3 H), 0.81 - 0.90 (m, 6 H). **MS (ESI):** MS calculated: 1131.6, MS observed: $[M+H]^+$ = 1132.1. **Purity (HPLC, 254 nm):** 97.3%

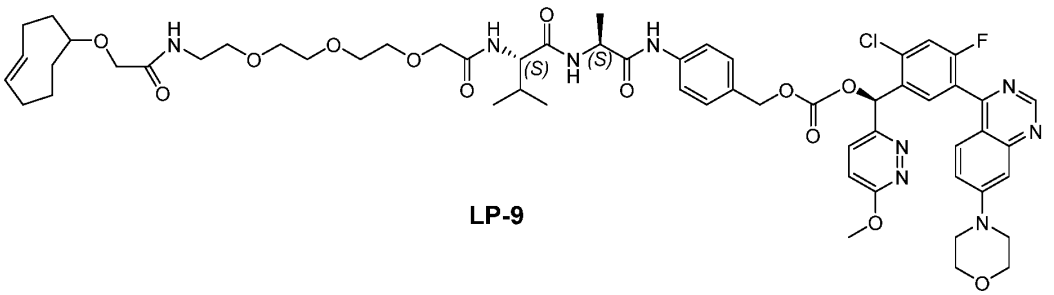
xxiv) N-(4-((2S,5S)-20-(((E)-cyclooct-4-en-1-yl)oxy)-5-isopropyl-2-methyl-4,7,19-trioxo-9,12,15-trioxa-3,6,18-triazaicosanamido)benzyl)-N,N-dimethyl-3-((5-(3-methyl-2-oxo-1-(tetrahydro-2H-pyran-4-yl)-2,3-dihydro-1H-imidazo[4,5-c]quinolin-8-yl)pyridin-2-yl)oxy)propan-1-aminium (LP-8)



LP-8

LP-8 was synthesized according to General Procedure 3B from **I19e**. **Yield:** 32% (obtained as a mixture of quaternary salts). **^{1}H NMR (400 MHz, DMSO-*d*$_6$)** δ ppm 10.17 - 10.33 (m, 1 H), 8.90 - 8.95 (m, 1 H), 8.69 (d, J=2.75 Hz, 1 H), 8.41 - 8.47 (m, 2 H), 8.22 - 8.27 (m, 1 H), 8.14 - 8.19 (m, 1 H), 7.95 - 8.00 (m, 1 H), 7.71 - 7.76 (m, 2 H), 7.39 - 7.50 (m, 4 H), 6.97 - 7.04 (m, 1 H), 5.47 - 5.57 (m, 1 H), 5.29 - 5.38 (m, 1 H), 5.11 - 5.21 (m, 1 H), 4.50 - 4.54 (m, 2 H), 4.42 - 4.46 (m, 2 H), 4.35 - 4.40 (m, 1 H), 4.27 - 4.32 (m, 1 H), 4.03 - 4.09 (m, 2 H), 3.92 - 3.96 (m, 2 H), 3.69 - 3.72 (m, 1 H), 3.51 - 3.58 (m, 10 H), 3.21 - 3.26 (m, 4 H), 2.97 - 3.00 (m, 6 H), 2.64 - 2.77 (m, 4 H), 2.30 - 2.38 (m, 4 H), 2.24 - 2.29 (m, 2 H), 2.14 - 2.21 (m, 1 H), 1.85 - 2.04 (m, 7 H), 1.69 - 1.77 (m, 2 H), 1.41 - 1.47 (m, 2 H), 1.30 - 1.34 (m, 3 H), 1.23 - 1.26 (m, 1 H), 0.87 - 0.90 (m, 3 H), 0.79 - 0.83 (m, 3 H). **MS (ESI):** MS calculated: 1092.6, MS observed: $[M+H]^+$ =1093.0. **Purity (HPLC, 254 nm)**: 99.9% (mixture of quaternary salts)

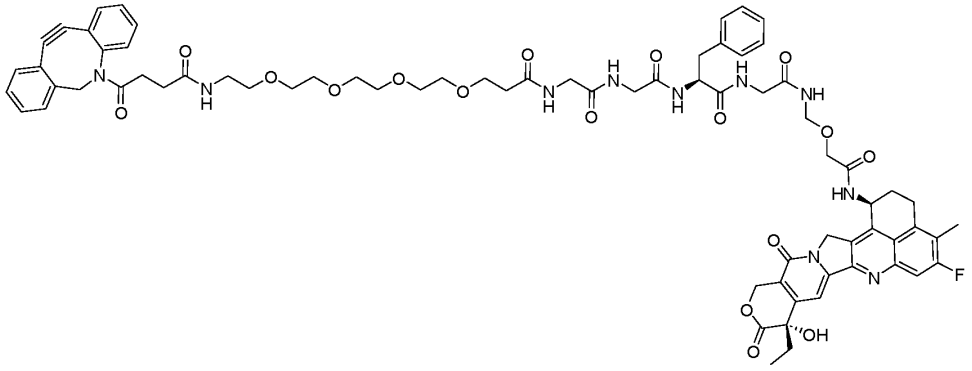
xxv) (S)-(2-chloro-4-fluoro-5-(7-morpholinoquinazolin-4-yl)phenyl)(6-methoxypyridazin-3-yl)methyl (4-((2S,5S)-20-(((E)-cyclooct-4-en-1-yl)oxy)-5-isopropyl-2-methyl-4,7,19-trioxo-9,12,15-trioxa-3,6,18-triazaicosanamido)benzyl) carbonate (LP-9)



LP-9

LP-9 was synthesized according to General Procedure 3B from **I19f. Yield:** 39%. **^{1}H NMR (400 MHz, DMSO-*d*$_6$)** δ ppm 10.04 (s, 1H), 9.11 (s, 1H), 8.40 (d, *J* = 6.8 Hz, 1H), 7.85 (d, *J* = 9.3 Hz, 1H), 7.83 - 7.77 (m, 2H), 7.60 - 7.55 (m, 2H), 7.53 (dd, *J* = 2.5, 6.6 Hz, 1H), 7.47 - 7.42 (m, 2H), 7.29 (dd, *J* = 8.9, 12.5 Hz, 3H), 7.20 (d, *J* = 1.9 Hz, 1H), 7.18 (s, 1H), 5.56 - 5.46 (m, 1H), 5.37 - 5.26 (m, 1H), 5.14 (s, 2H), 4.39 (t, *J* = 7.1 Hz, 1H), 4.29 (dd, *J* = 6.5, 8.9 Hz, 1H), 4.01 (s, 3H), 3.94 (s, 2H), 3.80 - 3.73 (m, 4H), 3.70 (d, *J* = 8.8 Hz, 2H), 3.62 - 3.50 (m, 8H), 3.46 - 3.38 (m, 6H), 3.26 - 3.20 (m, 2H), 3.01 (br dd, *J* = 3.2, 9.9 Hz, 1H), 2.31 - 2.13 (m, 3H), 2.06 - 1.95 (m, 2H), 1.91 - 1.80 (m, 2H), 1.78 - 1.70 (m, 2H), 1.48 - 1.39 (m, 2H), 1.30 (d, *J* = 7.0 Hz, 3H), 1.23 (s, 1H), 0.90 - 0.80 (m, 6H). **MS (ESI):** MS calculated: 1155.48, MS observed: $[M+H]^+$ = 1156.5. **Purity (HPLC, 254 nm):** 95.2%

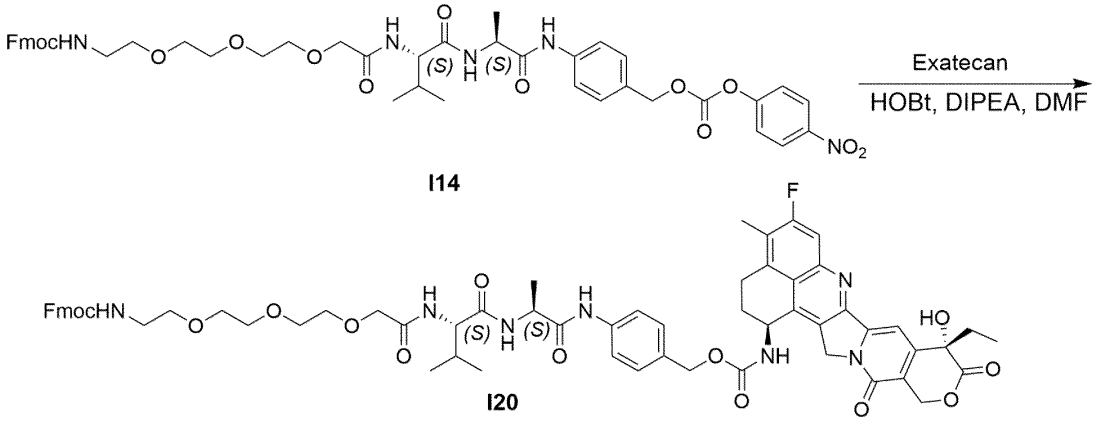
xxvi) N-[20-(11,12-Didehydrodibenzo[b,f]azocin-5(6H)-yl)-17,20-dioxo-4,7,10,13-tetraoxa-16-azaicosan-1-oyl]glycylglycyl-L-phenylalanyl-N-[(2-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)methyl]glycinamide (LP-10)



N-[20-(11,12-Didehydrodibenzo[b,f]azocin-5(6H)-yl)-17,20-dioxo-4,7,10,13-tetraoxa-16-azaicosan-1-oyl]glycylglycyl-L-phenylalanyl-N-[(2-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3′,4′:6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)methyl]glycinamide) is available commercially (CAS No. : 2694856-51-2) from suppliers such as Key Organics Ltd, UK.

Example 6 – Synthesis of Linker-payload molecule LP-11

i) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (I20)

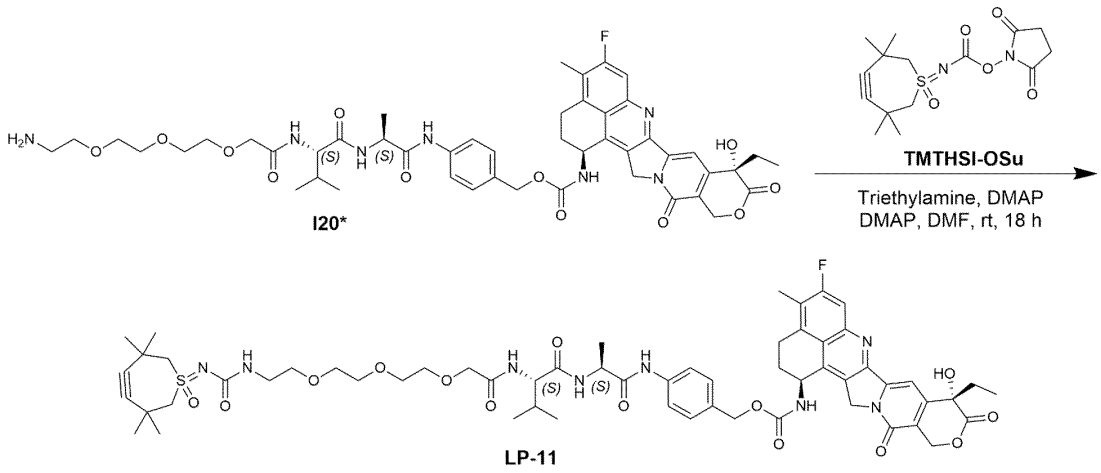


To a solution of compound **I14** (2.5 g, 2.8 mmol, 1.0 eq.) and Exatecan (1.49 g, 2.8 mmol, 1.0 eq.) in DMF (25 mL) was added HOBt (416 mg, 3.08 mmol, 1.1 eq.) and DIPEA (725 mg, 5.61 mmol, 927 µL, 2.0 eq.). The mixture was stirred at 25 °C for 2 h. LCMS analysis indicated the completion of the reaction. Then isopropyl ether (25 mL) was added to the reaction mixture and the precipitate formed was collected by centrifugation to obtain compound **I20** (4.0 g, crude) as a brown oil and directly used for the next step.
MS (ESI): MS calculated: 1165.4, MS observed: $[M+H]^+$ = 1166.1

ii) 4-((2S,5S)-17-amino-5-isopropyl-2-methyl-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanamido)benzyl ((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamate (I20)*

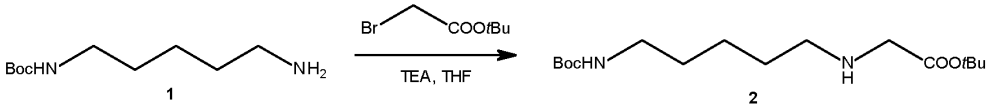
To a solution of compound **I20** (4.0 g, 1.0 eq.) in DMF (28 mL) was added triethylamine (12 mL). The mixture was stirred at 25 °C for 12 h. Upon completion, isopropyl ether (400 mL) was added to the reaction mixture and centrifuged to obtain the intermediate amine **I20*** (4g, crude). The crude product was characterized using LC-MS analysis and used directly for the next step. MS (ESI): MS calculated: 943.4, MS observed: [M+H]+ = 944.2.

iii) [4-[[(2S)-2-[[(2S)-3-methyl-2-[[2-[2-[2-[2-[(3,3,6,6-tetramethyl-1-oxo-1λ⁶-thiacyclohept-4-yn-1-ylidene)carbamoylamino]ethoxy]ethoxy]ethoxy]acetyl]amino]butanoyl]amino]propanoyl]amino]phenyl]methyl N-[(10S,23S)-10-ethyl-18-fluoro-10-hydroxy-19-methyl-5,9-dioxo-8-oxa-4,15-diazahexacyclo[14.7.1.0^{2,14}.0^{4,13}.0^{6,11}.0^{20,24}]tetracosa-1,6(11),12,14,16(24),17,19-heptaen-23-yl]carbamate (LP-11):

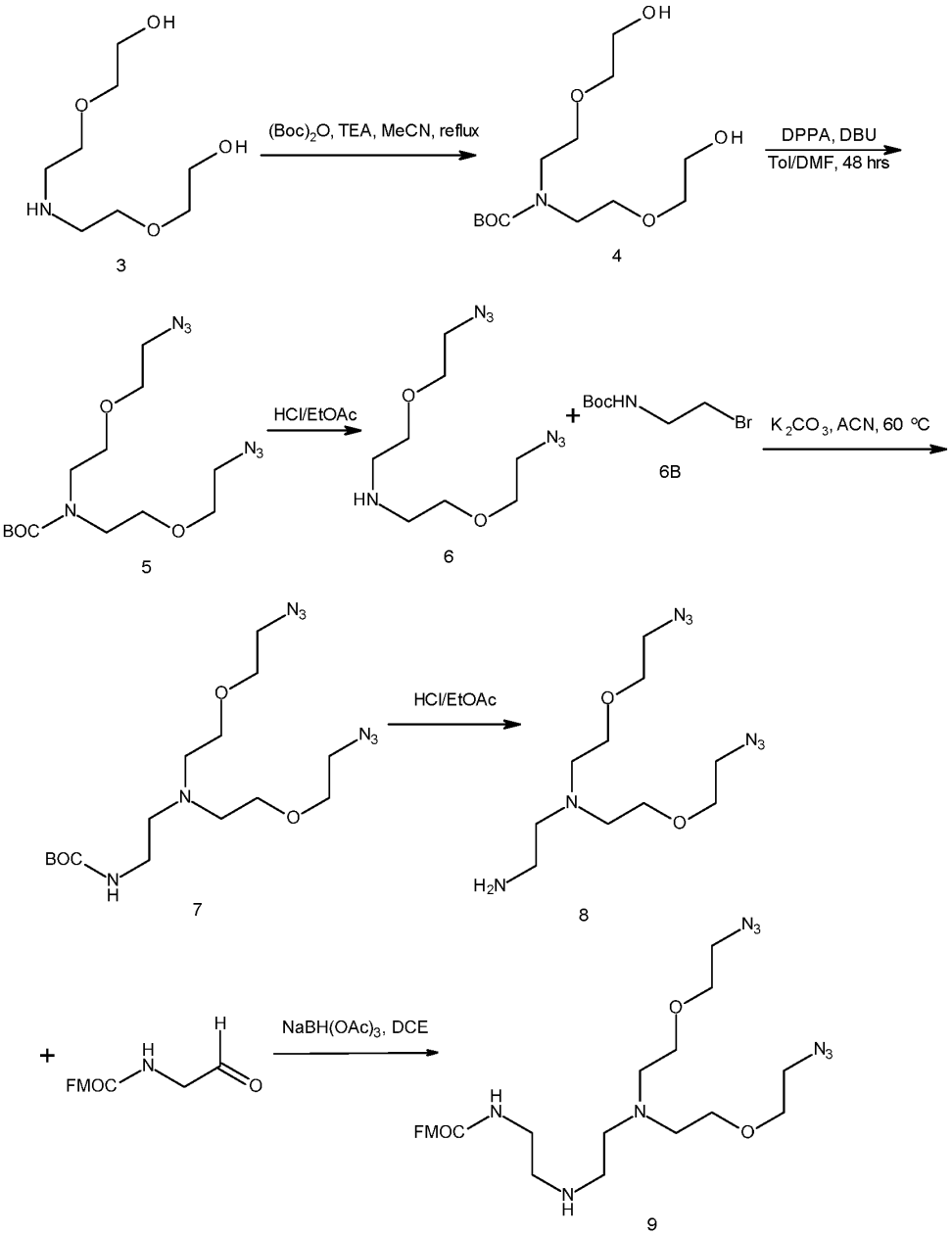


Triethylamine (23.59 µL, 37.2 µmol, 2.5 equiv.) and DMAP (10.5 mg, 85.95 µmol, 0.81 equiv.) were added successively to a solution of compound **I20*** (100 mg, 105.9 µmol, 1.0 equiv.) and compound TMTHSI-OSu (37.86 mg, 111.2 µmol, 1.05 equiv.) in DMF (1 mL). The mixture was stirred at 25 °C for 18 h. Upon completion (as observed by LC-MS analysis), the solvent was removed under reduced pressure and the residue was purified by prep-HPLC to give **LP-11** (23 mg) as a yellow solid. **Purity by HPLC (220 nm)**: 95.6%, Rt = 10.720 min; **MS (ESI)**: $[(M+H)]^+$=1169.4, $[(M+2H)]^{2+}$=585.5. **^{1}H NMR (400 MHz, DMSO-d_6)** δ ppm 9.99 (s, 1 H), 8.37 (d, J=7.00 Hz, 1 H), 8.06 (d, J=8.63 Hz, 1 H), 7.78 (d, J=10.38 Hz, 1 H), 7.59 (d, J=8.63 Hz, 2 H), 7.44 (d, J=9.13 Hz, 1 H), 7.36 (d, J=8.00 Hz, 2 H), 7.31 (s, 1 H), 6.52 (d, J=6.63 Hz, 2 H), 5.45 (s, 2 H), 5.29 (br s, 3 H), 5.08 (s, 2 H), 4.29 - 4.39 (dd, J=8.63, 6.88 Hz, 2 H), 3.95 (s, 2 H), 3.84 - 3.87 (d, 2 H), 3.42 - 3.65 (m, 10 H), 3.07 (d, J=5.25 Hz, 2 H), 2.89 (s, 1 H), 2.73 (s, 1 H), 2.38 (d, J=1.13 Hz, 4 H), 2.15 - 2.24 (m, 2 H), 1.95 - 2.04 (m, 2 H), 1.82 - 1.92 (m, 2 H), 1.28 - 1.34 (m, 12 H), 1.18 (s, 3 H), 0.88 (d, J=7.13 Hz, 6 H), 0.83 (d, J=6.63 Hz, 3 H).

Example 7 – Synthetic route to Linker 1



a) Compound 2 may be made from compound 1 by coupling tert-butyl 2-bromoacetate, for example using triethanolamine (TEA) in THF.



b) Compound 4 – Boc protection

Compound 4 may be made from compound 3 by treatment with Boc_2O and a base (e.g. TEA) in anhydrous conditions, such as in acetonitrile.

c) Compound 5 – Alcohol to azide SN2 conversion

Compound 5 may be made from compound 4 by treating with diphenylphosphoryl azide (DPPA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in a dry aprotic solvent (such as toluene/DMF), for example, for 48 hours.

d) Compound 6 – Boc deprotection

Compound 6 may be made from compound 5 by removal of the Boc group, for example using HCl in ethyl acetate.

e) Compound 7

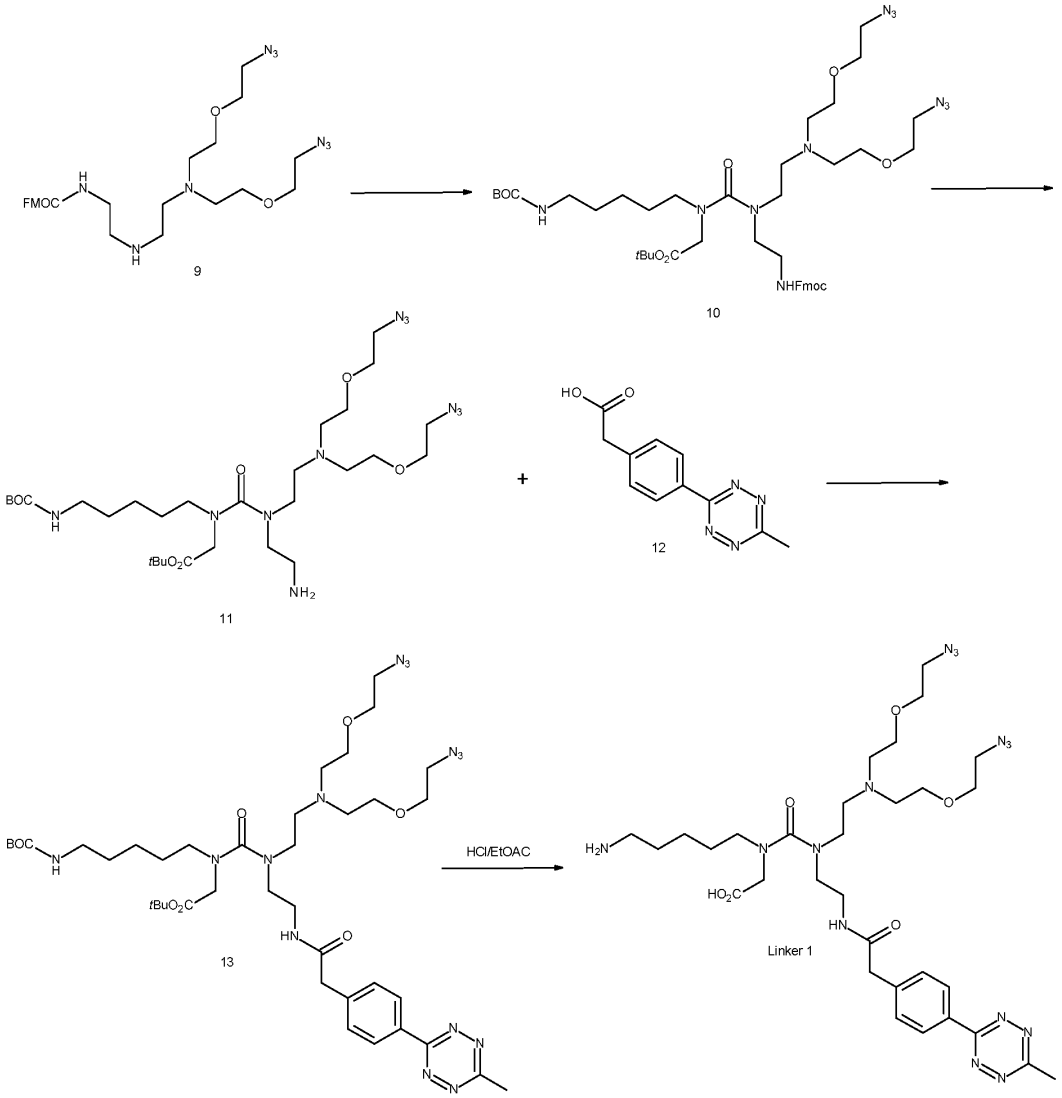
Compound 7 may be made from compound 6 by linking compound 6B, for example using potassium carbonate in acetonitrile, such as at 60°C.

f) Compound 8

Compound 8 may be made from compound 7 by removal of the Boc group, for example using HCl in ethyl acetate.

g) Compound 9

Compound 9 may be made from compound 8 by linking 9H-fluoren-9-ylmethyl N-(2-oxoethyl)carbamate, for example using $NaBH(OAc)_3$ in DCE.



h) Compound 10

Compound 10 may be made from compound 9 by using a urea formation reaction with Compound 2.

i) Compound 11

Compound 11 may be made from compound 10 by removal of the Fmoc group.

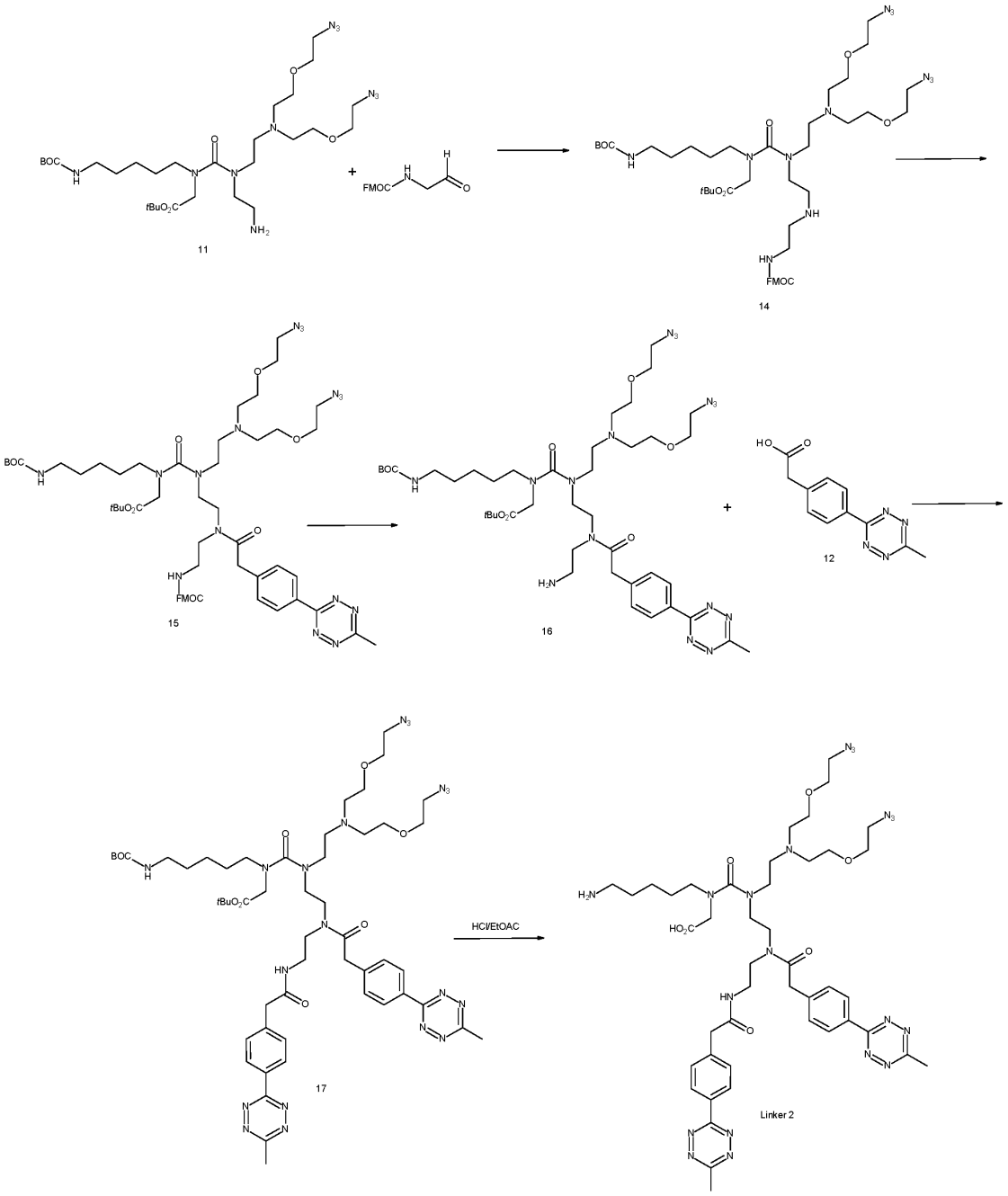
j) Compound 13

Compound 13 may be made from compound 11 by coupling 2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetic acid.

k) Linker 1

Linker 1 may be made from compound 13 by removal of the Boc and t-Butoxy groups, for example using HCl in ethyl acetate.

Example 8 – Synthetic Route to Linker 2



a) Compound 14

Compound 14 may be made from compound by linking 9H-fluoren-9-ylmethyl N-(2-oxoethyl)carbamate, for example using $NaBH(OAc)_3$ in DCE

b) Compound 15

Compound 15 may be made from compound 14 by coupling 2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetic acid.

c) Compound 16

Compound 16 may be made from compound 15 by removal of the Fmoc protecting group

d) Compound 17

Compound 17 may be made from compound 16 by coupling 2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetic acid.

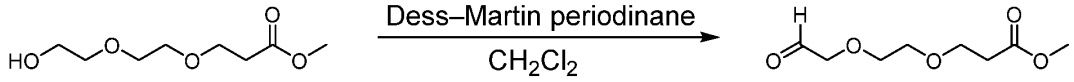
e) Linker 2

Linker 2 may be made from compound 17 by removal of the Boc and t-Butoxy groups, for example using HCl in ethyl acetate.

Example 9 –Synthesis of linkers 3 and 4

i) Synthesis of common intermediate AA_9:

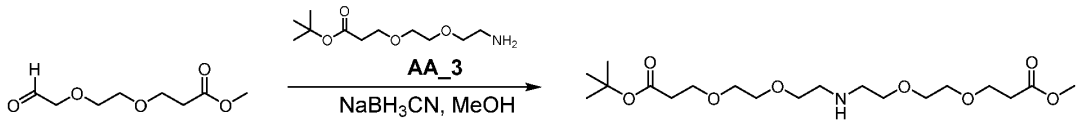
i (a) Methyl 3-(2-(2-oxoethoxy)ethoxy)propanoate (Compound AA_2)



AA_1 **AA_2**

To a solution of compound **AA_1** (37 g, 192 mmol, 1.0 eq.) in CH_2Cl_2 (100 mL) was added a solution of Dess-Martin periodinane (DMP) (122 g, 288 mmol, 1.5 eq.) in CH_2Cl_2 (200 mL). The mixture was stirred at 25 °C for 4 h. Then the reaction contents were poured into saturated aqueous $NaHCO_3$ (100 mL) and extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with saturated aqueous $Na_2S_2O_3$ (100 mL) and brine (3 x 50 mL), dried over anhydrous Na_2SO_4, and concentrated to give a residue. The residue was triturated with MeOH (200 mL) for 2 h, and the filtrate was concentrated to give compound **AA_2** (>38 g, crude) as a yellow oil. The crude material was used directly for the next step without any further purification.

i (b) 1-(tert-butyl) 19-methyl 4,7,13,16-tetraoxa-10-azanonadecanedioate (Compound AA_4)

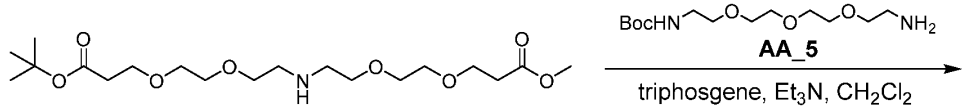


AA_2 **AA_4**

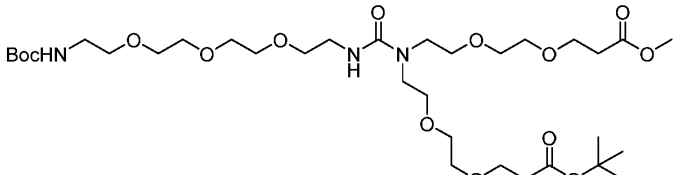
To a solution of compound **AA_2** (36.6 g, 194 mmol, 1.0 eq.) in MeOH (500 mL) was added compound **AA_3** (68 g, 291 mmol, 1.5 eq.), AcOH (16.7 mL, 291 mmol, 1.5 eq.) and $NaBH_3CN$ (24.4 g, 389 mmol, 2.0 eq.). The mixture was stirred at 25 °C for 4 h. Then the reaction mixture was concentrated under reduced pressure and purified by preparative HPLC to afford compound **AA_4** (24 g, 58.9 mmol, 30% yield) as a yellow oil. **^{1}H NMR (400 MHz, $CDCl_3$)** δ ppm 3.84 (br t, J = 4.14 Hz, 2 H), 3.71 - 3.80 (m, 6 H),

3.70 (m, 4 H), 3.66 (br d, *J* = 2.51 Hz, 4 H), 3.62 (s, 3 H), 3.57 (d, *J* = 5.27 Hz, 1 H), 3.49 (s, 1 H) 3.47 (d, *J* = 4.77 Hz, 1 H), 3.30 (br s, 2 H), 2.58 - 2.65 (m, 2 H), 2.51 (m, 2 H), 1.46 (s, 9 H). **MS (ESI)**: MS calculated: 407, MS observed: $[M+H]^+$ = 408.

i (c) 1-(tert-butyl) 19-methyl 10-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)carbamoyl)-4,7,13,16-tetraoxa-10-azanonadecanedioate (Compound AA_6)



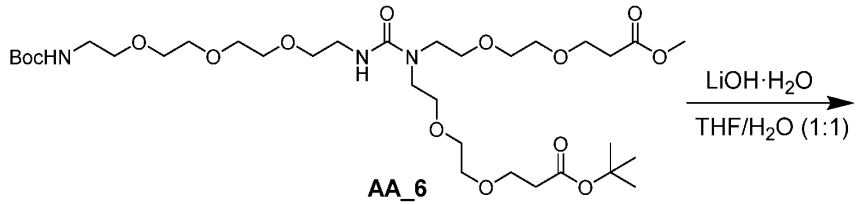
AA_4



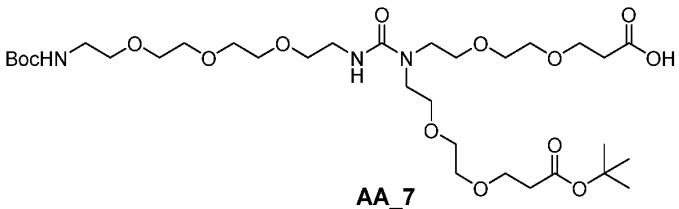
AA_6

A solution of compound **AA_5** (24.7 g, 84.6 mmol, 1.5 eq.) and triethylamine (47 mL, 338 mmol, 6.0 eq.) in CH_2Cl_2 (200 mL) was added slowly to an ice-cold solution of triphosgene (12.5 g, 42.3 mol, 0.70 eq.) in CH_2Cl_2 (600 mL). The mixture was stirred at 0 °C for 1 h. Then amine **AA_4** (23 g, 56.4 mmol, 1.0 eq) in CH_2Cl_2 (100 mL) was added slowly at 0 °C for over 10 min. The mixture was stirred at 0 °C for another 2 h. The reaction mixture was poured into saturated aqueous $NaHCO_3$ (100 mL) and extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with brine (3 x 50 mL), dried over anhydrous Na_2SO_4, concentrated and purified by prep-HPLC to afford compound **AA_6** (22 g, 54% yield) as a yellow solid. **^{1}H NMR (400 MHz, $CDCl_3$)** δ ppm 5.94 - 6.06 (m, 1 H), 5.08 (br s, 1 H), 3.75 (t, *J* = 6.40 Hz, 2 H), 3.70 (s, 3 H), 3.58 - 3.67 (m, 22 H), 3.55 (br d, *J* = 4.77 Hz, 4 H), 3.47 (t, *J* = 5.02 Hz, 4 H), 3.34 - 3.39 (m, 2 H), 3.29 - 3.34 (m, 2 H), 2.57 - 2.64 (m, 2 H) ,2.48 - 2.53 (m, 2 H), 1.46 (d, *J* = 1.51 Hz, 18 H). **LCMS**: MS calculated: 725, MS observed: $[M+H]^+$ = 726.

i (d) 19-(2-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)ethyl)-2,2-dimethyl-4,18-dioxo-3,8,11,14,22,25-hexaoxa-5,17,19-triazaoctacosan-28-oic acid (Compound AA_7):



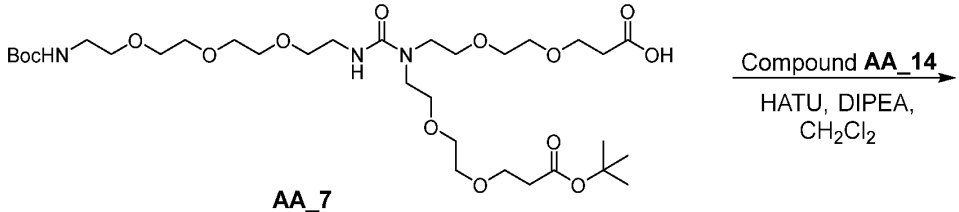
AA_6



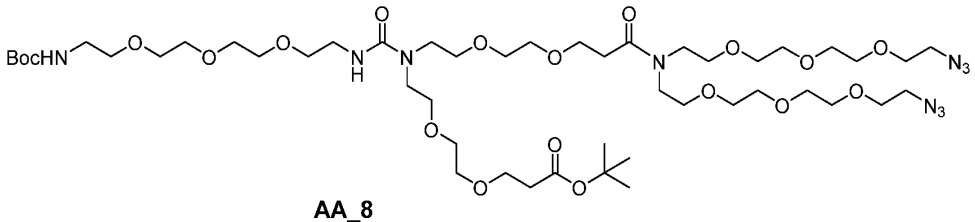
AA_7

To a solution of compound **AA_6** (22 g, 30.3 mmol, 1.0 eq.) in THF/H_2O (1:1, 220 mL) was added $LiOH·H_2O$ (1.9 g, 45.4 mmol, 1.5 eq.). The mixture was stirred at 0 °C for 2 h. Upon completion, the reaction was acidified with 1N HCl to pH 3 and extracted with CH_2Cl_2 (3 x 500 mL). The combined organic phase was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to furnish compound **AA_7** (20 g, crude) as a white solid that was used without further purification. **LCMS**: MS calculated: 711, MS observed: $[M+H]^+$ = 712.

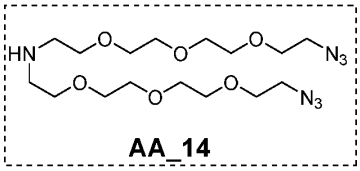
i (e) tert-butyl 1-azido-12-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-22-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)carbamoyl)-13-oxo-3,6,9,16,19,25,28-heptaoxa-12,22-diazahentriacontan-31-oate (Compound AA_8)



AA_7



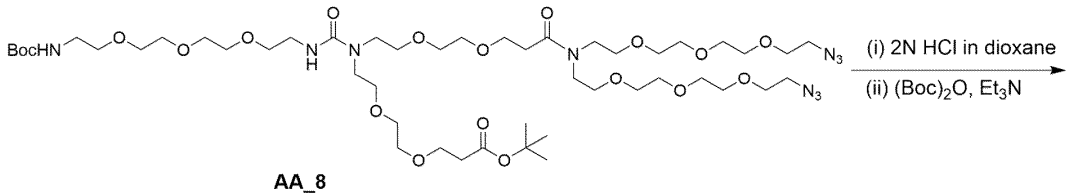
AA_8



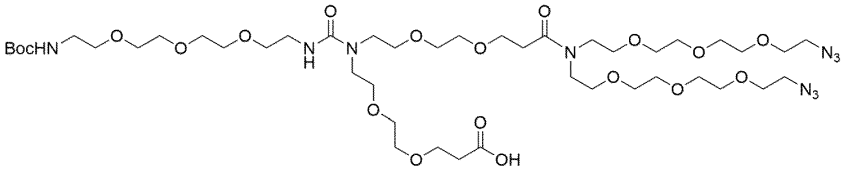
AA_14

To a solution of compound **AA_7** (20 g, 30.9 mol, 1.0 eq.) in CH_2Cl_2 (200 mL) was added compound **AA_14** (12.9 g, 30.9 mmol, 1.1 eq.), HATU (14 g, 42.1 mmol, 1.5 eq.) and DIPEA (6.96 g, 42.1 mmol, 1.5 eq.). The mixture was stirred at 25 °C for 2 h. Then it was added to H_2O (100 mL) and extracted with CH_2Cl_2 (3 x 100 mL). The combined organic phase was washed with brine (50 mL x 3), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford compound **AA_8** (26 g, crude) as a yellow solid and used for the next step without further purification. **^{1}H NMR (400 MHz, $CDCl_3$)** δ ppm 6.01 - 6.16 (m, 1 H), 5.02 - 5.26 (m, 1 H), 3.76 - 3.82 (m, 4 H), 3.53 - 3.72 (m, 56 H), 3.44 - 3.52 (m, 4 H), 3.41 (br s, 4 H), 2.67 - 2.77 (m, 2 H) ,2.54 - 2.61 (m, 2 H) ,1.41 - 1.47 (m, 18 H).

i (f) 1-azido-12-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-22-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)carbamoyl)-13-oxo-3,6,9,16,19,25,28-heptaoxa-12,22-diazahentriacontan-31-oic acid (Compound AA_9)



AA_8

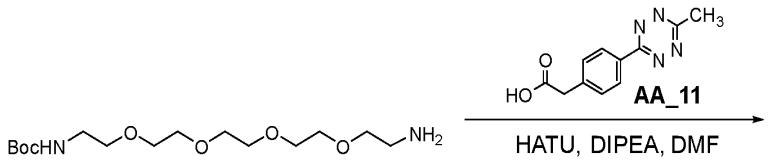


AA_9

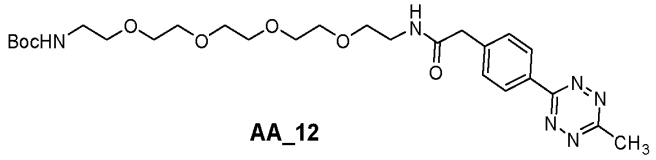
A solution of compound **AA_8** (26 g, 32.3 mmol, 1.0 eq.) in 2N HCl in 1,4-dioxane (260 mL) was stirred at 0 °C for 2 h. Then the reaction mixture was concentrated under reduced pressure to give intermediate acid (25 g, crude) that was redissolved in CH_2Cl_2 (250 mL) followed by the addition of $(Boc)_2O$ (8.55 g, 39.1 mmol, 1.5 eq.) and triethylamine (5.51 mL, 39.1 mmol, 1.5 eq.). The mixture was stirred at 25 °C for 2 h. Then the reaction mixture was concentrated under reduced pressure and further purified by silica gel column chromatography (CH_2Cl_2/MeOH=20:1,) to afford compound **AA_9** (18.6 g, 17.5 mmol, 58% yield for 2 steps) as a white solid. **^{1}H NMR (400 MHz, DMSO-d_6)** δ ppm 6.63 - 6.81 (m, 1 H), 6.22 - 6.31 (m, 1 H), 5.76 (s, 1 H), 3.34 - 3.61 (m, 64 H), 3.05 (s, 4 H), 2.57 - 2.62 (m, 2 H), 2.44 (t, J = 6.27 Hz, 2 H), 1.38 (s, 9 H). **MS (ESI):** calculated: 1057, MS observed: $[M+H]^+$ =1058.

ii) Synthesis of intermediates AA_13 and AA_18

ii (a) tert-butyl (1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12,15-tetraoxa-3-azaheptadecan-17-yl)carbamate (Compound AA_12)



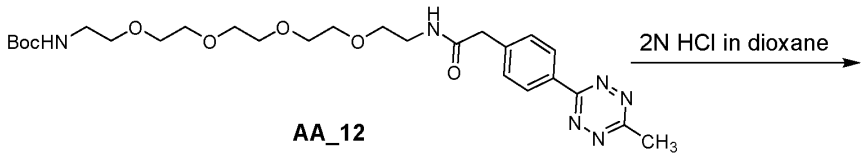
AA_10



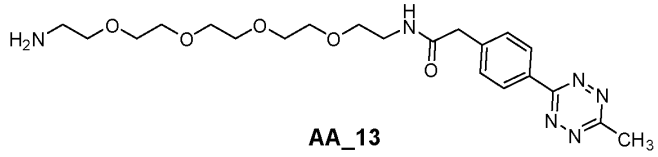
AA_12

To a solution of compound **AA_11** (6.7 g, 29 mmol, 1.0 eq.) in DMF (67 mL) was added DIPEA (13.1 mL, 79.4 mmol, 2.73 eq.), HATU (30.2 g, 79.4 mmol, 2.73 eq.) and compound **AA_10** (13.4 g, 39.68 mmol, 1.36 eq.). The mixture was stirred at 25 ℃ for 2 h. After completion, the reaction mixture was concentrated under reduced pressure and purified by preparative HPLC to afford compound **AA_12** (10 g, 63% yield) as a pink solid. **MS (ESI)**: Exact mass: 548.3, MS observed: $[M+H]^+$ = 549.1

ii (b) N-(14-amino-3,6,9,12-tetraoxatetradecyl)-2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetamide (Compound AA_13)



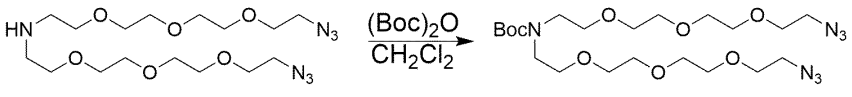
AA_12



AA_13

To compound **AA_12** (10 g, 18.2 mmol, 1.0 eq.) was added 2N HCl in 1,4-dioxane (100 mL). The reaction mixture was stirred at 25 ℃ for 2 h. Then the solvent was concentrated under reduced pressure to give compound **AA_13** (8.1 g, 84% yield) as a pink solid which was used without further purification. **MS (ESI):** Exact mass: 448.3, MS observed: $[M+H]^+$ = 449.

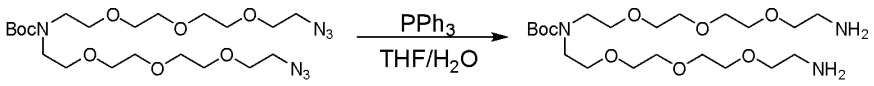
ii (c) tert-butyl bis(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)carbamate (Compound AA_15)



AA_14 **AA_15**

To a solution of compound **AA_14** (12 g, 28.6 mmol, 1.0 eq.) in CH_2Cl_2 (120 mL) was added $(Boc)_2O$ (12.5 g, 57.2 mmol, 2.0 eq.) and triethylamine (9.9 mL, 71.2 mmol, 2.5 eq.). The mixture was stirred at 25 °C for 15 h. After completion the reaction mixture was diluted with water (200 mL) and extracted with CH_2Cl_2 (3 x 200 mL). The combined organic layers were washed with brine (3 x 50.0 mL), dried over anhydrous Na_2SO_4, filtered, concentrated and purified by flash silica gel chromatography to furnish compound **AA_15** (13.6 g, 87% yield) as a colourless oil. **^{1}H NMR (400 MHz, $CDCl_3$)** δ ppm 3.53 - 3.72 (m, 24 H), 3.32 - 3.49 (m, 8 H), 1.42 - 1.48 (m, 9 H). **MS (ESI)**: MS calculated: 519.6, MS observed: $[M+H]^+$ = 520.2

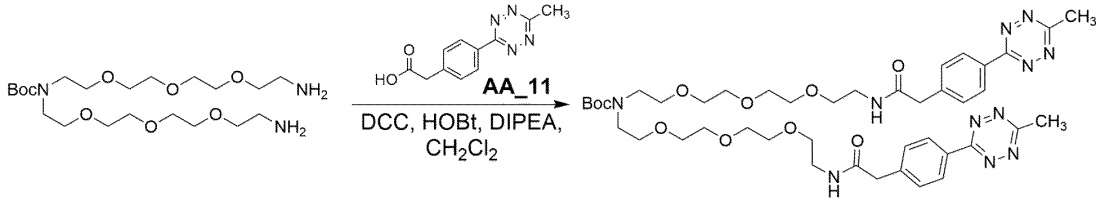
ii (d) tert-butyl bis(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)carbamate (Compound AA_16)



AA_15 **AA_16**

To a solution of compound **AA_15** (13.6 g, 26.2 mmol, 1.0 eq.) in THF (100 mL) was added triphenylphosphine (20.6 g, 78.7 mmol, 3.0 eq.) and the reaction mixture was stirred at 25 °C for 3 h. Then H_2O (100 mL) was added, and the mixture was stirred at 80 °C for further 12 h. Upon completion, the reaction was diluted with CH_2Cl_2 (500mL) and stirred for 5 min. The organic layer was discarded, and the aqueous layer was concentrated and lyophilized to afford compound **AA_16** (12 g, crude) as a colourless oil and used without further purification. **^{1}H NMR (400 MHz, D_2O)** δ ppm 1.38 - 1.45 (m, 9 H), 3.09 - 3.17 (m, 4 H), 3.39 - 3.49 (m, 4 H), 3.61 - 3.73 (m, 24 H). **LCMS**: MS calculated: 467.6, MS observed: $[M+H]^+$ = 468.3.

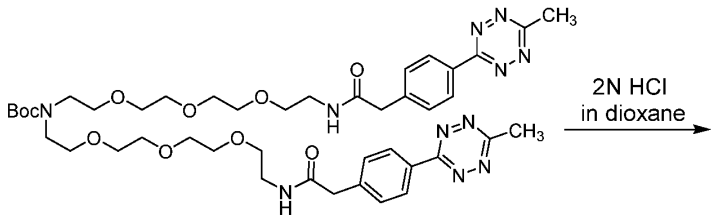
ii (e) tert-butyl bis(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)carbamate (Compound AA_17)



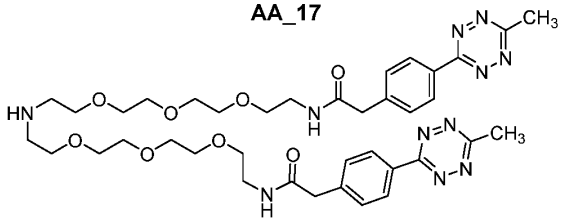
AA_16 **AA_17**

To a solution of compound **AA_16** (1.8 g, 3.85 mmol, 1.0 eq.) in CH_2Cl_2 (40 mL) was added compound **AA_11** (2.21 g, 9.63 mmol, 2.5 eq.), DCC (1.98 g, 9.63 mmol, 2.5 eq.), HOBt (1.56 g, 11.5 mmol, 3.0 eq.) and DIPEA (3.82 mL, 23.1 mmol, 6.0 eq.). The reaction mixture was stirred for 6 h at room temperature. Upon completion, the contents were diluted with CH_2Cl_2 (200 mL) and washed with water (400 mL). The aqueous layer was extracted with CH_2Cl_2 (2 x 200 mL). The combined organic layers were washed with brine (3 x 50 mL), dried over anhydrous Na_2SO_4, filtered, concentrated and purified by flash silica gel chromatography to give compound **AA_17** (2.79 g, 81% yield) as a purple oil. **^{1}H NMR (400 MHz, $CDCl_3$):** δ ppm 8.51 - 8.57 (m, 4 H), 7.50 - 7.55 (m, 4 H), 3.65 - 3.69 (m, 4 H), 3.53 - 3.61 (m, 24 H), 3.47 (s, 8 H), 3.08 - 3.12 (m, 6 H), 1.42 - 1.46 (m, 9 H). **MS (ESI)**: MS calculated: 891.5, MS observed: $[M+Na]^+$ = 914.7.

ii (f) N,N'-(3,6,9,15,18,21-hexaoxa-12-azatricosane-1,23-diyl)bis(2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetamide) (Compound AA_18)



AA_17

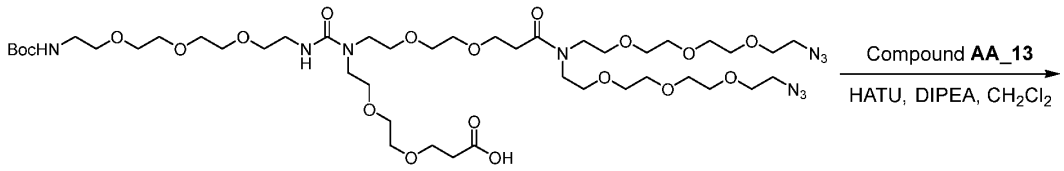


AA_18

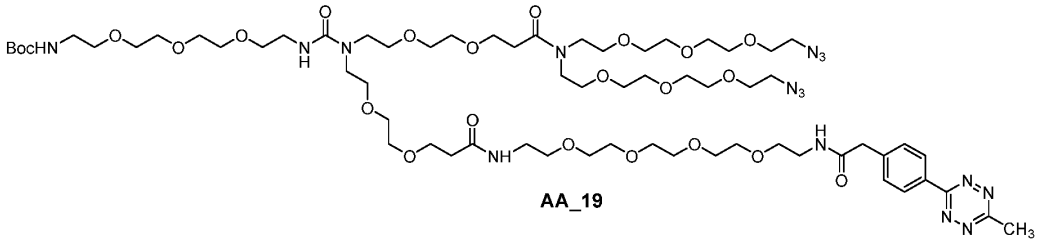
Compound **AA_17** (1.41 g, 1.59 mmol, 1.0 eq.) was taken in 2N HCl in 1,4-dioxane (15 mL) and the mixture was stirred at 25 °C for 2 h. Upon completion, the reaction mixture was concentrated under reduced pressure to afford compound **AA_18** (1.3 g, crude) as a purple oil and used without any further purification.

iii) Synthesis of linkers 3 and 4

iii (a) tert-butyl (14-(1-azido-12-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-13-oxo-3,6,9,16,19-pentaoxa-12-azahenicosan-21-yl)-41-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-13,23,40-trioxo-3,6,9,17,20,27,30,33,36-nonaoxa-12,14,24,39-tetraazahentetracontyl)carbamate (Compound AA_19)



AA_9

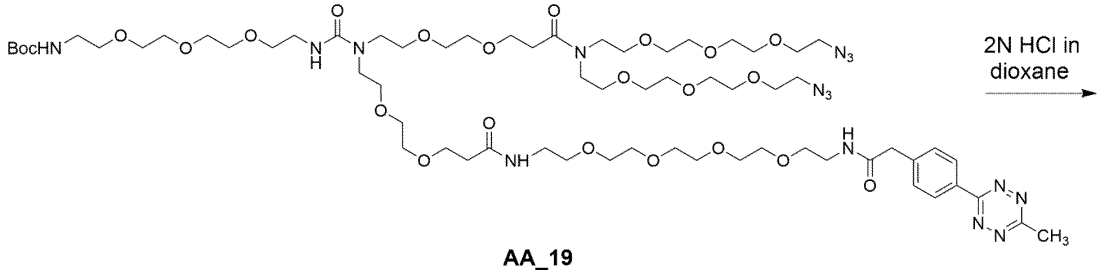


AA_19

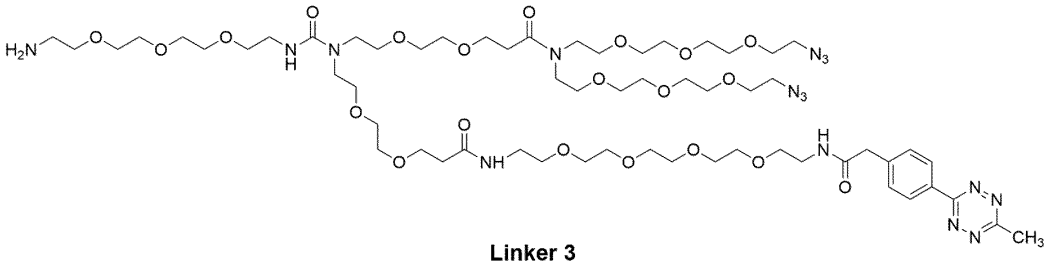
To a solution of compound **AA_9** (7.7 g, 7.28 mmol, 1.0 eq.) in CH_2Cl_2 (80 mL) was added compound **AA_13** (3.92 g, 8.7 mmol, 1.2 eq.), HATU (4.14 g, 10.9 mmol, 1.5 eq.) and DIPEA (2.41 mL, 14.5 mmol, 2.0 eq.). The mixture was stirred at 25 °C for 30 min. Then the reaction mixture was concentrated and

passed through a silica plug to give compound **AA_19** (10 g, crude) as a purple solid and used directly for the next step. **LCMS**: MS calculated: 1487, MS observed: $[M+H]^+$ =1488.

iii (b) 10-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)carbamoyl)-N1,N1-bis(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-N19-(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12,15-tetraoxa-3-azaheptadecan-17-yl)-4,7,13,16-tetraoxa-10-azanonadecanediamide (Linker 3)



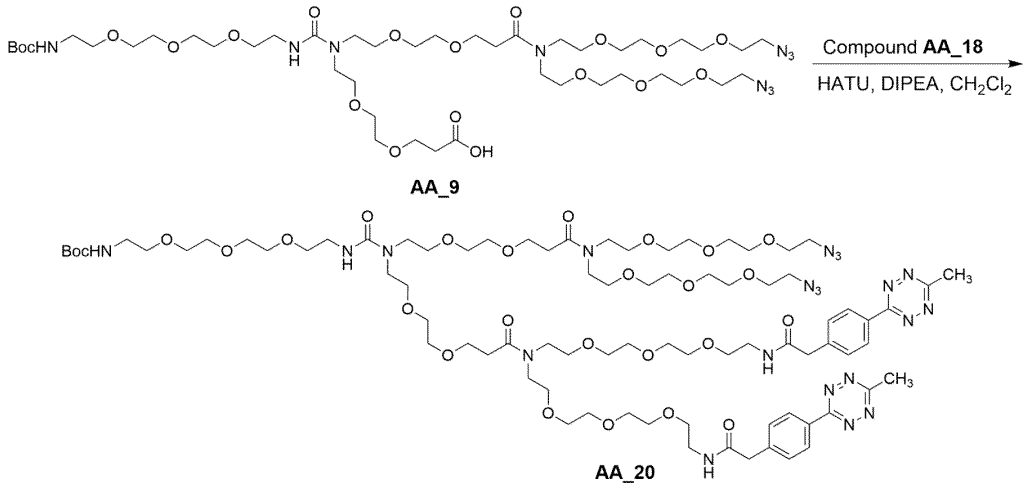
AA_19



Linker 3

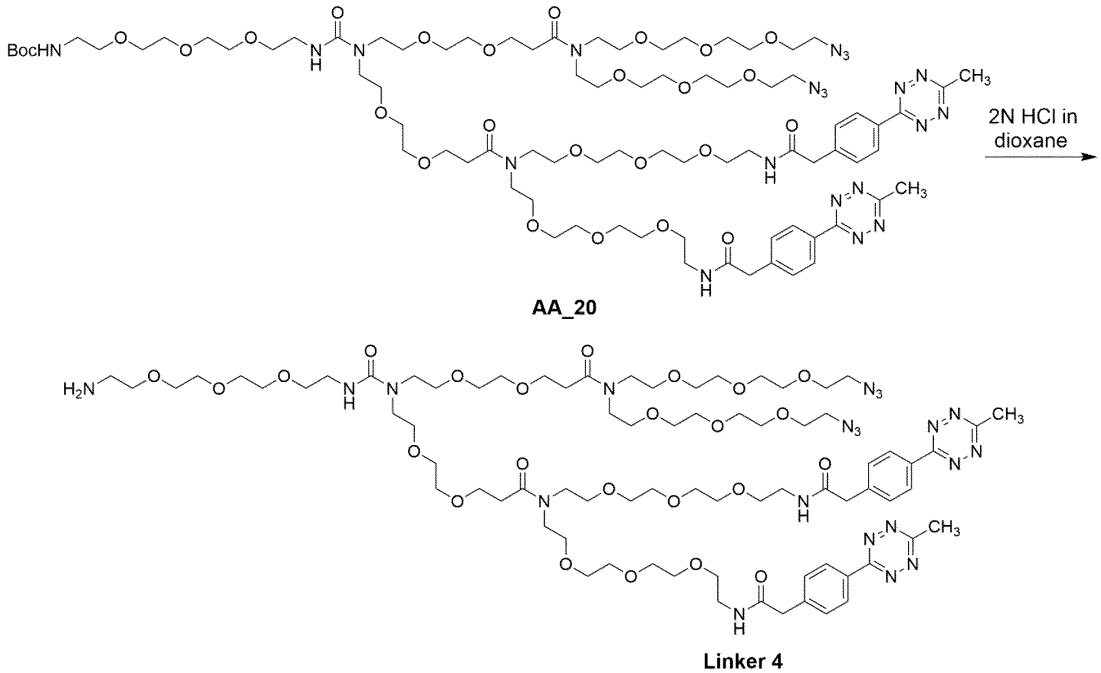
A solution of compound **AA_19** (10 g, 6.7 mmol, 1.0 eq.) in 2N HCl in 1,4-dioxane (90 mL) was stirred at 0 °C for 30 min. Then the reaction mixture was concentrated under reduced pressure and purified by preparative HPLC (0.01% TFA) to afford **linker 3** (3.9 g, 42%) as a purple solid after lyophilization. **^{1}H NMR (400 MHz, DMSO-d_6)** δ ppm7.90 (s, 1 H), 7.69 - 7.86 (m, 3 H), 7.54 (d, J = 8.28 Hz, 2 H), 6.28 (br t, J = 5.52 Hz, 1 H), 3.33 - 3.65 (m, 82 H), 3.11 - 3.27 (m, 6 H), 3.00 (s, 3 H), 2.98 (br s, 2 H), 2.60 (s, 2 H), 2.32 (t, *J* = 6.53 Hz, 2 H). **MS (ESI)**: MS calculated: 1836, MS observed: $[M+H]^+$ = 1837. **HPLC purity (254 nm)** = 98.6%

iii (c) tert-butyl (14-(1-azido-12-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-13-oxo-3,6,9,16,19-pentaoxa-12-azahenicosan-21-yl)-38-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-24-(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)-13,23,37-trioxo-3,6,9,17,20,27,30,33-octaoxa-12,14,24,36-tetraazaoctatriacontyl)carbamate (Compound AA_20)



To a solution of compound **AA_9** (7.7 g, 7.28 mmol, 1.0 eq.) in CH_2Cl_2 (77 mL) was added compound **AA_18** (7.25 g, 8.75 mmol, 1.2 eq.), HATU (4.16 g, 10.9 mmol, 1.5 eq.) and DIPEA (2.41 mL, 14.6 mmol, 2.00 eq.). The mixture was stirred at 25 °C for 30 min. Then the reaction mixture was concentrated under reduced pressure and the resulting residue was purified by silica plug column to give compound **AA_20** (14.7 g, crude) as a purple solid and used directly without further purification. **^{1}H NMR (400 MHz, DMSO-*d*$_6$)** δ ppm 8.57 - 8.60 (m, 1 H), 8.39 (d, J = 8.28 Hz, 4 H), 8.23 (br s, 2 H), 7.53 (d, J = 8.28 Hz, 4 H), 6.73 (br s, 1 H), 3.37 - 3.59 (m, 96 H), 3.23 (br d, J = 5.77 Hz, 4 H), 3.01 - 3.11 (m, 4 H), 2.99 (s, 6 H), 2.58 (br t, J = 6.78 Hz, 4 H), 1.35 - 1.38 (m, 9 H). **LCMS**: MS calculated: 1831, MS observed: $[M+H]^+$ =1832.

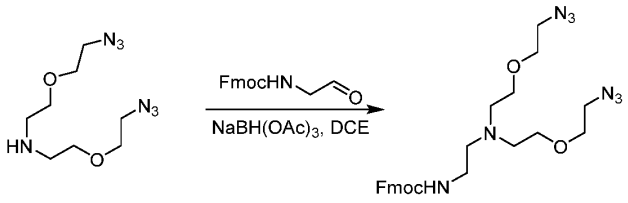
iii (e) 10-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)carbamoyl)-N1,N1-bis(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-N19,N19-bis(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)-4,7,13,16-tetraoxa-10-azanonadecanediamide (Linker 4)



A solution of compound **AA_20** (14.7 g, 8.02 mmol, 1.0 eq.) in 2N HCl in 1,4-dioxane (150 mL) was stirred at 0 °C for 30 min. The reaction was monitored by LCMS analysis. Upon completion, the reaction mixture was concentrated under reduced pressure, purified by preparative HPLC (0.01% TFA), and lyophilized to obtain **linker 4** (4.9 g, 39% yield for two steps, 96.7% purity) as a purple solid. **^{1}H NMR (400 MHz, DMSO-d_6)** δ ppm 8.40 (d, *J* = 8.28 Hz, 4 H), 8.19 - 8.29 (m, 2 H), 7.68 - 7.89 (m, 3 H), 7.54 (d, J = 8.28 Hz, 4 H), 6.16 - 6.35 (m, 1 H), 3.35 - 3.61 (m, 96 H), 3.24 (br d, J = 5.77 Hz, 4 H), 3.11 - 3.18 (m, 2 H), 2.99 (s, 6 H), 2.94 - 2.99 (m, 2 H), 2.59 (br t, *J* = 6.78 Hz, 4 H). **MS (ESI)**: MS calculated: 1729, MS observed: $[(M+2H)/2]^{2+}$ = 866. **HPLC purity (254 nm)** = 96.7%

Example 10 - Synthesis of linker 5

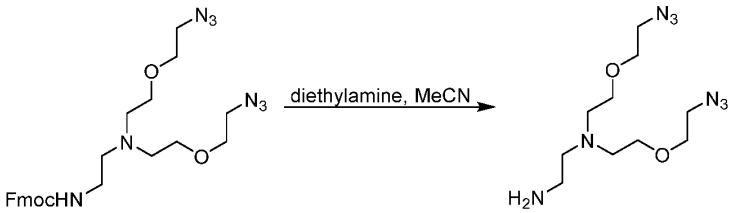
i) Intermediate AA_57



AA_56 **AA_57**

To a solution of **AA_56** (5.0 g, 20.6 mmol, 1.0 *eq*), was added FmocHN-CH_2-CHO (6.07 g, 21.6 mmol, 1.05 *eq*) in DCE (75.0 mL) and was stirred for 5 min and then $NaBH(OAc)_3$ (8.71 g, 41.1 mmol, 2.0 *eq*) was added to the mixture at 0 °C. The mixture was allowed to warm to room temperature, and stirred at 25 °C for 16 h. Upon completion, the reaction mixture was quenched by addition of H_2O (10.0 mL) and concentrated the resultant under reduced pressure to give a residue. The residue was purified by pre-HPLC (TFA condition) to give **AA_57** (6.74 g, 10.8 mmol, 52.7% yield, TFA salt) as colorless oil. **LCMS:** R_t: 0.88 min, MS cal.: 508.3, MS observed: $[M+Na]^+$ =531.4. **HPLC:** ES27056-40-p1cc, R_t: 4.08 min, purity: 97.27%. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 7.76 (d, *J* = 7.53 Hz, 2 H), 7.62 (d, *J* = 7.28 Hz, 2 H), 7.37 - 7.45 (m, 2 H), 7.29 - 7.37 (m, 2 H), 6.76 (br s, 1 H), 4.35 (d, *J* = 7.28 Hz, 2 H), 4.18 - 4.26 (m, 1 H), 3.92 (br d, *J* = 4.27 Hz, 4 H), 3.57 - 3.69 (m, 6 H), 3.46 - 3.53 (m, 6 H), 3.38 (t, *J* = 4.77 Hz, 4 H).

ii) Intermediate AA_58

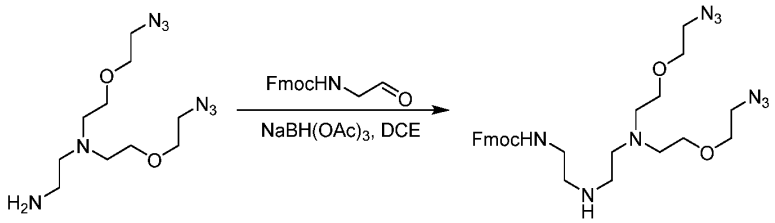


AA_57 **AA_58**

Diethylamine (50.0 mL) was added to a solution of **AA_57** (6.74 g, 13.3 mmol, 1.00 eq.) in MeCN (100 mL), and stirred at 25 °C for 4 h. Upon completion of the reaction, the solvent was concentrated to give a residue. The residue was purified by column chromatography (SiO_2, dichloromethane: MeOH = 100/1 to 20/1) to give **AA_58** (2.73 g, 9.53 mmol, 71.9% yield) as yellow oil.

LCMS: R_t: 0.45 min, MS cal.: 286.2, MS observed: 287.1 $[M+H]^+$. **HPLC:** ES27056-60-p1c1, R_t: 2.27 min, purity: 84.56%. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 3.61 - 3.66 (m, 4 H), 3.57 (t, J = 5.77 Hz, 4 H), 3.35 - 3.43 (m, 4 H), 2.75 - 2.84 (m, 6 H), 2.67 - 2.70 (m, 2 H).

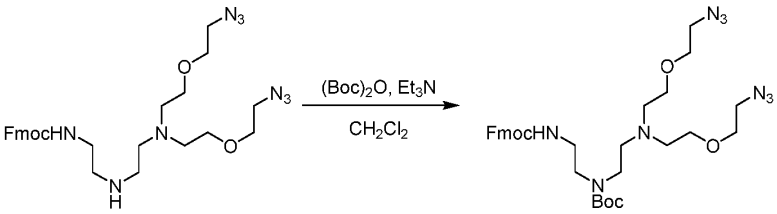
iii) Intermediate AA_59



AA_58 **AA_59**

To a solution of **AA_58** (1.0 g, 3.49 mmol, 1.0 eq) in DCE (30.0 mL) was added FmocHN-CH_2-CHO (491 mg, 1.75 mmol, 0.50 eq.) and $NaBH(OAc)_3$ (1.11 g, 5.24 mmol, 1.50 eq.) at 0 °C. The mixture was slowly allowed to reach room temperature, and stirred at 25 °C for 16 h. Upon completion, the mixture was quenched with H_2O (5.0 mL), and concentrated under reduced pressure to give a residue. The residue was purified by prep-HPLC (TFA condition) to give **AA_59** (560 mg, 841 µmol, 24% yield, TFA salt) as yellow oil. **LCMS:** ES27056-83-p1a, R_t: 1.07 min, MS cal.: 551.3, MS observed: 569.3 $[M+H_2O]^+$. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 7.76 (d, J = 7.53 Hz, 2 H), 7.62 (br d, J = 7.53 Hz, 2 H), 7.37 - 7.44 (m, 2 H), 7.28 - 7.36 (m, 2 H), 6.57 (br s, 1 H), 4.34 (br d, J = 7.03 Hz, 2 H), 4.17 - 4.25 (m, 1 H), 3.79 (br d, J = 4.27 Hz, 4 H), 3.56 - 3.66 (m, 8 H), 3.38 - 3.44 (m, 6 H), 3.31 (br s, 4 H), 3.22 (br s, 2 H).

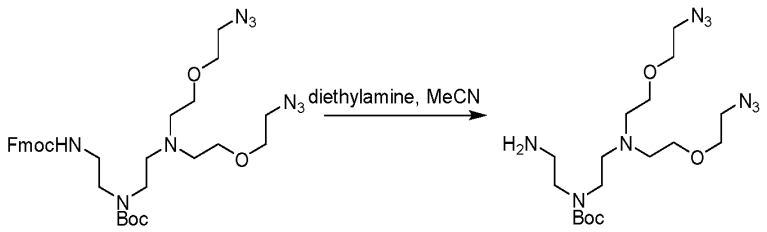
iv) Intermediate AA_60



AA_59 **AA_60**

To a solution of **AA_59** (743 mg, 953 µmol, 1.00 eq) in dichloromethane (18.5 mL) was added $(Boc)_2O$ (438 µL, 1.91 mmol, 2.00 eq) and triethylamine (133 µL, 953 µmol, 1.0 eq). The mixture was stirred at 25 °C for 2 h. Upon completion, the rection mixture was diluted with H_2O (50.0 mL), and extracted with dichloromethane (25.0 mL x 3). The combined organic layers were washed with brine (~25 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was further purified by column chromatography (SiO_2, dichloromethane/MeOH=100/1 to 20/1) to give **AA_60** (609 mg, 934 µmol, 98% yield) as a white solid. **LCMS:**, R_t: 0.95 min, MS cal.: 651.4, MS observed: 652.3 $[M+H]^+$. **^{1}H NMR:**, 400 MHz, $CDCl_3$ δ: 7.77 (d, J = 7.53 Hz, 2 H), 7.60 (br d, J = 7.28 Hz, 2 H), 7.37 - 7.45 (m, 2 H), 7.29 - 7.36 (m, 2 H), 4.54 (br s, 1 H), 4.42 (br d, J = 4.52 Hz, 1 H), 4.21 (br t, J = 6.40 Hz, 1 H), 3.37 - 3.67 (m, 10 H), 3.33 (br s, 6 H), 3.24 (br t, J = 6.27 Hz, 2 H), 2.54 - 2.84 (m, 6 H), 1.41 - 1.50 (m, 9 H).

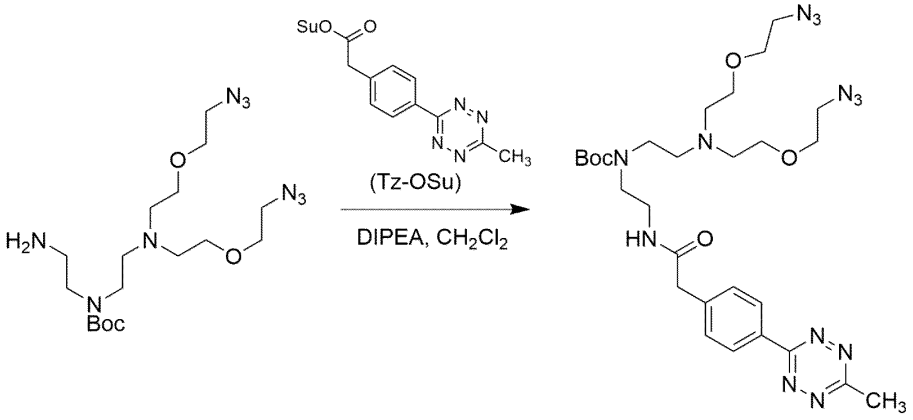
v) Intermediate AA_61



AA_60 **AA_61**

Diethylamino (4.81 mL) was added to a solution of **AA_60** (609 mg, 934 µmol, 1.0 eq) in acetonitrile (9.0 mL). The mixture was stirred at 25 °C for 2 h. Upon completion of the reaction, the solvent was concentrated in vacuum to give a residue. The residue was further purified by column chromatography (SiO_2, dichloromethane/MeOH=100/1 to 15/1) to give **AA_61** (328 mg, 764 µmol, 82% yield) as a milky white solid. **LCMS:** R_t: 1.03 min, MS cal.: 429.3, MS observed: 430.2 $[M+H]^+$. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 3.60 - 3.66 (m, 4 H), 3.57 (t, *J* = 5.90 Hz, 4 H), 3.39 (t, *J* = 4.89 Hz, 4 H), 3.29 (br s, 4 H), 2.85 (t, *J* = 6.53 Hz, 2 H), 2.80 (t, *J* = 5.77 Hz, 4 H), 2.74 (br s, 2 H), 1.47 (s, 9 H).

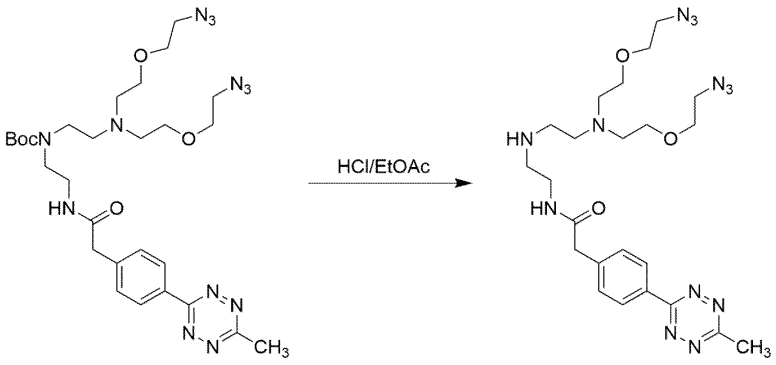
vi) Intermediate AA_62



AA_61 **AA_62**

To a solution of **AA_61** (328 mg, 764 µmol, 1.0 eq) and **Tz-OSu** (375 mg, 1.15 mmol, 1.50 eq) in CH_2Cl_2 (5.0 mL) was added DIPEA (200 µL, 1.15 mmol, 1.50 eq). The mixture was stirred at 25 °C for 3 h. Upon completion, the reaction mixture was diluted with H_2O (5.0 mL), and extracted with dichloromethane (5.0 mL x 3). The combined organic layers were washed with brine (~5.0 mL), dried over Na_2SO_4, filtered and concentrated under vacuum to give a residue. The residue was then purified by column chromatography (SiO_2, Petroleum ether/Ethyl acetate=10/1 to 0/1) to give **AA_62** (485 mg, 756 µmol, 99.0% yield) as a purple oil. **LCMS:** R_t: 0.87 min, MS cal.: 641.4, MS observed: 642.5 $[M+H]^+$. **^{1}H NMR:** ES27056-100-p1b1, 400 MHz, $CDCl_3$ δ: 8.52 - 8.60 (m, 2 H), 7.49 - 7.55 (m, 2 H), 3.63 (br s, 2 H), 3.58 - 3.62 (m, 4 H), 3.55 (br s, 2 H), 3.40 (br s, 4 H), 3.36 (t, *J* = 4.89 Hz, 4 H), 3.23 (br s, 1 H), 3.10 (s, 3 H), 2.79 (br s, 2 H), 2.69 (s, 1 H), 2.65 - 2.73 (m, 1 H), 2.10 - 2.39 (m, 5 H), 1.44 (s, 9 H).

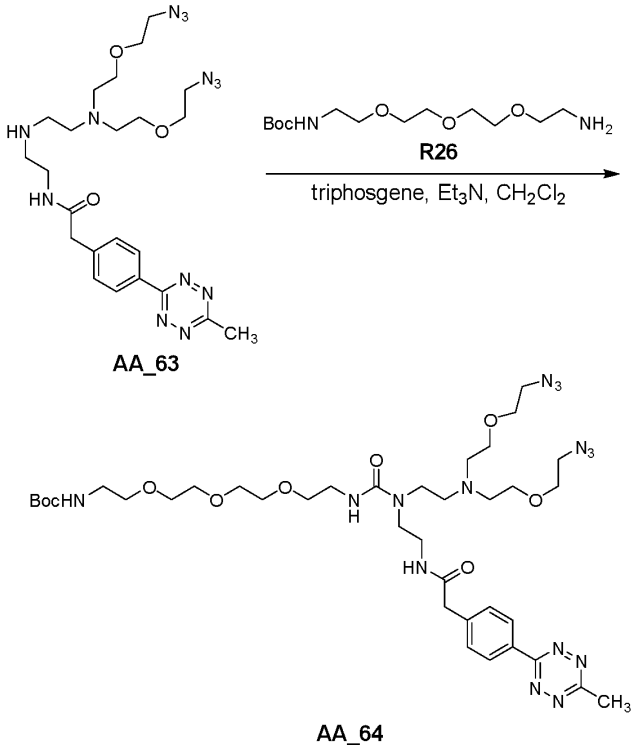
vii) Intermediate compound AA_63



AA_62 **AA_63**

AA_62 (485 mg, 756 µmol, 1.0 eq) was dissolved in 2N HCl/EtOAc (10 mL), and stirred at 25 °C for 2 h. Upon completion of the reaction, the solvent was concentrated under reduced pressure to give a residue. The residue was further purified by prep-HPLC (TFA condition) to give **AA_63** (456 mg, 592 µmol, 78.4% yield, TFA salt) as purple oil. **LCMS:** R_t: 0.76 min, MS cal.: 541.3, MS observed: 542.4 $[M+H]^+$.**HNMR:** 400 MHz, $CDCl_3$ δ: 8.53 (d, J = 8.28 Hz, 2 H), 7.91 (br s, 1 H), 7.55 (d, J = 8.53 Hz, 2 H), 3.77 - 3.85 (m, 4 H), 3.57 - 3.70 (m, 10 H), 3.40 - 3.46 (m, 4 H), 3.27 - 3.37 (m, 6 H), 3.18 - 3.24 (m, 2 H), 3.10 (s, 3 H).

viii) Intermediate compound AA_64

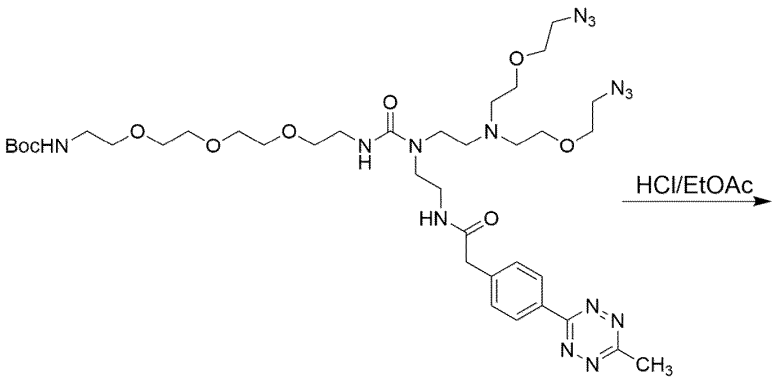


AA_63 **AA_64**

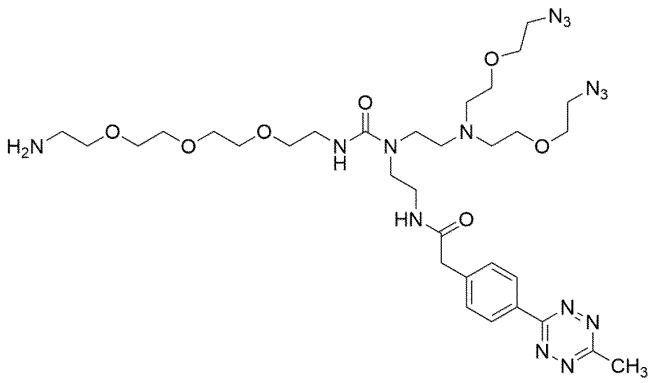
To a solution of triphosgene (114 mg, 385 µmol, 0.65 eq) in dichloromethane (7.5 mL) was added a solution of **R26** (225 mg, 770 µmol, 1.30 eq) and triethylamine (495 µL, 3.55 mmol, 6.00 eq) in dichloromethane (7.50 mL) at 0 °C. The mixture was stirred for 1 h at 0 °C. Then the resultant mixture

was added dropwise to a solution of **AA_63** (456 mg, 592 µmol, 1.0 eq) in dichloromethane (10.0 mL) at 0 °C. The mixture was stirred at 25 °C for 2 h. Upon completion, the reaction mixture was diluted with dichloromethane (~25 mL). Further it was washed with H_2O (~20 mL) and extracted with CH_2Cl_2 (50.0 mL x 3). The combined organic layers were washed with brine (50.0 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was further purified by column chromatography (SiO_2, DCM/MeOH=100/1 to 20/1) to give **AA_64** (573 mg, 666 µmol) as purple oil. **LCMS:** R_t: 0.88 min, MS cal.: 859.5, MS observed: 860.8 $[M+H]^+$. **HPLC:** R_t: 3.48 min, purity: 93.87%. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 8.55 (d, J = 8.28 Hz, 2 H), 7.45 - 7.56 (m, 3 H), 7.38 (br s, 1 H), 5.08 (br s, 1 H), 3.49 - 3.72 (m, 28 H), 3.29 - 3.42 (m, 14 H), 3.20 (br s, 2 H,) 3.10 (s, 3 H), 2.71 - 2.85 (m, 6 H), 1.45 (s, 9 H).

ix) Linker 5



AA_64



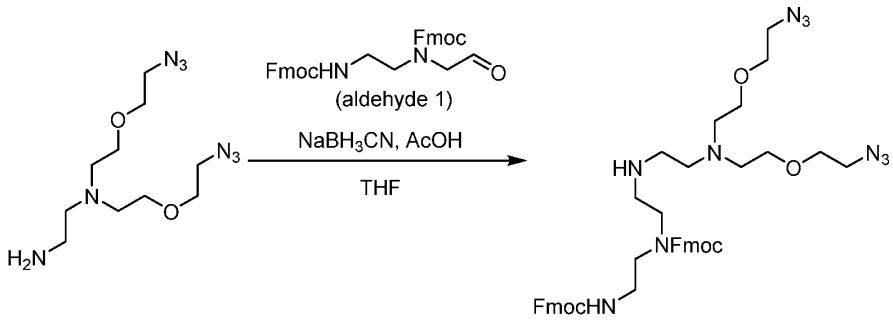
Linker 5

AA_64 (509 mg, 592 µmol, 1.00 eq) was dissolved in 2N HCl/EtOAc (10.0 mL), and stirred at 25 °C for 2 h. Upon completion of the reaction, the solvent was concentrated under vacuum to give a residue. The residue was purified by prep-HPLC (TFA condition). The elute was washed with 1% $NaHCO_3$ (50 mL) and extracted with dichloromethane (100 mL x 3). The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4, filtered and concentrated in vacuum to give **Linker 5** (180 mg, 237 µmol, 40% yield) as purple oil. **LCMS:** ES27056-112-p1a1, R_t: 0.78 min, MS cal.: 759.4, MS observed: 760.6 $[M+H]^+$. **HPLC:** ES27056-112-p1cc, RT: 2.18 min, purity: 95.37%. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 8.55 (d, J = 8.53 Hz, 2 H), 7.53 (d, J = 8.53 Hz, 3 H), 3.62 - 3.67 (m, 9 H), 3.60 (d, J = 4.77 Hz, 4 H), 3.49 - 3.59 (m, 10 H),

3.32 - 3.43 (m, 10 H), 3.21 (br d, *J* = 5.52 Hz, 2 H), 3.10 (s, 3 H), 2.87 (t, *J* = 5.27 Hz, 2 H), 2.79 (t, *J* = 5.52 Hz, 4 H), 2.75 (br d, *J* = 4.77 Hz, 2 H).

Example 11 - Synthesis of Linker 6

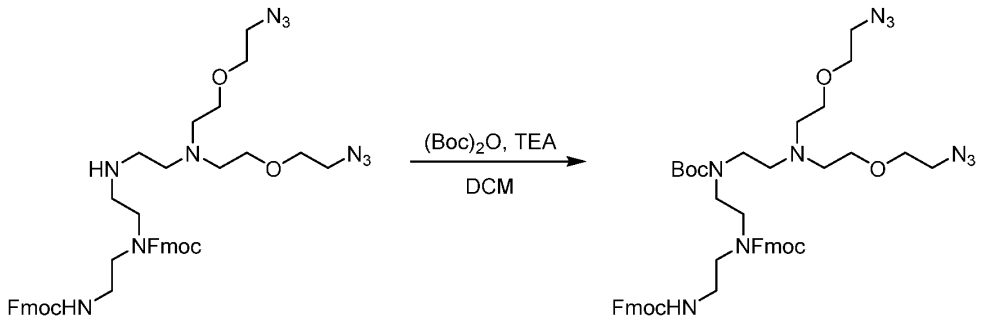
i) Intermediate AA_65



AA_58 **AA_65**

To a solution of **AA_58** (1.3 g, 4.54 mmol, 2.0 eq) in THF (25 mL) was added AcOH (780 µL, 13.6 mmol, 6.0 eq), then **aldehyde 1** (1.24 g, 2.27 mmol, 1.0 eq) and $NaBH_3CN$ (571 mg, 9.08 mmol, 4.00 eq) were added to the mixture. The mixture was stirred at 45 °C for 72 h. After which the mixture was diluted with H_2O (~5 mL) and then concentrated under vacuum to give a residue. The residue was further purified by prep-HPLC (TFA condition) to give **AA_65** (1.06 g, 1.30 mmol, 57% yield) as colorless oil. **LCMS:** ES27056-114-p2a5, R_t: 0.97 min, MS cal.: 816.4, MS observed: 839.6 $[M+Na]^+$. **HPLC:** ES27056-114-p1c, R_t: 4.756 min, purity: 90%. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 7.70 - 7.84 (m, 4 H), 7.59 (br s, 2 H), 7.31 - 7.50 (m, 8 H), 7.21 - 7.27 (m, 2 H), 4.51 (br s, 4 H), 4.12 - 4.24 (m, 2 H), 3.84 (br s, 4 H), 3.66 (br d, *J* = 4.02 Hz, 6 H), 3.21 - 3.50 (m, 12 H), 2.68 - 3.18 (m, 6 H), 2.25 (br d, *J* = 12.55 Hz, 1 H).

ii) Intermediate compound AA_66

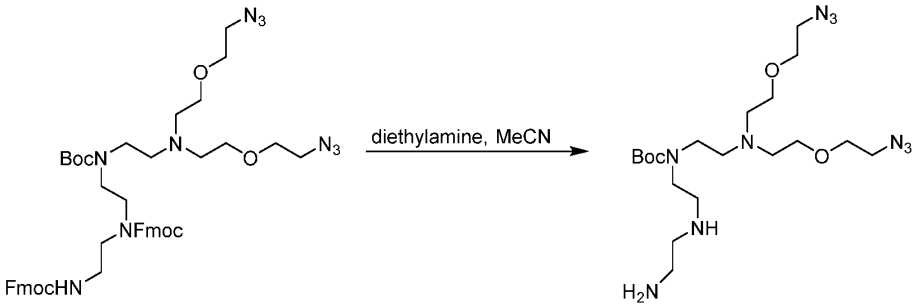


AA_65 **AA_66**

To a solution of **AA_65** (393 mg, 422 µmol, 1.0 eq) in dichloromethane (4.00 mL) was added $(Boc)_2O$ (388 µL, 1.69 mmol, 4.00 eq) and triethylamine (88.1 µL, 633 µmol, 1.50 eq). The mixture was stirred at 25 °C for 4 h, after which the mixture was diluted with dichloromethane (2.0 mL), and purified by column chromatography (SiO_2, Petroleum ether/Ethyl acetate=100/1 to 20/1) to give **AA_66** (354 mg, 386 µmol, 91% yield) as colorless oil. **LCMS:** ES27056-123-p1a9, R_t: 1.05 min, MS cal.: 916.5, MS observed: 917.8 $[M+H]^+$. **HPLC:** ES27056-123-p1c, R_t: 5.27 min, purity: 92.87%. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 7.68 - 7.85

(m, 4 H), 7.48 - 7.63 (m, 4 H), 7.28 - 7.47 (m, 8 H), 4.29 - 4.71 (m, 5 H), 4.19 (br s, 2 H), 3.87 (br s, 2 H), 3.22 - 3.73 (m, 18 H), 2.51 - 3.22 (m, 7 H), 2.04 (br d, *J* = 12.05 Hz, 1 H), 1.41 (br t, *J* = 7.28 Hz, 9 H).

ii) Intermediate compound AA_67



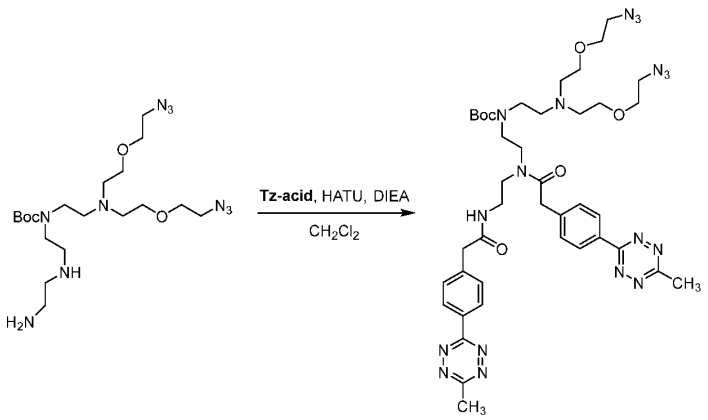
AA_66 **AA_67**

To a solution of **AA_66** (657 mg, 716 µmol, 1.0 eq) in MeCN (15.0 mL) was added dimethylamine (6.64 mL). The mixture was stirred at 25 °C for 2 h after which the reaction mixture was then concentrated under vacuum to give a residue. The residue was purified by column chromatography (SiO_2, dichloromethane/MeOH=50/1 to 5/1) to give **AA_67** (242 mg, 512 µmol, 71.5% yield) as colorless oil.

LCMS:, R_t: 1.61 min, MS cal.: 472.3, MS observed: 473.3 $[M+H]^+$.

HNMR:, 400 MHz, $CDCl_3$ *δ*: 3.63 (t, *J* = 5.02 Hz, 4 H), 3.57 (t, *J* = 5.90 Hz, 4 H), 3.21 - 3.42 (m, 8 H), 2.76 - 2.89 (m, 8 H), 2.73 (br s, 4 H), 1.47 (s, 9 H).

iii) Intermediate compound AA_68

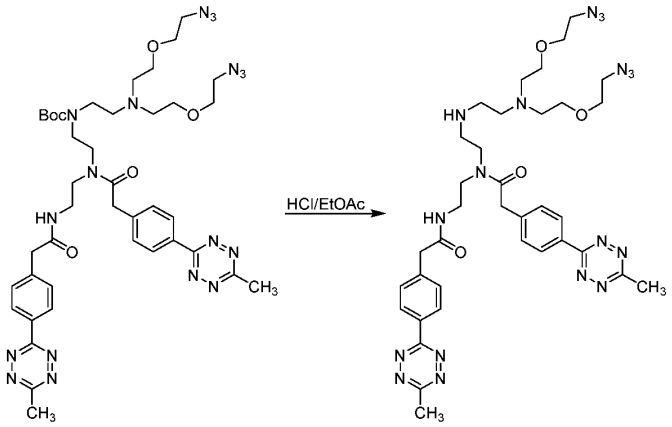


AA_67 **AA_68**

To a solution of **Tz-acid** (methyltetrazine acid (CAS: 1380500-88-8)) (377 mg, 1.64 mmol, 3.2 eq) in dichloromethane (2.5 mL) was added DIPEA (446 µL, 2.56 mmol, 5.0 eq) and HATU (623 mg, 1.64 mmol, 3.2 eq) at 0 °C. Then the mixture was added to a solution of **AA_67** (242 mg, 512 µmol, 1.0 eq) in dichloromethane (2.5 mL). The mixture was stirred at 25 °C for 2 h. Then the mixture was diluted with H_2O (~2.0 mL), and extracted with dichloromethane (5.0 mL x 3). Combined organic layers were washed with brine (~5.0 mL), dried over Na_2SO_4, filtered and concentrated under vacuum to give a residue. The residue was further purified by prep-HPLC (TFA condition) to give **AA_68** (351 mg, 391 µmol, 76% yield) as purple oil. **LCMS:** R_t: 0.94 min, MS cal.: 896.5, MS observed: 898.5 $[M+H]^+$. **HPLC:** R_t: 10.37 min, purity: 93.28%. **1H NMR:** 400 MHz, $CDCl_3$ *δ*: 8.38 - 8.63 (m, 4 H), 7.34 - 7.63 (m, 4 H), 3.88 (s, 1 H), 3.68

- 3.83 (m, 3 H), 3.52 - 3.67 (m, 9 H), 3.29 - 3.52 (m, 12 H), 3.17 - 3.29 (m, 1 H), 3.09 (s, 6 H), 2.57 - 2.99 (m, 4 H), 1.44 - 1.51 (m, 9 H).

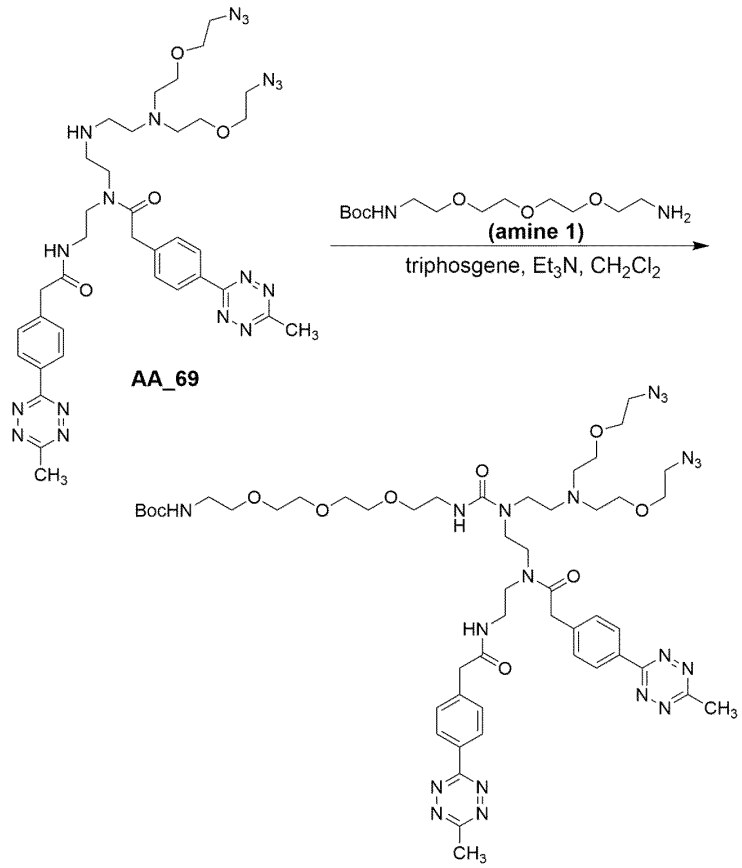
iv) Intermediate compound of AA_69



AA_68 AA_69

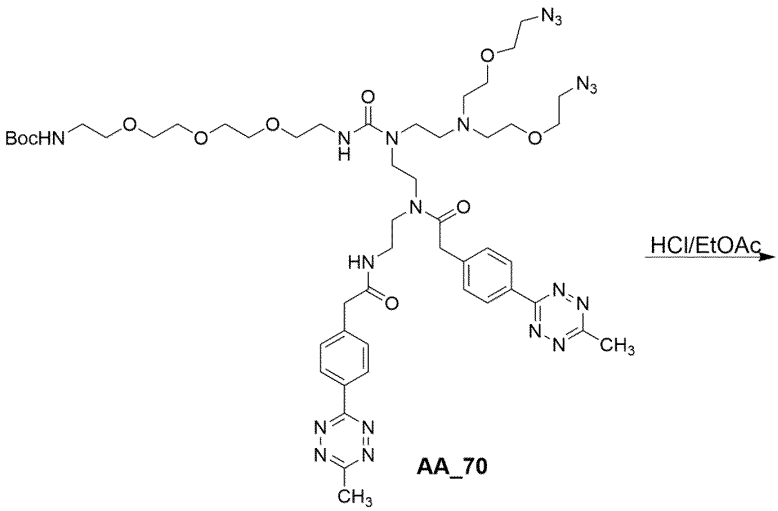
AA-68 (91.6 mg, 102 µmol, 1.0 eq) was dissolved in 2N HCl/EtOAc (500 µL), and stirred at 25 °C for 2 h. LC-MS showed **AA_68** was consumed completely, and identified the desired mass peak. The mixture was concentrated under vacuum to give a residue. The residue was purified by prep-HPLC (TFA condition) to give **AA_69** (81.0 mg, 102 µmol, 99.5% yield) as purple oil. **LCMS:** ES27056-125-p3a1, R_t: 0.85 min, MS cal.: 796.4, MS observed: 819.8 $[M+Na]^+$. **HPLC:** R_t: 2.87 min, purity: ~100%

v) Intermediate compound AA_70

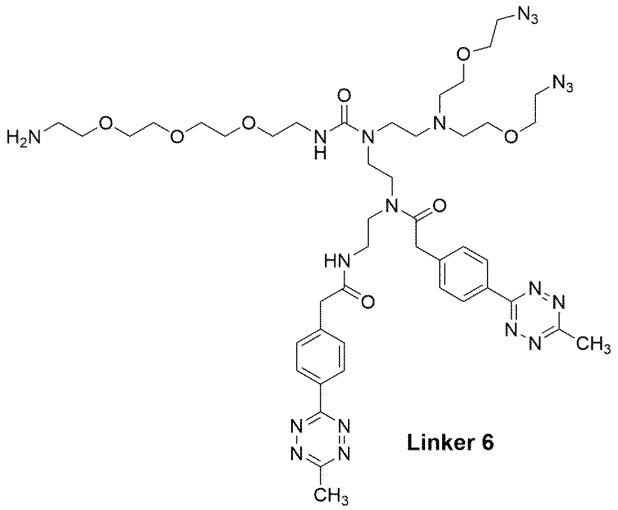


To a solution of triphosgene (19.4 mg, 65.3 µmol, 0.65 eq) in DCM (1.50 mL) was added a solution of **amine 1** (38.2 mg, 131 umol, 1.3 eq) and triethylamine (83.8 µL, 602 µmol, 6.0 eq) in dichormethane (1.5 mL) at 0 °C. The mixture was stirred for 1 h at 0 °C. TLC indicated that completion of the reaction. Then this mixture was added into a solution of **AA_69** (80.0 mg, 100 µmol, 1.0 eq) in dichloromethane (1.0 mL). The mixture was stirred at 25 °C for 2 h. After this the reaction mixture was diluted with dichloromethane (2.0 mL), washed with H_2O (2.0 ml) and extracted with CH_2Cl_2 (15 mL x 3). The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, dichloromethane/MeOH=100/1 to 20/1) to give **AA_70** (118 mg, 106 µmol) as purple oil. **LCMS:** R_t: 4.47 min, MS cal.: 1115.3, MS observed: 1116.6 $[M+H]^+$. **HPLC:** purity: ~100%. **^{1}H NMR:** 400 MHz, $CDCl_3$ δ: 8.33 - 8.59 (m, 4 H), 7.64 (br d, J = 8.03 Hz, 2 H), 7.38 (br s, 2 H), 3.24 - 4.08 (m, 67 H), 3.10 (d, J = 2.26 Hz, 6 H), 1.40 - 1.53 (m, 21 H).

vi) Linker 6



AA_70

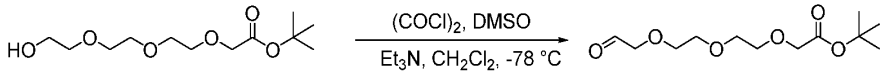


Linker 6

AA_70 (111 mg, 99.5 µmol, 1.0 eq) was dissolved in 2N HCl/EtOAc (1.50 mL), and stirred at 25 °C for 2 h. Upon completion of the reaction, the solvent was concentrated under vacuum to give a residue which was purified by prep-HPLC (TFA condition). The eluate was washed with 1% $NaHCO_3$ (50.0 mL) and extracted with dichloromethane (250 mL x 3). The combined organic layers were washed with brine (250 mL), dried over Na_2SO_4, filtered and concentrated under vacuum to give **Linker 6** (50 mg, 49.3 µmol, 49% yield) as purple oil. **LCMS:** MS cal.: 1015.1, MS observed: 1015.5 $[M+H]^+$. **HPLC:** Purity: 96.74%. **1H NMR:** 400 MHz, $CDCl_3$ *δ*: 8.43 - 8.60 (m, 4 H), 7.35 - 7.68 (m, 5 H), 3.91 (s, 1 H), 3.31 - 3.81 (m, 40 H), 3.18 - 3.30 (m, 2 H), 3.09 (s, 6 H), 2.85 - 3.00 (m, 2 H), 2.71 - 2.82 (m, 6 H).

Example 12 – Synthesis of 4-((2S,5S,31S,34S)-18-(6-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl)-35-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5,31-diisopropyl-2,34-dimethyl-4,7,29,32,35-pentaoxo-9,12,15,21,24,27-hexaoxa-3,6,18,30,33-pentaazapentatriacontanamido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (LP-13)

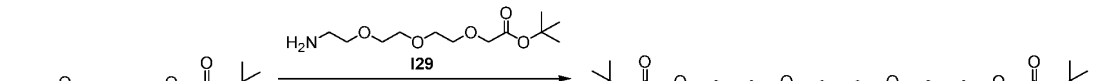
i) Tert-butyl 2-[2-[2-(2-oxoethoxy)ethoxy]ethoxy]acetate (compound I28)



I27 **I28**

To a solution of oxalyl chloride (5.9 mL, 136 mmol, 4.0 equiv.) in CH_2Cl_2 (90.0 mL), DMSO (9.6 mL, 68.1 mmol, 2.0 equiv.) was added at -78 °C followed by the addition of compound **I27** (9.0 g, 34.0 mmol, 1.0 equiv.) and the reaction mixture was stirred at the same temperature for 1 h. Thereafter triethylamine (28.4 mL, 204 mmol, 6.0 equiv.) was added at -78 °C and stirred for further 1 h and allowed to warm to room temperature. Upon completion, the reaction mixture was filtered and concentrated under reduced pressure to give compound **I28** (12.0 g, crude) as a colorless oil, which was used without any further purification. **^{1}H NMR (400 MHz, DMSO-d_6)**: δ ppm 9.54 (s, 1 H), 4.14 (s, 2 H), 3.55 - 3.60 (m, 2 H), 3.44 - 3.55 (m, 8 H), 1.38 (s, 9 H).

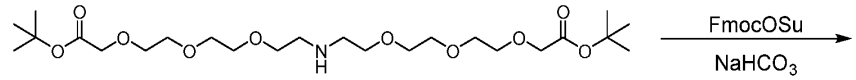
ii) Tert-butyl 2-[2-[2-[2-[2-[2-[2-(2-tert-butoxy-2-oxo-ethoxy)ethoxy]ethoxy]ethylamino]ethoxy]ethoxy]ethoxy]acetate (compound I30):



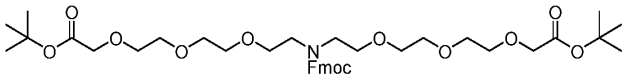
I28 **I30**

To a solution of compound **I28** (12.0 g, 1.0 equiv.) and compound **I29** (17.9 g, 681 µmol, 1.5 equiv.) in MeOH (120 mL), $NaBH_3CN$ (2.85 g, 454 mmol, 1.0 equiv.) was added. The mixture was stirred at 20-25 °C for 1 h. Upon completion, the reaction mixture was extracted with CH_2Cl_2 (200 mL) and H_2O (100 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give compound **I30** (18.0 g, crude) as a yellow oil. **MS (ESI)**: $[M+H]^+$:510.6.

iii) Tert-butyl 2-[2-[2-[2-[2-[2-[2-(2-tert-butoxy-2-oxo-ethoxy)ethoxy]ethoxy]ethyl-(9H-fluoren-9-ylmethoxycarbonyl)amino]ethoxy]ethoxy]ethoxy]acetate (compound I31):



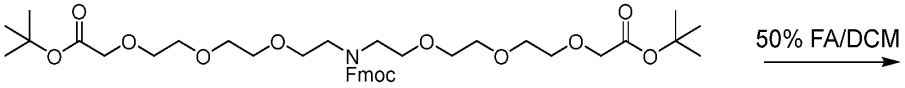
I30



I31

To a solution of compound **I30** (18.0 g, 1.0 equiv.), FmocOSu (17.9 g, 681 µmol, 1.5 equiv.) in THF (90 mL) and H_2O (90 mL), $NaHCO_3$ (5.9 g, 70.6 mmol, 2.0 equiv.) was added. The mixture was stirred at 20-25 °C for 1 h. Upon completion, the reaction LCMS showed compound **I30** was consumed completely. The reaction was extracted with DCM (200 mL) and H_2O (100 mL). The combined organics were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, Petroleum ether/Ethyl acetate=100/1 to 5/1) to give compound **I31** (2.1 g, 2.87 mmol, 98.1% purity, and 2.2 g, 3.01 mmol, 92.4% purity. 16.6% yield) as a white solid. **Purity by HPLC (220 nm)**: 98.1%; **MS (ESI)**: $[M+H]^+$:732.3; **^{1}H NMR (400 MHz, DMSO-d_6)**: δ ppm 7.88 (d, *J* = 7.4 Hz, 2 H), 7.63 (d, *J* = 7.4 Hz, 2 H), 7.37 - 7.44 (m, 2 H), 7.30 - 7.36 (m, 2 H), 4.45 (d, *J* = 5.6 Hz, 2 H), 4.27 (br t, *J* = 5.4 Hz, 1 H), 3.95 - 3.99 (m, 4 H), 3.46 - 3.56 (m, 16 H), 3.38 - 3.44 (m, 4 H), 3.13 (br s, 4 H), 1.40 - 1.42 (m, 18 H).

iv) Tert-butyl 2-[2-[2-[2-[2-[2-[2-(2-tert-butoxy-2-oxo-ethoxy)ethoxy]ethoxy]ethyl-(9H-fluoren-9-ylmethoxycarbonyl)amino]ethoxy]ethoxy]ethoxy]acetate (compound I32):



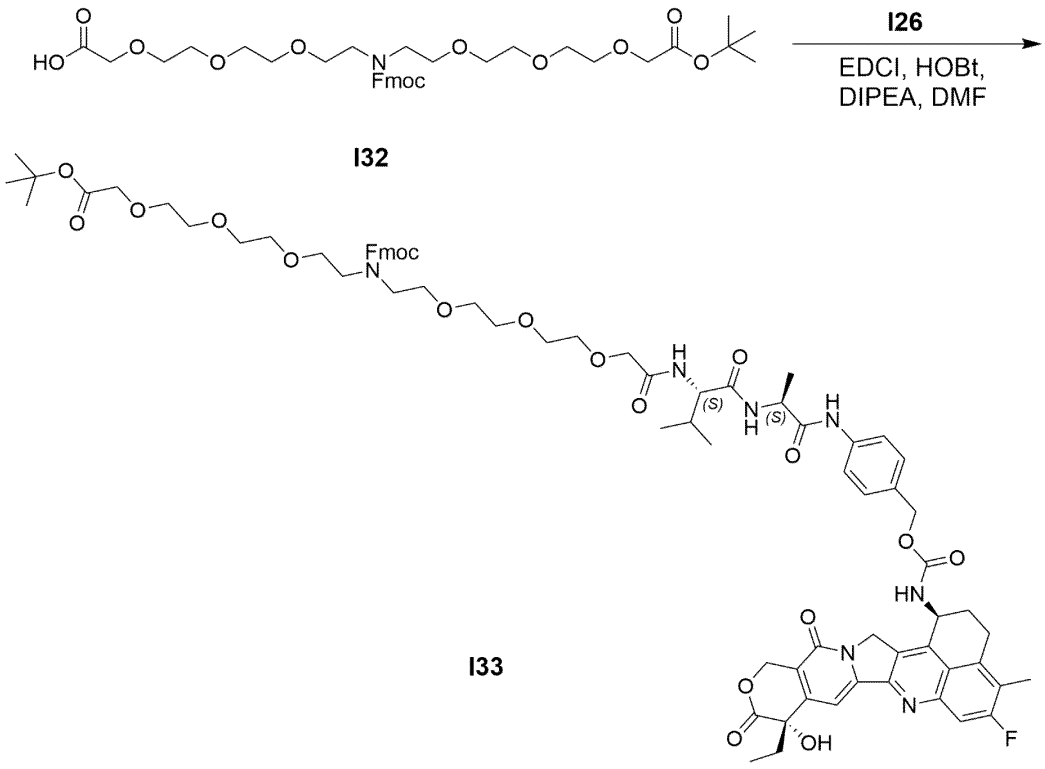
I31



I32

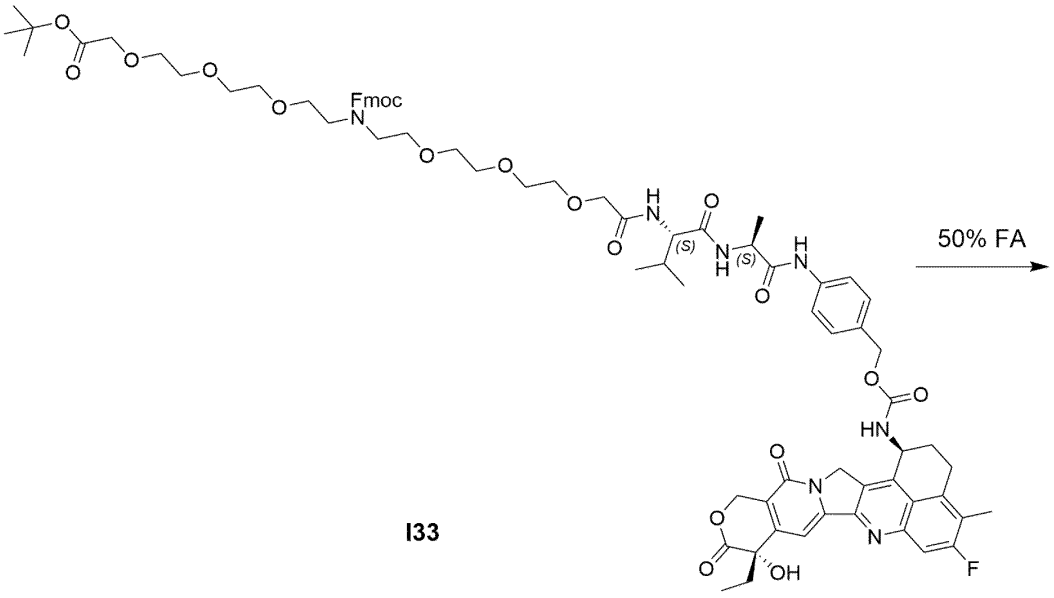
Compound **I31** (2.1 g, 2.87 mmol, 1.0 equiv.) was dissolved in 50% FA/DCM (21.0 mL) and stirred at 20-25 °C for 16 hrs. LCMS analysis showed compound **I31** was consumed completely. The reaction mixture was quenched by adding $NaHCO_3$ (100 mL) at 0-5 °C. The resultant mixture was purified directly by prep-HPLC (TFA condition) to give compound **I32** (700 mg, 1.03 mmol, 35.9% yield) as a colourless oil. **Purity by HPLC (220 nm)**: 98.2%; **MS (ESI)**: $[M+H]^+$:676.3; **^{1}H NMR (400 MHz, DMSO-d_6)**: δ ppm 7.88 (d, *J* = 7.5 Hz, 2 H), 7.64 (d, *J* = 7.4 Hz, 2 H), 7.38 - 7.43 (m, 2 H), 7.30 - 7.36 (m, 2 H), 4.45 (d, *J* = 5.6 Hz, 2 H), 4.23 - 4.32 (m, 1 H), 3.96 (s, 2 H), 3.92 (s, 2 H), 3.44 - 3.58 (m, 20 H), 3.12 (br s, 4 H), 1.40 (s, 9 H)

v) (2S,5S)-18-(((9H-luoren-9-yl)methoxy)carbonyl)-1-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15,21,24,27-hexaoxa-3,6,18-triazanonacosan-29-oic acid (compound I33):

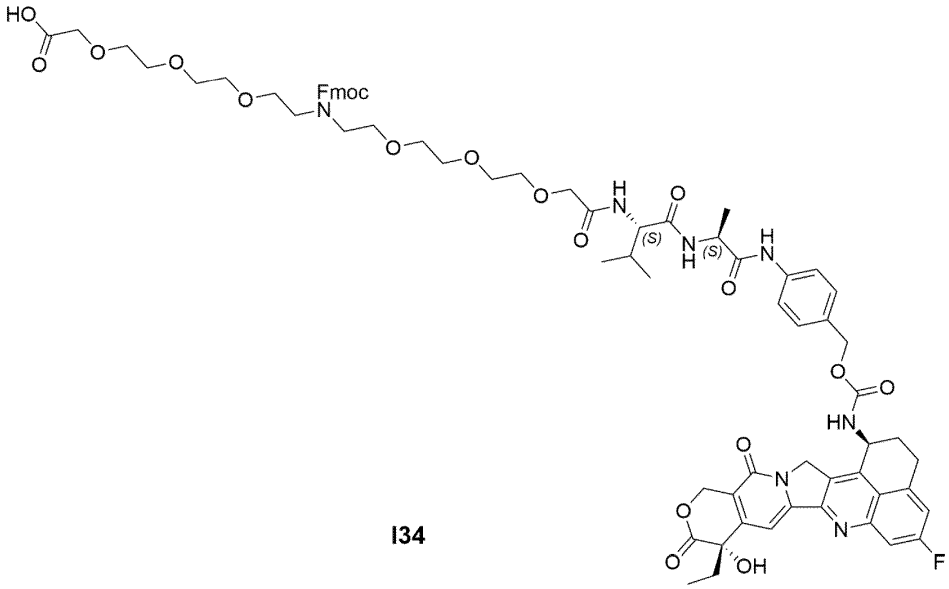


To a solution of compound **I32** (200 mg, 295 μmol, 1.0 equiv.) and compound **I26** (CAS: 2845164-91-0) (308 mg, 377 μmol, 1.3 equiv.) in DMF (2.0 mL) DIPEA (58.6 uL, 354 μmol, 1.2 equiv.), HOBt (47.9 mg, 354 μmol, 1.2 equiv.) and EDCI (67.9 mg, 354 μmol, 1.2 equiv.) were added respectively. The mixture was stirred at 20-25 °C for 3 hrs. LCMS analysis showed compound **I32** was consumed completely. The resultant mixture was purified directly by prep-HPLC (TFA condition) to give compound **I33** (900 mg, 637 μmol, 68.3% yield) as a white solid. **Purity by HPLC (220 nm)**: 97.3%; **MS (ESI)**: $[M+H]^+$:1413.5. The reactions were performed in parallel to obtain a total of 900 mg of **I33**

vi) (2S,5S)-18-(((9H-Fluoren-9-yl)methoxy)carbonyl)-1-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15,21,24,27-hexaoxa-3,6,18-triazanonacosan-29-oic acid (compound I34)



I33

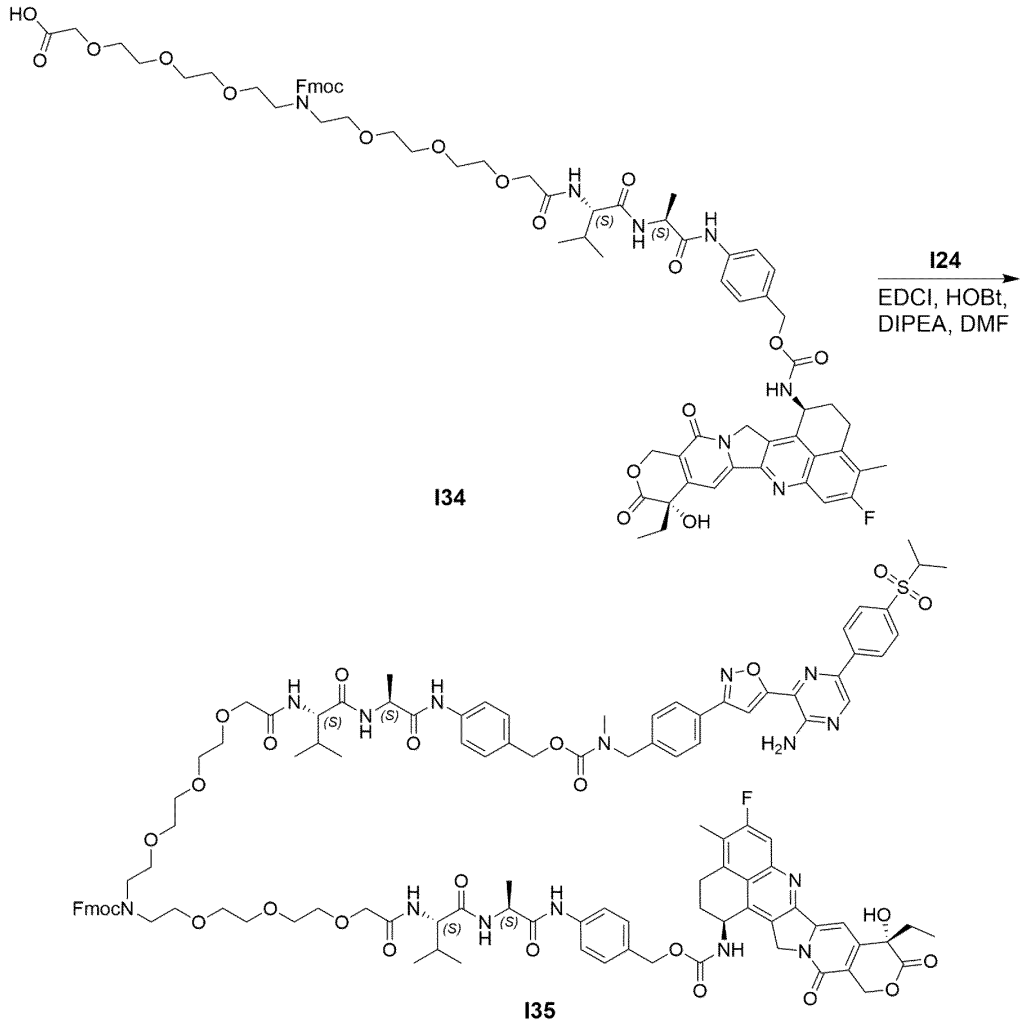


I34

Compound **I33** (900 mg, 637 μmol, 1.0 equiv.) was dissolved in 50% FA/DCM (9.0 mL) and stirred at 20-25 °C for 72 hrs. LCMS analysis showed compound **I33** was consumed completely. The resultant was triturated with isopropyl ether (10 mL) at 0-5 °C to give compound **I34** (500 mg, crude) as a colourless oil. **MS (ESI)**: $[M+H]^+$:1356.8.

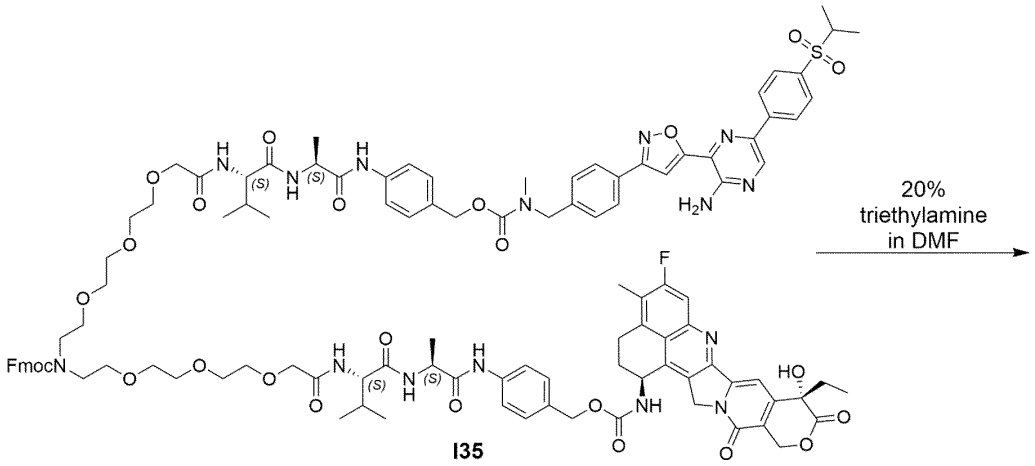
vii) (9H-fluoren-9-yl)methyl ((2S,5S)-1-((4-((((4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamoyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-

9,12,15-trioxa-3,6-diazaheptadecan-17-yl)((2S,5S)-1-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5-isopropyl-2-methyl-1,4,7-trioxo-9,12,15-trioxa-3,6-diazaheptadecan-17-yl)carbamate (compound I35)

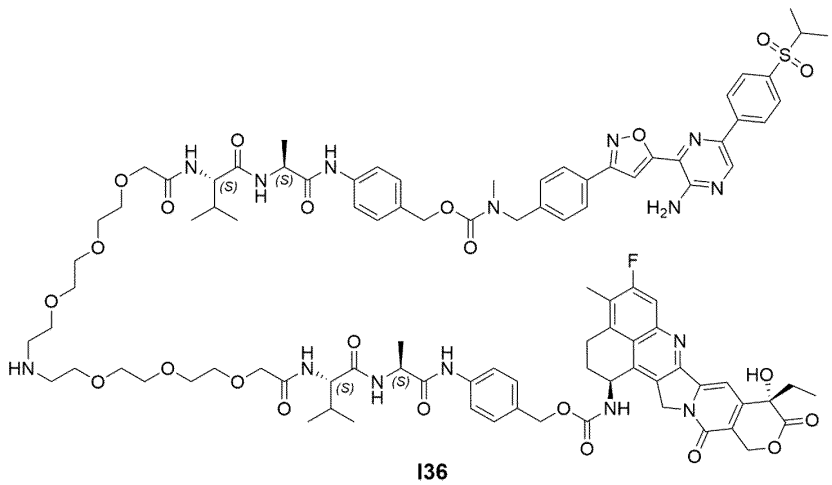


To a solution of compound **I34** (500 mg, 368 μmol, 1.0 equiv.) and compound **I24** (392 mg, 479 μmol, 1.3 equiv.) in DMF (5.0 mL), DIPEA (152 uL, 921 μmol, 2.5 equiv.), HOBt (74.7 mg, 552 μmol, 1.5 equiv.) and EDCI (105 mg, 552 μmol, 1.5 equiv.) were added successively. The mixture was stirred at 20-25 °C for 2 h. LC-MS analysis showed compound **I35** was consumed, and the desired mass was identified. The reaction mixture was directly used to the next step without further purifications. **MS (ESI)**: $[(M+2H)/2]^{2+}$:1062.5.

viii) 4-((2S,5S,31S,34S)-35-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5,31-diisopropyl-2,34-dimethyl-4,7,29,32,35-pentaoxo-9,12,15,21,24,27-hexaoxa-3,6,18,30,33-pentaazapentatriacontanamido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (compound I36):



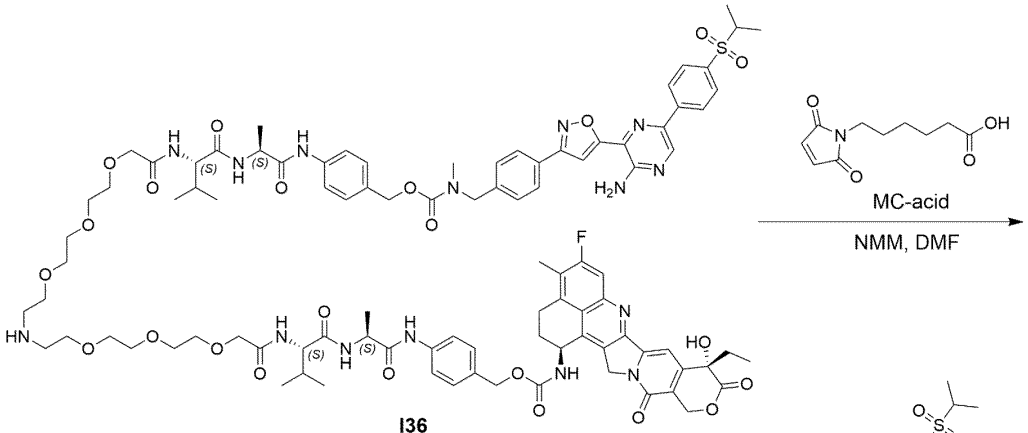
I35



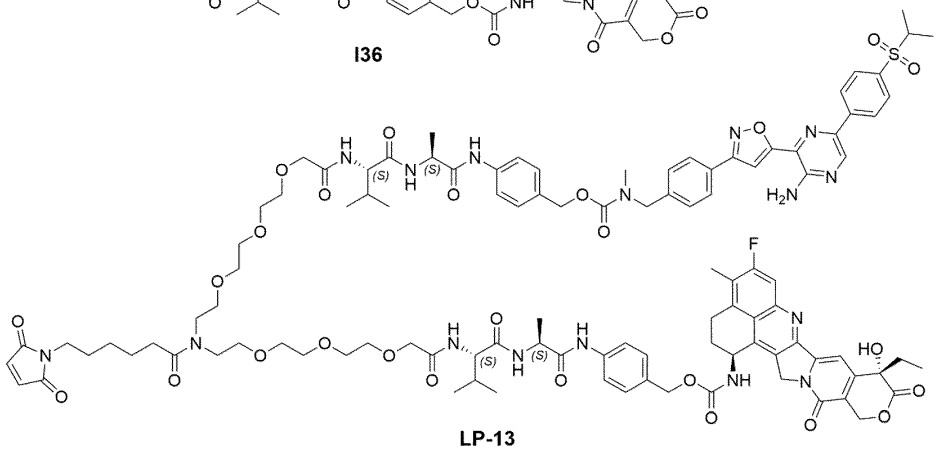
I36

To the above solution of compound **I35,** triethylamine (1.0 mL) was added. The reaction mixture was stirred at 20-25 °C for 4 h. LC-MS showed compound **I35** was consumed completely, and the desired mass was detected. The resultant mixture was directly purified by prep-HPLC (TFA condition) to give compound **I36** (200 mg, 105 μmol, 28.5% purity) as a white solid. **Purity by HPLC (220 nm)**: 92.9%; **MS (ESI)**: $[(M+2H)/2]^{2+}$:950.5.

ix) 4-((2S,5S,31S,34S)-18-(6-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl)-35-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5,31-diisopropyl-2,34-dimethyl-4,7,29,32,35-pentaoxo-9,12,15,21,24,27-hexaoxa-3,6,18,30,33-pentaazapentatriacontanamido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (LP-13)



I36

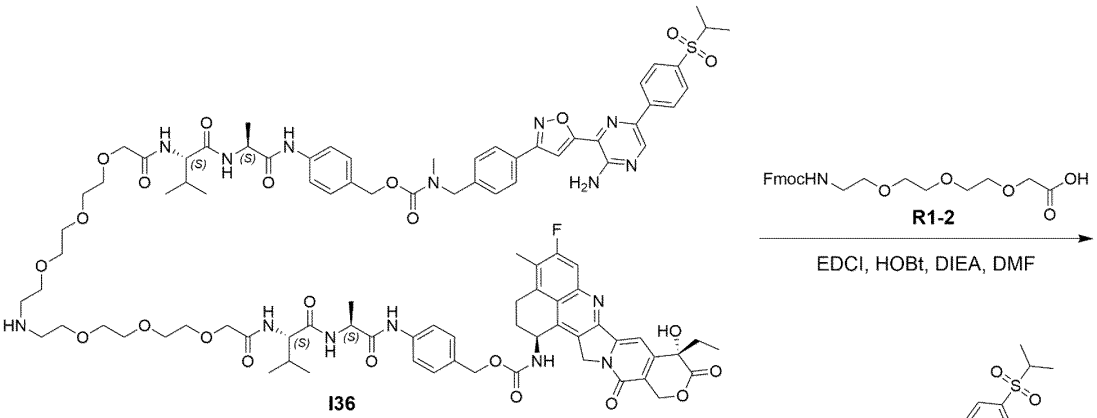


LP-13

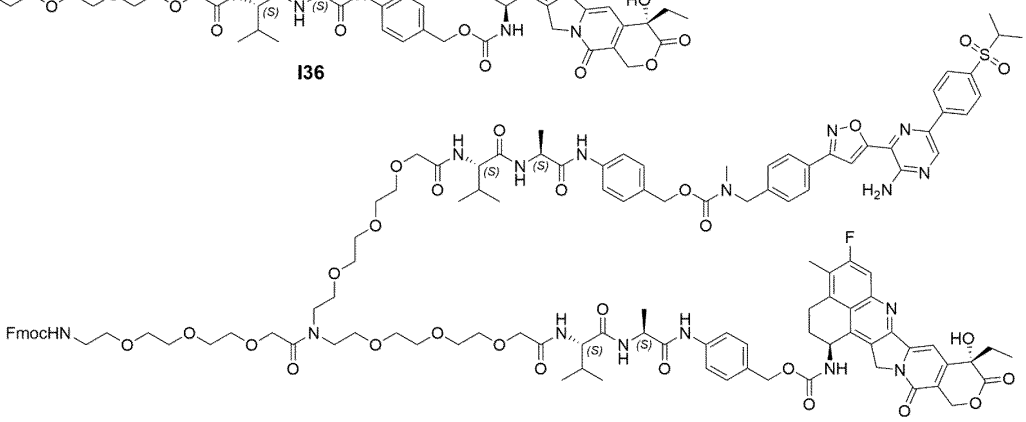
To a solution of compound **I36** (30 mg, 14.9 μmol, 1.0 equiv.) and compound MC-acid (6.2 mg, 29.3 μmol, 2.0 equiv.) in DMF (300 μL) were added DIPEA (6.10 μL, 37.2 μmol, 2.5 equiv.), HOBt (4.0 mg, 29.8 μmol, 2.0 equiv.) and EDCI (5.7 mg, 29.8 μmol, 2.0 equiv.). The mixture was stirred at 20-25 °C for 1 h. LC-MS indicated the completion consumption of compound **I36**, and the desired product peak was detected. The solvent was removed under reduced pressure and the residue was purified by prep-HPLC (AcOH condition) to give **LP-13** (13.1 mg) as a white solid. **Purity by HPLC (220 nm)**: 96.9%; **MS (ESI)**: $[(M+2H)/2]^{2+}$:1047.1. **^{1}H NMR (400 MHz, DMSO-d_6)** δ ppm 9.99 (s, 1 H), 9.91 - 10.06 (m, 1 H), 8.93 (s, 1 H), 8.88 - 8.96 (m, 1 H), 8.34 - 8.41 (m, 3 H), 8.34 - 8.41 (m, 1 H), 7.91 - 8.00 (m, 4 H), 7.74 (s, 1 H), 7.59 (br d, *J* = 8.6 Hz, 4 H), 7.20 - 7.50 (m, 10 H), 7.18 (br d, *J* = 3.8 Hz, 1 H), 7.14 - 7.20 (m, 1 H), 6.97 (s, 1 H), 5.44 (s, 1 H), 5.27 (br d, *J* = 4.0 Hz, 2 H), 5.05 - 5.09 (m, 3 H), 4.52 (s, 2 H), 4.35 - 4.44 (m, 2 H), 4.24 - 4.32 (m, 2 H), 3.93 (s, 3 H), 3.40 - 3.61 (m, 28 H), 2.87 (s, 3 H), 2.67 (s, 2 H), 2.35 (s, 2 H), 2.36 (br s, 1 H), 2.33 (br d, *J* = 1.6 Hz, 3 H), 2.21 - 2.28 (m, 3 H), 1.94 - 2.06 (m, 4 H), 1.69 (s, 2 H), 1.40 - 1.48 (m, 4 H), 1.26 - 1.34 (m, 8 H), 1.23 (br s, 3 H), 1.19 (d, *J* = 6.8 Hz, 8 H), 0.85 - 0.90 (m, 9 H), 0.81 (br d, *J* = 6.5 Hz, 6 H).

Example 13: Synthesis of [4-[[(2S)-2-[[(2S)-2-[[2-[2-[2-[2-[2-[2-[2-[[(1S)-1-[[(1S)-2-[4-[[(10S,23S)-10-ethyl-18-fluoro-10-hydroxy-19-methyl-5,9-dioxo-8-oxa-4,15-diazahexacyclo[14.7.1.02,14.04,13.06,11.020,24]tetracosa-1,6(11),12,14,16(24),17,19-heptaen-23-yl]carbamoyloxymethyl]anilino]-1-methyl-2-oxo-ethyl]carbamoyl]-2-methyl-propyl]amino]-2-oxo-ethoxy]ethoxy]ethoxy]ethyl-[2-[2-[2-[2-[(3,3,6,6-tetramethyl-1-oxo-1λ^6-thiacyclohept-4-yn-1-ylidene)carbamoylamino]ethoxy]ethoxy]ethoxy]acetyl]amino]ethoxy]ethoxy]ethoxy]acetyl]amino]-3-methyl-butanoyl]amino]propanoyl]amino]phenyl]methyl N-[[4-[5-[3-amino-6-(4-isopropylsulfonylphenyl)pyrazin-2-yl]isoxazol-3-yl]phenyl]methyl]-N-methyl-carbamate (LP-12)

i) 4-((2S,5S,31S,34S)-18-(1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-oyl)-35-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5,31-diisopropyl-2,34-dimethyl-4,7,29,32,35-pentaoxo-9,12,15,21,24,27-hexaoxa-3,6,18,30,33-pentaazapentatriacontanamido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (compound I37):



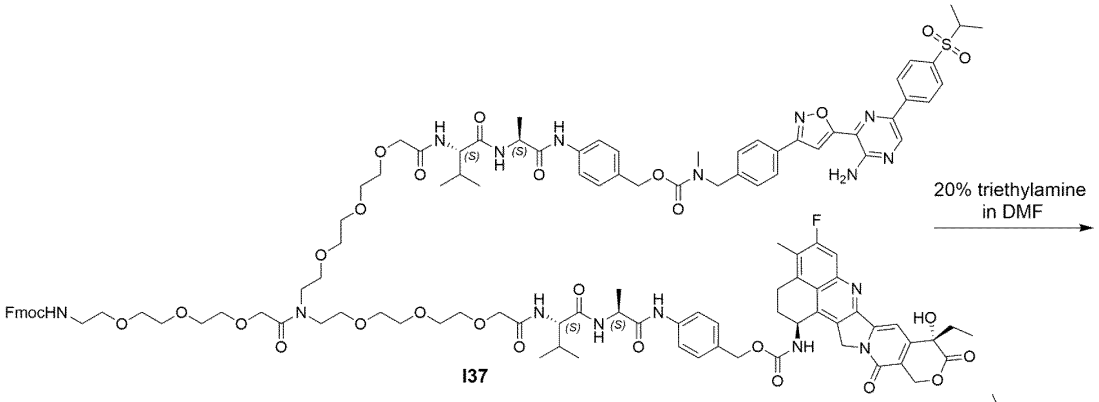
I36



I37

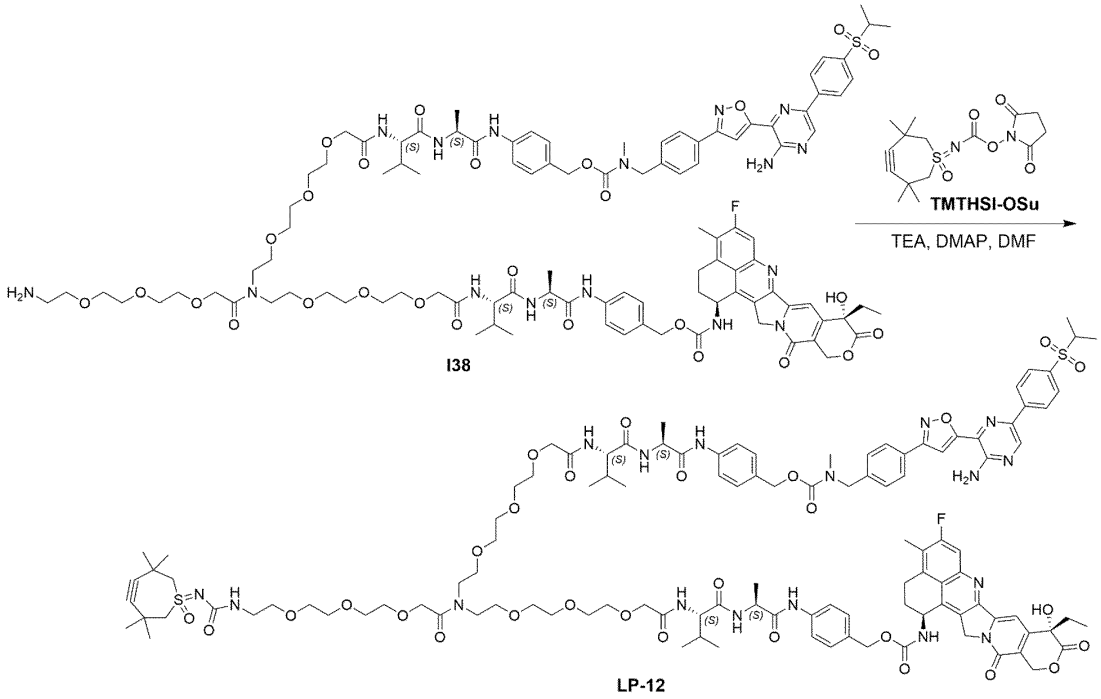
To a solution of compound **I36** (85 mg, 42.2 μmol, 1.0 equiv.) and compound **R1-2** (36.4 μL, 82.4 μmol, 2.0 equiv.) in DMF (1.0 mL) DIEA (17.4 μL, 105 μmol, 2.5 equiv.), HOBt (11.4 mg, 84.4 μmol, 2.0 equiv.) and EDCI (16.1 mg, 84.4 μmol, 2.0 equiv.) were added successively. The mixture was stirred at 20-25 °C for 1 hr. LC-MS showed compound **I36** was consumed completely and detected the desired mass. The reaction mixture was directly used for the next step. **MS (ESI)**: $[(M+2H)/2]^{2+}$:1155.9.

ii) 4-((2S,5S,31S,34S)-18-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)acetyl)-35-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-5,31-diisopropyl-2,34-dimethyl-4,7,29,32,35-pentaoxo-9,12,15,21,24,27-hexaoxa-3,6,18,30,33-pentaazapentatriacontanamido)benzyl (4-(5-(3-amino-6-(4-(isopropylsulfonyl)phenyl)pyrazin-2-yl)isoxazol-3-yl)benzyl)(methyl)carbamate (compound I38):



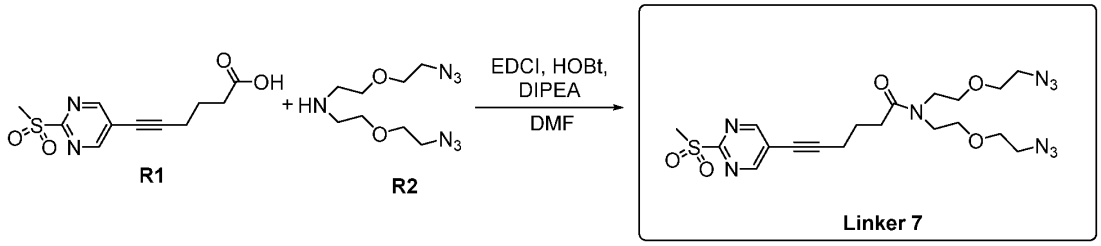
To the solution of compound **I37**, in DMF (0.8 mL), triethylamine (0.2 mL) was added. The mixture was stirred at 20-25 °C for 1 hr. LCMS showed compound **I37** was consumed completely, and detected the desired mass. The resultant reaction mixture was purified directly by prep-HPLC (TFA condition) to give compound **I38** (55 mg, 26.3 μmol, 62% yield) as a white solid. **Purity by HPLC (220 nm)**: 98.5%; **MS (ESI)**: $[(M+2H)/2]^{2+}$:1045.1.

iii) [4-[[(2S)-2-[[(2S)-2-[[2-[2-[2-[2-[2-[2-[2-[2-[[(1S)-1-[[(1S)-2-[4-[[(10S,23S)-10-ethyl-18-fluoro-10-hydroxy-19-methyl-5,9-dioxo-8-oxa-4,15-diazahexacyclo[14.7.1.02,14.04,13.06,11.020,24]tetracosa-1,6(11),12,14,16(24),17,19-heptaen-23-yl]carbamoyloxymethyl]anilino]-1-methyl-2-oxo-ethyl]carbamoyl]-2-methyl-propyl]amino]-2-oxo-ethoxy]ethoxy]ethoxy]ethyl-[2-[2-[2-[2-[(3,3,6,6-tetramethyl-1-oxo-1λ⁶-thiacyclohept-4-yn-1-ylidene)carbamoylamino]ethoxy]ethoxy]ethoxy]acetyl]amino]ethoxy]ethoxy]ethoxy]acetyl]amino]-3-methyl-butanoyl]amino]propanoyl]amino]phenyl]methyl N-[[4-[5-[3-amino-6-(4-isopropylsulfonylphenyl)pyrazin-2-yl]isoxazol-3-yl]phenyl]methyl]-N-methyl-carbamate
(LP-12):



To a solution of compound **I38** (40 mg, 19.1 μmol, 1.0 equiv.) in DMF, triethylamine (10.6 μL, 76.6 μmol, 4.0 equiv.) .), TMTHSI-OSu (39 mg, 114.6 μmol, 6.0 equiv.), and DMAP (4.68 mg, 38.3 μmol, 2.0 equiv.) were added respectively. The mixture was stirred at 20-25 °C for 2 h and monitored by LC-MS analysis. Upon completion of the reaction, the reaction mixture was concentrated, and the residue was purified by prep-HPLC (HCOOH condition) to give **LP-12** (20 mg, 8.64 μmol, 45.1% yield) as a white solid. **Purity by HPLC (220 nm)**: 97.7%; **MS (ESI)**: $[(M+2H)/2]^{2+}$:1157.8; **^{1}H NMR (400 MHz, DMSO-d_6)**: δ ppm 10.0 (s, 2 H), 8.93 (s, 1 H), 8.35 - 8.41 (m, 4 H), 8.05 (br d, *J* = 8.4 Hz, 1 H), 7.98 (br d, *J* = 7.4 Hz, 2 H), 7.93 (d, *J* = 8.5 Hz, 2 H), 7.73 - 7.78 (m, 2 H), 7.55 - 7.63 (m, 4 H), 7.31 - 7.47 (m, 8 H), 7.30 (s, 1 H), 7.18 (br s, 2 H), 6.64 (br t, *J* = 5.2 Hz, 1 H), 6.52 (s, 1 H), 5.44 (s, 2 H), 5.27 (br d, *J* = 4.8 Hz, 3 H), 5.07 (br s, 4 H), 4.52 (s, 2 H), 4.39 (br t, *J* = 6.7 Hz, 2 H), 4.25 - 4.33 (m, 2 H), 4.16 (s, 2 H), 3.93 (s, 4 H), 3.87 (s, 1 H), 3.84 (s, 1 H), 3.36 - 3.66 (m, 42 H), 3.06 - 3.13 (m, 3 H), 2.87 (s, 3 H), 2.35 (s, 3 H), 2.33 (br s, 1 H), 1.96 - 2.02 (m, 2 H), 1.83 - 1.92 (m, 2 H), 1.28 - 1.34 (m, 12 H), 1.17 - 1.21 (m, 12 H), 0.85 - 0.90 (m, 9 H), 0.82 (br d, *J* = 6.6 Hz, 6 H).

Example 14: Synthesis of N,N-bis(2-(2-azidoethoxy)ethyl)-6-(2-(methylsulfonyl)pyrimidin-5-yl)hex-5-ynamide (Linker 7):



R1 R2 Linker 7

To a solution of compound **R1** (40 mg, 149 µmol, 1.0 eq.) and compound **R2** (36.2 mg, 149 µmol, 1.00 eq.) in DMF (400 µL), HOBt (40.2 mg, 298 µmol, 2.0 eq.), DIPEA (57.8 mg, 447 µmol, 73.9 µL, 3.0 eq.) and EDCI (57.1 mg, 298 µmol, 2.0 eq.) were added. The mixture was stirred at 25 °C for 1 h. LC-MS indicated the consumption of staring materials. The reaction mixture was concentrated *in vacuo* and the residue was purified by prep-HPLC (TFA condition) to yield **linker 7** (59 mg, 119 µmol, 79% yield, 98.6% purity) in an oil form. **LCMS (ESI):** MS cal.: 493.5, MS observed: [M+H]+ =494.2. **HRMS**: MS cal.: 493.5, MS observed: [M+H]+ = 494.1. **HPLC**: Rt = 8.97 min, purity: 98.6%. **^{1}H NMR**: 400 MHz, $CDCl_3$. δ: 8.86 (s, 2 H), 3.59 - 3.70 (m, 12 H), 3.31 - 3.42 (m, 7 H), 2.56 - 2.64 (m, 4 H), 1.96 - 2.05 (m, 2 H).

Example 15 – ADC synthesis, monomeric purity, potency and DAR determination

A. Synthesis

The ADCs in table C were prepared according to the general method outlined below.

Antibodies were produced recombinantly by transient expression in CHO cells. Briefly, expression vectors encoding antibody heavy and light chains of the relevant antibody (*e.g.* trastuzumab (DrugBank Acc. No: DB00072; UNII: P188ANX8CK)) were transiently transfected into CHO cells at a 1:1 ratio. Expressed antibodies were subsequently purified from the culture supernatant using MabSelect SuRe Protein A column (Cytiva, #11003494).

The ADC synthesis consists of two steps, the enzymatic addition of a branched linker, followed by the conjugation of the payload-containing moieties. These events take place at the site of glutamine-295 (Q295), within the CH2 domain of the Fc region of the antibody (which additionally comprises the substitution N297A). The first step of the process is the microbial transglutaminase (MTG or MTGase)-mediated conjugation of an linker onto Q295 of the modified antibody. Having attached the clickable-handle, different combinations of DNA Topoisomerase I inhibitors (TOP1I) and DNA Damage Response inhibitors (DDRi) are introduced via metal-free click chemistry using orthogonal strain promoted azide–dibenzocyclooctyne (DBCO), tetramethylthiocycloheptyne sulfoximine (TMTHSI) and methyltetrazine–trans-cyclooctene (TCO) cycloaddition. In general, TOP1i payloads utilize the DBCO group to react with azide, while DDRi payloads utilize TCO group to react with methyltetrazine in a one-pot enzymatic reaction, giving the desired ADC.

Materials

The following materials were used in the synthesis:

- MTGase Enzyme: Activa® TI transglutaminase (Ajinomoto), unit activity 98.56 U/g
- PBS pH 7.45 (ThermoFisher)
- Activated carbon (Merck)
- Sodium deoxycholate (Merck)
- Propylene glycol (Merck)
- DMSO (Merck)
- Protein concentrators (Millipore)
- 0.22 µM syringe filter (Pall)

Enzymatic conjugation:

The reagents set out in table A were combined in PBS buffer (prepared according to the manufacturer's instructions) in a sterile glass bottle with a size of at least 2 times the reaction volume. The reaction was agitated using a magnetic stirrer bar and was incubated for 22 hours at room temperature. This reaction gives an ADC intermediate.

Table A: Concentration of MTGase reaction components

Reagent	**Approximate final concentration**
Antibody	7.6 mg/mL
Linker	40-60 mol eq/mAb (Mole equivalent per mole antibody)
Enzyme	7.9 U/mL
DMSO	4% v/v

Purification

The ADC intermediate formed is then cleaned using protein A chromatography via bind-elute mode to remove excess linker and residual enzyme at room temperature.

A loading level of 9-16 mg/mL resin was used, and pool collection was started at 45 mAU/cm at an absorbance of 280nm and ended at 35 mAU/cm absorbance at 280 nm. The affinity chromatography steps are as shown in table B below:

Table B: Affinity chromatography sequence of steps

Sequence of Steps	**Solution Description**	**Volume Required (CV)**	**Linear Velocity ±10% (cm/hr)**
Column Equilibration	50 mM Tris, 150 mM NaCl, pH 7.5	5.0	240

Load	ADC intermediate	As needed	240
Chase	50 mM Tris, 150 mM NaCl, pH 7.5	3.0	240
Wash I	50 mM Tris, 1 M NaCl, pH 7.5	5.0	240
Wash II	50 mM Tris, 150 mM NaCl, pH 7.5	5.0	240
Elution	100 mM Sodium Acetate, pH 3.5	As needed	240
Non-Denaturing Cleaning	1 M Acetic Acid	5.0	240
Flush	50 mM Tris, 150 mM NaCl, pH 7.5	3.0	240
Denaturing Periodic Cleaning	1 M NaOH	5.0	240
Flush	50 mM Tris, 150 mM NaCl, pH 7.5	3.0	240
Storage	20% EtOH	3.0	240

The eluted ADC intermediate was neutralized using 2M Tris at pH 7.4.

Click conjugation:

TOP1i and DDRi payloads were conjugated to the ADC intermediate in a one-pot reaction in a sequential addition manner. Specifically, TOP1 inhibitors which links to a strained alkyne click moiety is added first onto the ADC intermediate at the site of azide, via a typical SPAAC (Strain-promoted azide-alkyne cycloaddition) reaction. The reaction is set on a roller machine at room temperature, following overnight incubation, DDR inhibitor which links to a TCO click moiety is added onto the ADC intermediate at the site of tetrazine, via a typical IEDDA (Inverse-Electron-Demand Diels–Alder) reaction. The reaction was stopped 2-4 hours after adding linker-payload.

In an example method conjugation of the first payload was achieved by combining 1-3 mg/mL ADC intermediate with ca. 11 mM sodium deoxycholate, 0.28-28 wt.% propylene glycol in TBS pH 7.5. To the solution was added 7-14 mol. equivalents per antibody of TOP1 inhibitor or linker-payload. The reaction is set on a roller machine at room temperature for at least 12 hours.

Conjugation of the second payload was achieved by adding 7-10 mol. equivalents per antibody of linker-payload or TOP1 inhibitor. The mixture was then incubated for at least 2-4 hours at room temperature to allow for the click reaction to occur.

Free linker-payload removal

Activated carbon was used to remove the free drug from the reaction mixture. The reaction was carried out as follows:

Step 1: Dilute activated carbon powder into 100 mg/mL in PBS;
Step 2: Based on the amount of ADC in the reaction mix, add 1:1 ratio of activated carbon solution (weight : weight);

Step 3: Incubate reaction mixture with activated carbon at ambient temperature (25°C) and rotate for 1 hour.

Suspended activated carbon was removed from the mixture using a 0.22 µm PES filter and concentrated using protein concentrator with 50 kDa MWCO. The sample was filtered with 0.22 µm PES filter again before using it in subsequent examples.

The ADC is optionally further purified using HIC purification.

B. Analysis

i) Monomeric purity

Monomeric purity was determined using size exclusion chromatography.

Samples were prepared by diluting them to 1 mg/mL in PBS.

The samples were analysed on a Thermo Ultimate 3000 UPLC / Waters ACQUITY H-Class PLUS Bio System equipped with a ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 mm X 150 mm column and a ACQUITY UPLC Protein BEH SEC Guard Column, 200 Å, 1.7 µm, 4.6 mm X 30 mm guard column.

The analysis method was as follows:

- Mobile phase: 0.2M potassium phosphate buffer, 0.2M potassium Chloride, 15% (v/v) IPA, pH 6.8
- Flow rate: 0.35 mL/min
- Run time: 15 minutes
- Column temperature: Room temperature
- UV detection: 280 nm
- Injection load: 10 µg

The percentage purity results (% purity) were determined as

% purity = (Monomer peak area/Total peak area) x 100%.

ii) Potency determination

The binding potency to the target antigen relative to a refence material was evaluated by ELISA.

The potency determination was carried out on a Molecular Devices, SpectraMax iD3 plate reader, equipped with a BioTek 405 TS plate washer.

The following reagents were used during the potency determination:

- Wash buffer: PBS with 0.05% Tween 20

- Blocking buffer: PBS with 1% BSA
- Coating antigen: His tag target antigen, 1 ug/mL
- Secondary antibody: Anti-human IgG Fc antibody (HRP), 1:7000 (abcam, #ab97725)
- TMB solution: 1-Step™ turbo TMB-ELISA Substrate Solution (Thermo scientific, #34022)
- Stop solution: ELISA stop solution (Invitrogen, SS04)

The test was carried out as follows, briefly, a 96-well plate was coated with 100 µl/well of coating antigen overnight (or up to 72hrs) at 4°C. The plate was then blocked by washing the plate three times with wash buffer (200 µl/well) and then blocking the plate with blocking buffer (200 µl/well) and subsequently incubating at room temperature for 1 hour. Samples were then added to the wells by washing the plate three times with wash buffer (200 µl/well) and then adding serially diluted samples (100 µl/well) and subsequently incubating at room temperature for 1 hour. Secondary antibody addition was carried out by washing the plate three times with wash buffer (200 µl/well) and adding diluted secondary antibody (1:7000) to each well (100 µl/well) and incubating for 1 hour at room temperature in the dark. This was followed by TMB addition, which involved adding TMB equilibrated to room temperature (100 µl/well), the plate was then incubated at room temperature for 15 minutes in the dark. The reaction was then stopped by adding stop solution equilibrated to room temperature (100 µl/well) and the plates were read at 450 nm on a plate reader.

Results were generated by preparing a 4-parameter logistic dose-response curve to compute the EC_{50} values of the samples and reference. The relative potency of the samples was determined by EC_{50} of reference/EC_{50} of sample x 100%.

iii) Drug-to-Antibody Ratio (DAR)

DAR was determined by reversed phase liquid chromatography-mass spectrometry (RPLC-MS). DAR analysis was used to determine the average number of payloads and linkers attached to the Fc region of the ADC.

Samples were prepared by reducing 5 µg of ADC in 10 mM DTT at 40 ˚C for 30 min and injecting a 5 µL aliquot for analysis.

The samples were analysed on a Waters ACQUITY UPLC H-Class PLUS Bio System instrument equipped with a Waters TUV and Xevo® G2-XS QTof Mass Spectrometer detector and an ACQUITY UPLC Protein BEH C4 column, 300 Å, 1.7 µm, 2.1 mm X 50 mm (#186004495).

The analysis method was as follows:

- Column Temperature: 70 °C
- Mobile phase A: Water + 0.1 % formic acid
- Mobile phase B: Acetonitrile + 0.1 % formic acid
- Wavelength: 280 nm

- Effective gradient: linear increase from 20 to 80 % of solvent B within 1 to 3.5 min at the flow rate of 0.4 mL/min
- Xevo G2-XS: MS scan from 350 to 4000 m/z, ESI positive, sensitivity mode

The results were processed according to the following method. Analyte peaks time window: input time window range which covers the whole region of the peak and expected RT (i.e. mid-point of the time window range); background subtract results 5 % from baseline. The MaxEnt1 deconvolution parameters were as follows: input m/z range; output mass range; TOF resolution 20,000. The mass error tolerance was 100 ppm. The amino acid modifiers were input relevant modifiers such as -Lysine C-TERM, Pyroglutamic acid E N-TERM and linker-payload; select type as variable and maximum modification of 1. After processing and inspection of the deconvoluted spectra for identified species the overall DAR for each payload (e.g. exatecan and berzosertib), was calculated according to the following equation:

$$\text{DAR} = 2 \times \sum \left(DAR\ of\ payload \times \frac{relative\ abundance}{100} \right)$$

The results of this analysis are given in table C.

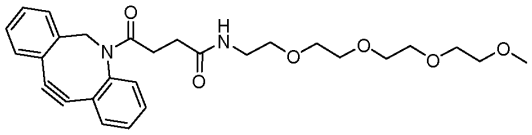
Table C: Description of ADCs along with their CMC data

ADC Number	Antibody	Linker	Linker payload 1	Linker payload 2	Purity (%)	Binding potency EC50 (ng/mL)	Relative potency (%)	DAR (Linker-Payload A : Linker-Payload B
1	T- N297A	Linker 5	LP-1	LP-2	99.03	6.082	86	2-3 : 1-2
2	T- N297A	Linker 5	LP-1	LP-3	93.66	6.597	77	2-3 : 1.5-2
4	T- N297A	Linker 6	LP-1	LP-3	96.77	282.3	78	2.5-4 : 2.5-4
5	T- N297A	Linker 3	LP-1	LP-3	98.84	330.8	88	3.5-4 : 1.5-2
6	T- N297A	Linker 4	LP-1	LP-3	96.83	172.0	88	2.5-4 : 2.5-4
7	T- N297A	Linker 4	LP-1	CP-2	94.64	302.1	86	2-4 :2-4
8	T- N297A	Linker 4	CP-1	LP-3	95.11	210.2	122	2-4 : 2-4
9	T- N297A	Linker 4	LP-1	LP-6	95.00	540.8	90	2-4 :2-4
10	T- N297A	Linker 4	CP-1	LP-6	91.33	213.8	91	2-4 : 2-4
11	T- N297A	Linker 4	LP-1	LP-7	92.91	462.0	83	2-4 :2-4
12	T- N297A	Linker 4	CP-1	LP-7	95.56	250.8	77	2-4 : 2-4
13	T- N297A	Linker 4	LP-1	LP-8	95.36	259.8	70	2.5-4: 2.5-4
14	T- N297A	Linker 4	LP-1	LP-9	97.04	293.7	104	2.5-4: 2.5-4
15	T- N297A	Linker 4	LP-10	LP-3	93.96	509.7	79	2-4 : 2-4
16	T- N297A	Linker 4	LP-10	CP-2	97.07	137.8	111	2.5-4: 2.5-4

17	T- N297A	Linker 4	LP-4	LP-3	96.11	466.0	78	2.5-4: 2.5-4
18	T- N297A	Linker 4	LP-5	LP-3	94.42	726.8	71	2.5-4: 2.5-4
19	T- N297A	Linker 7	LP-12	N/A	97.11	799.3	94	3.5-4: 3.5-4
23	T- N297A	Linker 4	LP-11	LP-3	94.95	568.9	73	2-4 : 2-4
24	T- N297A	Linker 3	LP-11	LP-3	98.04	755.2	78	3-4 : 1.5-2

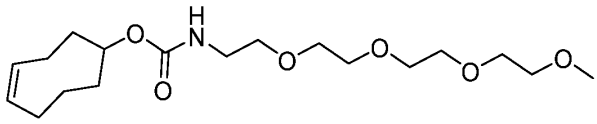
"T-N297A" is trastuzumab provided in N297A format (*i.e.* comprising the substitution N297A at position 297 (EU numbering) of the CH2 domains of the Fc region).

CP-1 is:



, known as m-PEG4-DBCO (CAS: 2228857-36-9), available for example from BroadPharm as BP-24030.

CP-2 is:



, known as m-PEG4-TCO, available for example from BroadPharm as BP-27872.

Example 16 – Affinity Capture for DAR Analysis

This method was used to capture antibody specific ADCs from a complex matrix of plasma for DAR analysis. f

ADCs 5 and 6 were synthesised as described in example 15 above.

Sample preparation was carried out as follows. Briefly, immunocapture was performed by adding 12 µL of biotinylated HER2 antigen (250 µg/mL) to 50 µL of plasma incubated ADC (0.1mg/mL). Subsequently, 50 µL of streptavidin beads (washed with DPBS) were added and the mixture was vortexed, finally the "Sample-Antigen-Beads complex" was rotated at 4 ºC for 2 hours at a rotation rate of approximately 20 rpm.

On bead reduction was carried out by washing the incubated complex three times with cold DPBS, followed by separating and transferring the flowthrough in microtubes with a magnetic stand for troubleshooting. 50 µl of 25 mM DTT was then added to the beads and they were incubated at 40°C for 30 min. The eluant was then collected on a magnetic stand and 5 µL of sample was injected for analysis.

DAR analysis was carried out as described in example 15 above.

The results are shown in figure 20. The results demonstrate that the ADCs show plasma stability for over 7 days in human plasma, with no significant drop in DAR.

Example 17 – Efficacy of dual payload ADCs with TMTHSI or DBCO moieties

The efficacy of dual payload ADCs with TMTHSI or DBCO moieties on NCI-N87 gastric cell line using 2D IVP was analysed using CellTiter-Glo® 2D Cell Viability Assay (Promega, #G9242).

Cells of the HER2-expressing cell line NCI-N87 (CRL-5822; gastric carcinoma) were seeded in 96-well white opaque plates in 175 µl of cell culture media, and incubated at 37°C, 5% CO_2 for 24 h. NCI-N87 were seeded at a density of 9000 cells/well.

The following agents were evaluated:

- ADC6
- ADC23

The agents were added to the cells in culture in aliquots of 25 µl., (from 0.33 uM, 10 points of 3-fold serial dilution).

The cells were then incubated for 5 days at 37°C and 5% CO_2. Post-incubation, 50 µl of CellTiter-Glo reagent was added to the plates and incubated for 25 mins with gentle agitation at 600 rpm. Cell viability was measured via luminescence using Victor Nivo, PerkinElmer (2 replicates).

Background luminescence was subtracted, based on luminescence detected from wells having media only (*i.e.* no cells). Percent inhibition was calculated using the formula 100- {(lum. of cells treated with test article / lum. of cells treated with buffer control) *100}, where lum.= luminescence. The average and SEM was calculated by GraphPad PRISM 10. Graphs were plotted using GraphPad PRISM 10, fitting the data points to a four-parameter logistic model.

The results are shown in figure 29. ADC6 is referred to as DBCO and ADC23 is referred to as TMTHSI in figure 29.

The data shows that ADCs with either TMTHSI or DBCO moieties show similar in vitro efficacy in killing NCI-N87 cells.

Example 18 – *In vitro* analysis of the efficacy of DDR inhibitor and TOP1 inhibitor dual payload ADCs

The efficacy of dual payload ADCs comprising various TOP1i and DDRi combinations - NCI-N87 gastric cell line using 2D IVP was analysed using the CellTiter-Glo® 2D Cell Viability Assay (Promega, #G9242).

Cells of the HER2-expressing cell line NCI-N87 (CRL-5822; gastric carcinoma) were seeded in 96-well white opaque plates in 175 µl of cell culture media, and incubated at 37°C, 5% CO_2 for 24 h. NCI-N87 were seeded at a density of 9000 cells/well.

The following agents were evaluated:

- ADC6 [T-(Exa+Ber)]
- ADC14 [T-(Exa+Ned)]
- ADC15 [T-(DXd+Ber)]

The agents were added to the cells in culture in aliquots of 25 µl, either alone or in combination, (from 0.33 uM, 10 points of 3-fold serial dilution).

The cells were then incubated for 5 days at 37°C and 5% CO_2. Post-incubation, 50 µl of CellTiter-Glo reagent was added to the plates and incubated for 25 mins with gentle agitation at 600 rpm. Cell viability was measured via luminescence using Victor Nivo, PerkinElmer (2 replicates. Background luminescence was subtracted, based on luminescence detected from wells having media only (*i.e.* no cells). Precent inhibition was calculated using the formula 100- {(lum of cells treated with test article / lum of cells treated with buffer control) *100}, where lum= luminescence. The average and SEM was calculated by GraphPad PRISM 10. Graphs were plotted using GraphPad PRISM 10, fitting the data points to a four-parameter logistic model.

The results are shown in figure 21. The data shows that dual payload ADCs with various TOP1i and DDRi combinations show comparable in vitro efficacy in the killing of NCI-N87 cells.

In further experiments the following agents were evaluated:

- ADC6 [T-(Exa+Ber)]
- ADC7 [T-Exa]
- ADC8 [T-Ber]
- ADC11 [T-(Exa+Ada)]
- ADC12 [T-Ada]

The analysis was carried out as described above. [T-Exa +T-Ber is ADC numbers 7 and 8]

The results are shown in figures 22 and 23. The results show that dual payload ADCs with TOP1i and DDRi show superior *in vitro* efficacy than single payload ADCs and their combination in the killing of NCI-N87.

Example 19 – In vivo efficacy comparing T-(exa+ber) vs single payload ADCs and their combination

The *in vivo* anti-cancer efficacy of dual and single payload ADCs was assessed in a mouse model.

In a first experiment, 6-8 week old, female NSG (NOD-*scid* IL2Rgamma^{null}) mice were injected subcutaneously at the right flank with 3 million JIMT-1 cells in admixture with Matrigel (#354234; Corning, USA). Treatment was initiated when tumors reached approximately 150 mm^3.

Mice were administered with vehicle, or with a single dose of one of the following agents, intravenously, at a dose of 1.5 mg/kg bodyweight:

- ADC7 [T-Exa]
- ADC8 [T-Ber]
- ADC5 [T-(Exa+Ber)]

Tumor volume was measured every 3 days using a Vernier-Caliper. Tumor growth inhibition (TGI) was calculated using the formula:

$$\frac{Tumor\ volume\ of\ treatment\ on\ day\ 27 - day\ 0}{Tumor\ volume\ of\ vehicle\ on\ day\ 27 - day\ 0} \times 100 = TGI\ \%$$

Average TGI, average body weight and SEM were calculated by GraphPad PRISM 10.

The results are shown in figure 24. The data demonstrates that the dual payload ADC is significantly more potent than the single payload ADCs and their combination in preventing the growth of JIMT-1 tumours *in vivo*.

Example 20 – *In vivo* efficacy; ADC assessment in JIMT-1 CDX model

The *in vivo* anti-cancer efficacy of dual payload ADCs with berzosertib (ATRi) or prexasertib (CHK1i) as DDRi was analyzed in a mouse model.

In a first experiment, 6-8 week old, female NSG (NOD-*scid* IL2Rgamma^{null}) mice were injected subcutaneously at the right flank with 3 million JIMT-1 in admixture with Matrigel (#354234; Corning, USA). Treatment was initiated when tumors reached approximately 150 mm^3.

Mice were administered with vehicle, or with a single dose of one of the following agents, intravenously, at a dose of 3 mg/kg bodyweight:

- ADC6 [T-(Exa+Ber)]
- ADC9 [T-(Exa+Prex)]

Tumor volume was measured every 3 days using a Vernier-Caliper. Tumor growth inhibition (TGI) was calculated using the formula:

$$\frac{Tumor\ volume\ of\ treatment\ on\ day\ 27 - day\ 0}{Tumor\ volume\ of\ vehicle\ on\ day\ 27 - day\ 0} \times 100 = TGI\ \%$$

Average TGI, average body weight and SEM were calculated by GraphPad PRISM 10

The results are shown in figure 25.

Example 21 – *In vivo* efficacy of dual payload ADCs with different target DAR ratios

The *in vivo* anti-cancer efficacy of dual payload ADCs with different target DAR ratios was analyzed in a mouse model.

In a first experiment, 6-8 week old, female NSG (NOD-*scid* IL2Rgamma^{null}) mice were injected subcutaneously at the right flank with 3 million JIMT-1 in admixture with Matrigel (#354234; Corning, USA). Treatment was initiated when tumors reached approximately 150 mm^3.

Mice were administered with vehicle, or with a single dose of one of the following agents, intravenously, at a dose of 3 mg/kg bodyweight:

- ADC6 [Tz-(Exa(4)+Ber(4))]
- ADC5 [Tz-(Exa(4)+Ber(2))]

Tumor volume was measured every 3 days using a Vernier-Caliper. Tumor growth inhibition (TGI) was calculated using the formula:

$$\frac{Tumor\ volume\ of\ treatment\ on\ day\ 27 - day\ 0}{Tumor\ volume\ of\ vehicle\ on\ day\ 27 - day\ 0} \times 100 = TGI\ \%$$

Average TGI, average body weight and SEM were calculated by GraphPad PRISM 10

The results are shown in figure 26.

Example 22– NHP tolerability and safety assessment

This experiment was performed according to the following study protocol (see below).

Briefly, four monkeys were assigned to 2 groups of 1/sex/group to determine the toxicity of the dual payload ADCs. ADC6 (T-(Exa+Ber) w administered once every three weeks by intravenous slow bolus injection (over 3-5 minutes) at 15 and 30 mg/kg/dose for two doses in total, on Days 1 and 22 followed by a 21-day recovery period. A complete necropsy was conducted on Study Day 44.

Start date	12 June 2024
Species/Dosing route	Cynomolgus monkey/IV (slow i.v. bolus, over 3-5 minutes)
Test article concentration	ADC6 at 15 and 30 mg/kg
Dosing schedule	Repeat dose: 2 total doses (Days 1 and 22) followed by a 3-week observation period

Number of animals/group	2 (1 male and 1 female)/group
In-life measurements	Daily observations of clinical signs, including local dosing site reactions and food consumption Post dose observations: 1, 4, and 8 hours post dose Body weight measured weekly
Clinical pathology	Haematology & Clinical chemistry: day -5, 4, 7, 15, 22(pre-dose), 26, 29, 44
Necropsy	Day 44
Microscopic pathology	Collect all tissues. Read heart, lung, liver, kidney, spleen, GI tract, bone marrow, gross lesions, injection site (for 30 mg/kg group). Read 15 mg/kg group if toxicity is observed in 30 mg/kg group

The body weight change results are shown in figure 27. Biochemistry and haematology results are shown in figure 28. The key findings are that after the 1st dose (day 1) no significant changes in body weight relative to pre-dose were observed. After the 1st dose, changes in biochemistry and hematology parameters were either transient or remained within the normal physiologic range and were not considered toxicologically adverse. After the 2nd dose (Day 22), data indicates no toxicologically adverse changes in biochemistry and hematology parameters, the results are shown in figure 28.

The key pathology findings were as follows:

Mortality

- There were no moribund or found dead animals during this study.

Organ Weights

- There were no test article-related organ weight changes in any treatment groups at the end of the study.
- All intergroup differences in organ weights in the study were not considered test article-related based on the absence of a dose-response, the absence of microscopic correlates, and the values being within normal biological variation in monkeys of this age

Gross Findings

- There were no test article-related macroscopic changes in any treatment groups at the end of the study.
- All macroscopic changes were not considered test article-related as they were considered common background or spontaneous findings consistent with the age and strain of this species.

Histopathology findings

- There were no test article-related microscopic findings at 30 mg/kg/dose at the end of study.
- The microscopic findings shown in individual animal data were considered common background or spontaneous findings consistent with the age and strain of this species.

Conclusion

There were no found dead or moribund animals during this study. There were no test article-related changes in organ weights, gross and histopathology (30 mg/kg/dose) examinations at the end of the study.

Data showing individual and absolute organ weights are given in tables D and E below.

Table D: Individual absolute and relative organ weights

Organ Weights	Group 1		Group 2	
	15 mg/kg/dose		30 mg/kg/dose	
	Male	Female	Male	Female
Terminal BW (kg)	2.540	2.310	3.310	2.77
Adrenals (g)	0.412	0.397	0.389	0.379
Adrenals/BW Ratio (10^{-3})	0.162	0.172	0.118	0.137
Adrenals/Brain Ratio	0.006	0.007	0.006	0.005
Brain (g)	64.251	60.256	68.787	72.128
Brain/BW Ratio (10^{-3})	25.296	26.085	20.782	26.039
Epididymides (g)	1.096	-	1.097	-
Epididymides/ BW Ratio (10^{-3})	0.431	-	0.331	-
Epididymides/Brain Ratio	0.017	-	0.016	-
Heart (g)	8.686	7.353	12.003	9.552
Heart/BW Ratio (10^{-3})	3.420	3.183	3.626	3.448
Heart/Brain Ratio	0.135	0.122	0.174	0.132
Kidneys (g)	10.814	10.616	14.473	11.991
Kidneys/ BW Ratio (10^{-3})	4.257	4.596	4.373	4.329
Kidneys/Brain Ratio	0.168	0.176	0.210	0.166
Liver (g)	44.420	46.061	61.586	59.385
Liver/BW Ratio (10^{-3})	17.488	19.940	18.606	21.439
Liver/Brain Ratio	0.691	0.764	0.895	0.823
Pituitary (g)	0.053	0.053	0.053	0.063
Pituitary/BW Ratio (10^{-3})	0.021	0.023	0.016	0.023
Pituitary/Brain Ratio	0.825	0.88	0.770	0.873

Table E: Individual absolute and relative organ weights

Organ Weights	Group 1		Group 2	
	15 mg/kg/dose		30 mg/kg/dose	
	Male	Female	Male	Female
Prostate (g)	0.353	-	0.542	-
Prostate/ BW Ratio (10^{-3})	0.139	-	0.164	-
Prostate/Brain Ratio	0.005	-	0.008	-
Spleen (g)	2.257	2.032	5.015	3.519
Spleen/BW Ratio (10^{-3})	0.889	0.880	1.515	1.270
Spleen/Brain Ratio	0.035	0.034	0.073	0.049
Testes (g)	2.730	-	1.947	-
Testes/ BW Ratio (10^{-3})	1.075	-	0.588	-
Testes/Brain Ratio	0.042	-	0.028	-
Thymus (g)	3.111	1.443	3.115	2.513
Thymus/8W Ratio (10^{-3})	1.225	0.625	0.941	0.907
Thymus/Brain Ratio	0.048	0.024	0.045	0.035
Thyroids (g)	0.276	0.188	0.258	0.237
Thyroids/ BW Ratio (10^{-3})	0.109	0.081	0.078	0.086
Thyroids/Brain Ratio	0.004	0.003	0.004	0.003
Ovaries (g)	-	0.211	-	0.277
Ovaries/ BW Ratio (10^{-3})	-	0.091	-	0.100
Ovaries/Brain Ratio	-	0.004	-	0.004
Uterus (g)	-	4.575	-	8.898
Uterus/ BW Ratio (10^{-3})	-	1.981	-	3.212
Uterus/Brain Ratio	-	0.076	-	0.123

The data in tables D and E demonstrate that there were no test article-related organ weight changes in any treatment groups at the end of the study. There were no test article-related macroscopic changes in any treatment groups or microscopic changes in the 30 mg/kg group at the end of the study. All values were within the normal biological variation in monkeys of this age.

Data for each of the individual animals is given in tables F-I below

Table F: Data for individual animals

Animal: 1001	Group: 1	Sex: Male
Species: Non-Human Primate (Macaca fascicularis)	Strain: Cynomolgus	Study Type: Intermittent Repeated Dose
	Dose: 15 mg/kg/dose	
Death Date: 2024-07-25	Removal Reason: Scheduled Euthanasia-Terminal	
Necropsy Date: 2024-07-25	Study Day (Week) of Death: 44 (7)	
		Gross Status: Complete
		Histo Status: Complete

Gross Pathology Observations [Correlation]:

Any remaining protocol required tissues, which have been examined, have no visible lesions.

Table G: Data for individual animals

Animal: 2001	Group: 2	Sex: Male
Species: Non-Human Primate (Macaca fascicularis)	Strain: Cynomolgus	Study Type: Intermittent Repeated Dose
	Dose: 30 mg/kg/dose	
Death Date: 2024-07-25	Removal Reason: Scheduled Euthanasia-Terminal	
Necropsy Date: 2024-07-25	Study Day (Week) of Death: 44 (7)	
		Gross Status: Complete
		Histo Status: Complete

Gross Pathology Observations [Correlation]:

Any remaining protocol required tissues, which have been examined, have no visible lesions.

Histopathology Observations [Correlation]:

Kidneys : Infiltration Mononuclear; Multifocal, Unilateral, Minimal

Liver : Infiltration, Mononuclear; Multifocal, Minimal

Any remaining protocol required tissues, which have been examined, have no visible lesions.

Table H: Data for individual animals

Animal: 1501	Group: 1 Strain: Cynomolgus	Sex: Female
Species: Non-Human Primate (Macaca fascicularis)		Study Type: Intermittent Repeated Dose
	Dose: 15 mg/kg/dose Removal Reason: Scheduled Euthanasia-Terminal	
Death Date: 2024-07-25		
Necropsy Date: 2024-07-25	Study Day (Week) of Death: 44 (7)	
		Gross Status: Complete
		Histo Status: Complete

Gross Pathology Observations [Correlation]:

Lungs With Mainstem Bronchi : Discoloration; Red, Focal : (Comment) Noted at the right caudal lobe, approximately 3.0 cm X 1.5 cm in size. (TLG) [Lungs With Mainstem Bronchi : Hemorrhage; Postmortem, Alveolus; Locally Extensive, Mild (H)]

Any remaining protocol required tissues, which have been examined, have no visible lesions.

Histopathology Animal Details:

No animal details found

Histopathology Observations [Correlation]:

Lungs With Mainstem Bronchi : Hemorrrhage; Postmortem, Alveolus; Locally Extensive, Mild [Lungs With Mainstem Bronchi : Discoloration; Red, Focal : (Comment) Noted at the right caudal lobe, approximately 3.0 cm X 1.5 cm in size. (G)]

Histopathology - The following Tissues were Not Examined:

None

Histopathology - The following Protocol Required Tissues were Not Processed:

None

Table I: Data for individual animals

Animal: 2501	Group: 2 Strain: Cynomolgus	Sex: Female
Species: Non-Human Primate (Macaca fascicularis)		Study Type: Intermittent Repeated Dose
	Dose: 30 mg/kg/dose Removal Reason: Scheduled Euthanasia-Terminal	
Death Date: 2024-07-25		
Necropsy Date: 2024-07-25	Study Day (Week) of Death: 44 (7)	
		Gross Status: Complete
		Histo Status: Complete

Gross Pathology Observations [Correlation]:

Any remaining protocol required tissues, which have been examined, have no visible lesions.

Histopathology Animal Details:

No animal details found

Histopathology Observations [Correlation]:

Kidneys: Infiltration Mononuclear; Multifocal, Unilateral, Minimal

Liver: Infiltration, Mononuclear; Multifocal, Minimal

Any remaining protocol required tissues, which have been examined, have no visible lesions.

Histopathology - The following Tissues were Not Examined:

None

Histopathology - The following Protocol Required Tissues were Not Processed:

None

Example 23 – *In vitro* analysis of efficacy of DDR inhibitor + TOP1 inhibitor dual payload ADC

The ability of ADCs comprising both DDR inhibitor + TOP1 inhibitor payloads to kill cells expressing the target antigen of the ADC was analyzed *in vitro*, using the CellTiter-Glo® 3D Cell Viability Assay (Promega, #G9682).

Cells of the HER2-expressing cell lines NCI-N87 (CRL-5822; gastric carcinoma), JIMT-1 (ACC 589; breast ductal carcinoma) or HEC-1-B (HTB-113; endometrial adenocarcinoma) cell lines were seeded in wells of a 96-well U bottom Ultra Low Attachment plate in 175 µl of cell culture media, and incubated at 37°C and 5% CO_2. Cells were incubated for 3 days to form spheroids prior treatment. JIMT-1 cells were seeded at 2500 cells/well, NCI-N87 cells were seeded at 5000 cells/well and HEC-1-B cells were seeded at 10,000 cells/well.

The following agents were evaluated:

- ADC1. Referred to in Figure 2 as 'T-(Exa+Ber)'.
- Trastuzumab deruxtecan (T-DXd) (DrugBank Acc. No. DB14962; marketed as Enhertu®). trastuzumab deruxtecan (T-DXd) comprises the TOP1 inhibitor DXD(1) conjugated to trastuzumab via a cathepsin-cleavable tetrapeptide linker. Referred to in Figure 2 as 'T-DXd'.
- Isotype-matched control antibody conjugated to exatecan. Referred to in Figure 2 as 'Isotype Control'.

The agents were added to the cells in culture at concentrations providing matched Ab concentrations, in a 25 µl volume. The antibody - concentration provided to the cells was 333 nM.

The cells were then incubated at 37°C and 5% CO_2 for 7 days. 50 µl of CellTiter-Glo reagent was subsequently added to the plates and incubated for 25 min, with gentle agitation at 600 rpm. 100 µl of the cell culture supernatant was then transferred to wells of 96-well, opaque white plates for analysis of luminescence, using a Victor Nivo plate reader (PerkinElmer).

Background luminescence was subtracted, based on luminescence detected from wells having media only (*i.e.* no cells). Percent cell death (as determined by reduction in luminescence, as a correlate of cell viability) was calculated using the formula 100- [(lum. reading for cells treated with drug / lum. reading for cells treated with buffer control) *100], where lum. = luminescence. Mean values and SEM were calculated using GraphPad PRISM 9 software.

The results are shown in Figure 2. The dual TOP1 inhibitor + DDR inhibitor ADC1 displayed superior cell killing efficacy against cells expressing the target antigen for ADC, as compared to the ADC comprising the same antibody and having only a TOPPO1 inhibitor payload (T-DXd). Importantly, the dual TOP1 inhibitor + DDR inhibitor ADC displayed dramatically increased cell killing efficacy against cells that were insensitive to treatment with the ADC comprising the same antibody and having only a TOP1 inhibitor payload.

In further experiments, cells of the following HER2-expressing human cancer cell lines were evaluated:

- BT-474 (HTB-20) – breast ductal carcinoma
- NCI-N87 (CRL-5822) – gastric carcinoma
- JIMT-1 (ACC 589) – breast ductal carcinoma
- HEC-1-B (HTB-113) – endometrial adenocarcinoma
- HCT116 (CCL-247) – colorectal carcinoma

And the following HER2-specific antigen-binding molecules were evaluated:

- Trastuzumab. Referred to in Figures 7A to 7E as 'T (naked)'.
- ADC1. Referred to in Figures 7A to 7J as 'T-(Exa+Ber)'.
- Trastuzumab deruxtecan (T-DXd) (see above). Referred to in Figures 7A to 7J as 'T-DXd'.
- Isotype-matched control antibody - conjugated to exatecan. Referred to in Figures 7A to 7J as 'Isotype Control'.

The cells were seeded in 96-well white opaque plates in 175 µl of media, and incubated at 37°C with 5% CO_2 for 24 hours. HCT-116 and JIMT-1 were seeded at 3000 cells/well, HEC-1-B, NCI-N87 and BT-474 were seeded at 5000 cells/well. The next day, different agents were added to the cells in culture at 333 nM concentration (for 'antibody concentration matched' experiments), or at concentrations providing equivalent concentrations of payloads of 0.857 nM (for 'payload concentration matched' experiments), The cells were then incubated at 37°C and 5% CO2 for 3 days. 50 µl of CellTiter-Glo reagent was subsequently added to the plates and incubated for 25 min, with gentle agitation at 600 rpm. 100 µl of the cell culture supernatant was then transferred to wells of 96-well, opaque white plates for analysis of luminescence, using a Victor Nivo plate reader (PerkinElmer).

The results are shown in Figures 7A to 7J. The dual TOP1 inhibitor + DDR inhibitor ADC (T-(Exa+Ber)) displayed superior cell killing efficacy against cells expressing the target antigen for ADC, as compared to the ADC comprising the same antibody and having only a TOP1 inhibitor payload (T-DXd). As above, the dual TOP1 inhibitor + DDR inhibitor ADC displayed dramatically increased cell killing efficacy against cells that were insensitive to treatment with the ADC comprising the same antibody and having only a TOP1 inhibitor payload.

Example 24 – In vivo analysis of efficacy of DDR inhibitor + TOP1 inhibitor dual payload ADC

The *in vivo* anti-cancer efficacy of an ADC comprising DDR inhibitor + TOP1 inhibitor payloads was analyzed in CDX mouse models.

In a first experiment, 4-5 week-old female NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice were injected subcutaneously at the right flank with 3 x 10^6 JIMT-1 cells, in admixture with Matrigel (#354234; Corning, USA). Treatment was initiated when tumors reached approximately 100 to 300 mm^3.

Mice were administered with vehicle, or with a single dose of one of the following agents, intravenously, at a dose of 3 mg/kg bodyweight:

- ADC1. Referred to in Figures 3 and 4 as 'T-(Exa+Ber)'.

- Trastuzumab deruxtecan (T-DXd) (see Example 23). Referred to in Figures 3 and 4 as 'T-DXd'.

Tumor volume was measured 2 times a week using a digital caliper, and calculated using the formula [L x W x W)/2]. Bodyweights of the mice were also monitored.

The results are shown in Figures 3 and 4. The DDR inhibitor + TOP1 inhibitor dual payload ADC was found to be extremely potent at inhibiting tumor growth, and much more so than the ADC comprising the same antibody and having only a TOP1 inhibitor payload (T-DXd) (Figure 3). The DDR inhibitor + TOP1 inhibitor dual payload ADC was also well-tolerated, with no significant change in bodyweight observed (Figure 4).

Example 25 – Effect of dual conjugation of DDR inhibitor + TOP1 inhibitor payloads on binding to cells expressing the target antigen of the ADC

The ability of an antibody, and different ADCs comprising the antibody and having different payloads, to bind to cells expressing the target antigen of the antibody was evaluated by flow cytometry.

Cells of the following HER2-expressing human cancer cell lines were evaluated:

- BT-474 (HTB-20) – breast ductal carcinoma
- NCI-N87 (CRL-5822) – gastric carcinoma
- JIMT-1 (ACC 589) – breast ductal carcinoma
- HEC-1-B (HTB-113) – endometrial adenocarcinoma
- HCT-116 (CCL-247) – colorectal carcinoma

Binding of the following HER2-specific antigen-binding molecules was evaluated:

- Trastuzumab (anti-HER2). Referred to in Figures 5A and 5B as 'T (naked)'.
- ADC1. Referred to in Figures 5A and 5B as 'T-(Exa+Ber)'.
- Trastuzumab deruxtecan (T-DXd) (see Example 23). Referred to in Figures 5A and 5B as 'T-DXd'.
- Isotype-matched control antibody - conjugated to exatecan. Referred to in Figures 5A and 5B as 'Isotype (Exa)'.
- Isotype-matched control antibody (Ultraleaf IgG1, Biolegend Cat #403502). Referred to in - Figures 5A and 5B as 'Isotype (Naked)'.

Briefly, cells were harvested and stained with Zombie NIR fixable live/dead stain in PBS at 4°C (1:3000, 15 minutes) and washed twice with FACS buffer (PBS+ 2mM EDTA+ 1% BSA). Cells were then seeded in wells of 96-well plates at 80,000 cell/well. The indicated antigen-binding molecules were added to wells in 12-point, 6-fold dilution series, starting from a concentration of 33.4 nM. Cells were incubated with the antigen-binding molecules for 1 h at 4°C. Cells were subsequently washed twice with FACS buffer, and stained with Alexa Fluor™ 647-conjguated Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody (Thermo Fisher Scientific, Cat #A21445) at a 1:1000 dilution, and incubated for 20 min at 4°C. Cells were subsequently washed twice with FACS buffer, and events were acquired using a Cytek

Northern Lights flow cytometer. The data were analyzed using FlowJo software (version 10.1). The percentage of live cells that were stained by the antigen-binding molecules was determined. Graphs were plotted using GraphPad PRISM 9, fitting the data points to a four-parameter logistic model. EC_{50} values for the % binding (*i.e.* the percentage of the live cells that are positive for HER2 as determined by staining with the relevant antigen-binding molecule) and mean fluorescence intensities (MFIs) were determined.

The results are shown in Figures 5A and 5B, and summarised in the tables below, in which T-DXd is Trastuzumab deruxtecan:

	EC_{50} (M, % Binding)		
Antibody	**ADC1**	**T-DXd**	**Trastuzumab (T)**
BT-474	1.143E-11	1.672E-11	1.906E-11
NCI-N87	2.151E-12	3.132E-12	2.771E-12
JIMT-1	4.558E-12	5.762E-12	4.745E-12
HEC-1-B	3.538E-12	4.708E-12	4.195E-12
HCT116	1.142E-11	~ 2.006e-011	1.342E-11

	EC_{50} (M, MFI)		
Antibody	**ADC1**	**T-DXd[2]**	**Trastuzumab (T)**
BT-474	3.128E-09	6.572E-09	8.755E-09
NCI-N87	1.69E-09	2.591E-09	3.294E-09
JIMT-1	1.556E-09	1.966E-09	1.433E-09
HEC-1-B	6.11E-10	6.909E-10	6.156E-10
HCT116	5.682E-10	7.306E-10	6.131E-10

Conjugation of the antibody to the different payloads was found not to influence the level of binding to cells expressing the target antigen for the antibody.

No difference in the level of binding to cells expressing the target antigen was observed between ADCs comprising DXd, and dual payload ADCs comprising both DDR inhibitor + TOP1 inhibitor payload moieties.

Example 26 – Effect of dual conjugation of DDR inhibitor + TOP1 inhibitor payloads on internalization by cells expressing the target antigen of the ADC

The ability of different ADCs comprising an antibody and having different payloads, to be internalized by cells expressing the target antigen of the antibody was evaluated by immunofluorescence microscopy.

Cells of the following HER2-expressing human cancer cell lines were evaluated:

- NCI-N87 (CRL-5822) – gastric carcinoma; 4 x 10^4 cells/well
- HEC-1-B (HTB-113) – endometrial adenocarcinoma; 4 x 10^4 cells/well

Internalization of the following HER2-specific antigen-binding molecules was evaluated:

- ADC1. Referred to in Figures 6A and 6B as 'T-(Exa+Ber)'.
- Trastuzumab deruxtecan (T-DXd) (see Example 23). Referred to in Figures 6A and 6B as 'T-DXd'.

Briefly, 12-well chamber slides (Ibidi, Cat # 81201) were coated with poly-D-lysine (Gibco, Cat # A3890401) in accordance with the manufacturer's instructions. Chamber slides were washed twice with PBS, and cells were plated in 100 µl of RPMI medium/well and incubated overnight at 37°C, 5% CO_2. Slides were then placed for 30 min on ice, and then treated with 0.5 µg/ml of the relevant antigen-binding molecule, reconstituted in 100 µl of RPMI medium for 30 min, 2 h, 6 h or 24 h at 37°C, 5% CO_2. Control slides (0 h) were incubated on ice for 45 min. Post-treatment, slides were fixed with 200 µl of ice-cold 4% PFA (Thermofisher scientific, Cat #J61899.AK) for 30 min on ice, and quenched with 0.1 M Glycine for 15 min at room temperature (RT). Slides were then washed twice with ice-cold PBS, and blocked and permeabilized using BP-staining buffer (0.4% Saponin, 1% BSA, 2% normal goat serum in PBS) for 1 h at RT. Subsequently, slides were incubated with 1 µg/ml Alexa Fluor™ 647-conjguated Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody (Thermo Fisher Scientific, Cat #A21445) for 1 h at RT.

Slides were then washed twice with BP-staining buffer and incubated with 100 µl of BP-staining buffer constituting primary antibody cocktail (5 µg/ml of RAB5A Monoclonal Antibody (Invitrogen, Cat # 2E8B11) for endosomal staining, and 1 µg/ml of FITC anti-human CD107a (LAMP-1) Antibody (Biolegend, Cat # 32806) for lysosomal staining). Slides were incubated overnight at 4°C and washed thrice with PBS. Subsequently, slides were incubated with 2 ug/ml of Alexa Fluor™ 594-conjguated Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Invitrogen Cat #A11032) for 1 h at RT in BP-staining buffer. Cells were then washed twice with PBS and incubated in DAPI staining solution (1:100 in BP-staining buffer) for 30 min at RT. Silicon separators were gently removed, and slides were left to airdry in the dark for 10 min. Slides were mounted using Prolonged Diamond mounting medium (Invitrogen, Cat #P36961) and stored overnight at 4°C. Slides were imaged using Leica DMi8 Microscope, using lasers 405, 488, 594 and 647. Images were analyzed using LAS X software.

The results are shown in Figures 6A and 6B. Both ADCs (ADC1) and T-DXd) were detected on the cell membrane at 0 h, and within the cells at other time points tested. Localization of both ADCs to the endosome (as determined by co-staining with anti-RAB5) was observed at 30 min and 2 h time points. Localization of both ADCs within the lysosome was also observed at 2 h time point (as indicated by co-staining with anti-LAMP-1).

No differences in internalization rate and intracellular trafficking were observed between ADCs comprising DXd, and dual payload ADCs comprising both DDR inhibitor + TOP1 inhibitor payload moieties.

Example 27 – Effect of DDR inhibitors and TOP1 inhibitors on the DNA damage response signaling pathway

The effects of DDR inhibitor and TOP1 inhibitors, alone and in combination, on DNA damage response (DDR) signalling was analysed *in vitro*.

JIMT-1 (lowTOP1 inhibitor sensitivity, breast cancer cell line) or HCT-116 (high TOP1 inhibitor sensitivity, CRC) cells were seeded in 10 cm plates at 1 and 0.8 x 10^6 cells per/well respectively, using complete growth media (RPMI and McCoy respectively). Following overnight incubation, cells were serum starved using serum free media for at least 18 h. Cells were then treated with varying concentrations of berzosertib and/or exatecan for 24 h and 72 h, using complete growth media. Following incubation, cells were harvested using accutase, and lysed in IP lysis buffer (ThermoScientific, cat no. 87788) according to manufacturer's protocol and quantified using BCA assay (ThermoScientific, cat no. 23225). 50 µg of each protein sample was fractionated by SDS-PAGE and transferred to PVDF membrane using Bio-Rad semi-dry transfer cell. Membranes were blocked with 5% milk in TBS and immunoblotted with the indicated primary antibodies: pATR S428 (CS2853) 1:1000, overnight incubation at 4°C; pCHK1 S317 (CS2344) 1:1000, overnight incubation at 4°C; CHK1 (CS2360) 1:1000, overnight incubation at 4°C; pH2AX S139 (CS9718) 1:1000, overnight incubation at 4°C; β-Actin (8H10D10) (CS3700S) 1:1000, overnight incubation at 4°C. Following incubation, blots were washed thrice with TBST and incubated with either anti-rabbit HRP (CS7074S) 1:1000 or anti-mouse HRP (CS7076S) secondary antibody used at 1:1000 dilution for 1 hr at room temperature. Blots were visualized with Bio-Rad Clarity Western ECL substrate (BioRad, cat no. 170-5061) and a ChemiDoc Imaging System (BioRad).

The exatecan and berzosertib combination significantly inhibits DNA damage response signaling (Figure 10, Figure 11). Exatecan alone induces pATR and pCHK1 of the DDR signaling pathway as well as DNA damage marker pH2AX. Addition of berzosertib to exatecan blocks DDR signaling pathway as seen by the decrease in pATR and pCHK1 and further increase in pH2AX indicating enhanced DNA damage.

Example 28 –Toxicology of DDR inhibitor + TOP1 inhibitor free payload combination

A free payload combination toxicology study was performed to assess synergistic or overlapping toxicities for the payloads in combination. The toxicological effects of exatecan and berzosertib in combination were investigated in female Sprague-Dawley rats aged 6 to 8 weeks old.

The following agents and concentrations were used:

- Vehicle (20% DMSO (MedChemExpress,#HY-Y0320), 45% PEG300 (Selleckchem,#S6704), 5% Tween 20 (MedChemExpress,#HY-Y1891), 35% PBS (Sigma, #D8537-500 ML))
- Exatecan (MedChemExpress,#HY-13631); Concentration: 1 mg/kg, 3 mg/kg, 10 mg/kg, 30 mg/kg
- Berzosertib (Selleckchem, #S7102); Concentration: 35 mg/kg
- Exatecan and berzosertib combination; Concentration: 1 mg/kg Exa + 35 mg/kg Ber, 3 mg/kg Exa +35 mg/kg Ber, 10 mg/kg Exa +35 mg/kg Ber, 30 mg/kg Exa +35 mg/kg Ber

Rats were administered with the agents intravenously on Day 1 and Day 8.

Mortality observations and clinical observations were taken for all animals once daily. Body weight was assessed for all animals once pre-study and weekly thereafter.

Clinical chemistry and haematology assessments were performed on Day 2, Day 7, Day 9 and Day 11. 50 µl of fresh blood for haematology was collected via cheek bleed using EDTA as anti-coagulant and analysed using Vetscan HM5 analyser (Zoetis). 100 µl of fresh blood for biochemistry was collected via cheek bleed using lithium heparin as anti-coagulant and analysed using Vetscan VS2 chemistry analyser (Abaxis) using VetScan Comprehensive Diagnostic Profile reagent rotor (ZOETIS #10023219). Data were analyzed using Excel and GraphPad PRISM.

The results show that exatecan alone or in combination with berzosertib was well tolerated up to 10 mg/kg, and that berzosertib alone or in combination with exatecan (up to 10 mg/kg), was well tolerated at 35 mg/kg. No animal deaths were observed at 35 mg/kg berzosertib and 1, 3, and 10 mg/kg exatecan doses, alone or in combination (Figure 12). No significant changes in body weight were observed for single or combination payload arms relative to vehicle (Figure 13).

At the tolerated doses, there were no significant differences in toxicity observed between the combination and single payload arms. The payload combination did not result in any additional/unexpected toxicity or exacerbation of toxicity signals over single payloads. No significant change in body weight were observed at tolerated doses for exatecan and/or berzosertib. At tolerated doses, all differences in haematology and clinical chemistry test results were either transient, negligible in magnitude, within the normal biological variation, and/or lacked a dose relationship (Figure 14, Figure 15).

The 10 mg/kg tolerated dose for exatecan provides a 5X safety margin relative to the total amount of exatecan delivered in an ADC format at the maximum dose routinely tested for ADCs in rodent toxicology studies. The 35 mg/kg tolerated dose for berzosertib provides a 15X safety margin relative to the total amount of berzosertib delivered in an ADC format at the maximum dose routinely tested for ADCs rodent toxicology studies.

Example 28 – Effect of dual conjugation of DDR inhibitor + TOP1 inhibitor on in vivo efficacy

The efficacy of different anti-HER2 ADCs was evaluated *in vivo* in a T-DXd (Enhertu) resistant HER2++ gastric cancer cell line NCI-N87-derived xenograft model.

Female NOD/SCID mice (6 to 8 weeks old) were injected (subcutaneous, right flank) with $8x10^6$ Enhertu-resistant NCI-N87 cells in admixture with Matrigel (Corning, Cat# 354234).

Mice were administered with vehicle (PBS) or with a single dose of one of the following agents, intravenously, at the indicated doses.

- Trastuzumab deruxtecan (T-DXd) (see hereinabove). Referred to in Figure 16 as ‘T-DXd’. Concentration: 3 mg/kg

- ADC2. Referred to in Figure 16 as 'T-(Exa+Ber)'. Concentration: 3 mg/kg (concentration matched), and 9 mg/kg (DAR matched).

Treatment was initiated once the tumour reached an average of 150 mm^3. Seven mice were used per arm. Tumour volume was measured every 3 days using Vernier-Caliper (Figure 16). Tumour growth inhibition (TGI) was calculated using the formula:

TGI = (Tumor volume of treatment on day 29 – Tumor volume of treatment on day 0) / (Tumor volume of vehicle on day 29 – Tumor volume of vehicle on day 0)

T-(Exa+Ber) showed superior *in vivo* efficacy over T-DXd across concentrations (Figure 16).

Example 29 – Bystander effect of DDR inhibitor + TOP1 inhibitor dual payload ADC

The ability of ADCs having different payloads to kill bystander cells was assessed *in vitro*. Bystander killing occurs when the drug from an ADC is released either from the target cell following internalization and degradation of the ADC or release of the drug within the extracellular space. In both cases, the drug is then taken up and kills surrounding or bystander cells, which themselves may or may not express the ADC target antigen.

HER2-positive NCI-N87-GFP and HER2-negative MDA-MB-231-mcardinal cells were co-cultured and tested with the following ADCs:

- ADC2. Referred to in Figure 17 as 'T-(Exa+Ber)'.
- Trastuzumab deruxtecan (T-DXd) (see Example 23). Referred to in Figure 17 as 'T-DXd'.

A 96 well plate was seeded with 1500 cell/well of MDA-MB-231 m-cardinal and 7,500 cell/well of NCI-N87 GFP in RPMI media with 10% FBS. The ratio of MDA-MB-231 : NCI-N87 used was 1:5. Cells were incubated for 24 hours at 37°C with 5% CO_2. Following incubation, cells were treated with serially diluted concentrations of the test ADCs. A 3-fold dilution of test ADC was used with either the exatecan payload concentration staring from 0.032 nM or antibody concentration starting from 12.3 nM. The plate was then incubated at 37°C with 5% CO_2 for 5 days within the Incucyte® S3 Live-Cell Analysis System machine. During the incubation period, 4 images/well were captured every 8 hours. Images were analyzed using Incucyte 2022revB software and Red Area Confluence (%) was measured. % Inhibition is relative to cells treated with only buffer (PBS) and was calculated as follows, with values plotted using GraphPad PRISM.

% Inhibition = 100 - {(OD of cells treated with drug / OD of cells treated with buffer control) *100}

T-(Exa+Ber) results in a bystander effect, causing the dose dependent cell death of HER2-negative MDA-MB-231 cells when co-cultured with HER2-postiive NCI-N87 cells (Figure 17). The observed bystander killing is comparable to that of T-DXd at both payload concentration matched and antibody concentration matched ADC doses.

Example 30 – *In vitro* analysis of ADC hydrophobicity and macropinocytosis by megakaryocytes

Macropinocytosis is implicated in clinical toxicities such as e.g. thrombocytopenia and interstitial lung disease (see Inhibition of Megakaryocyte Differentiation by Antibody-Drug Conjugates (ADCs) is Mediated by Macropinocytosis: Implications for ADC-induced Thrombocytopenia (PMID: 28655784) and Mechanisms of ADC Toxicity and Strategies to Increase ADC Tolerability (PMID: 36765668)). Macropinocytosis-mediated internalisation of an ADC comprising DDR inhibitor + TOP1 inhibitor payloads by human megakaryocytes was analyzed.

The following ADCs were tested:

- ADC2. Referred to in Figure 18 as 'T-(Exa+Ber)'.
- Trastuzumab deruxtecan (T-DXd) (see Example 23). Referred to in Figure 18 as 'T-DXd'.

CD34+ hematopoietic stem cells (HSCs) (STEMcell, # 70002) were differentiated into megakaryocytes using StemSpan SFEM II media (Stemcell, # 9655) supplemented with StemSpan megakaryocyte expansion supplement (STEMcell, #2696) at dilution of 1:100 with 10 ng/ml IL-1 (R&D, # 201-LB-010) and 10 ng/ml IL-3 (R&D, # 203-LB-010). After 3 weeks, differentiated megakaryocytes were used for the macropinocytosis assay. A 96-well plate was coated with poly-L-lysine (Sigma Aldrich, #P4707) and washed with PBS prior to seeding. Megakaryocytes were seeded in the 96-well plate (20,000 cells/well/80 µl) using SFEM II media (STEMcell technologies,#9655) + StemSpan CC100 (STEMcell technologies, #2690) for 24 hours at 37°C with 5% CO_2. 100 ng/ml of ADC was conjugated with Human Fabfluor-pH Red (Sartorius, #4722) at a ratio of 1:3. 20 µL of ADC was added into the wells. The plate was then incubated at 37°C with 5% CO_2 for 2 days within the Incucyte® S3 live-cell analysis machine. During the incubation period, 5 images/well were captured every 2 hours. Images were analyzed using cell-by-cell analysis software (Incucyte) and mean red fluorescence intensity per cell was measured. Values were normalized to the 0 timepoint and the ratio of normalized AUC of T-DXd to T-(Exa+Ber) was plotted using GraphPad PRISM.

Results showed that macropinocytosis-dependent uptake of T-(Exa+Ber) by megakaryocytes is significantly less than uptake of T-DXd. T-(Exa+Ber) uptake is 37% lower compared to T-DXd (Figure 18).

ADC hydrophobicity has been correlated with increased off-target uptake by macropinocytosis. Hydrophobicity of T-(Exa+Ber) was assessed using hydrophobic interaction chromatography (HIC). Hydrophobicity was measured as an increase in HIC retention time (RT) relative to the unconjugated antibody. Results showed a ~2 to 3-fold lower hydrophobicity compared to earlier clinical stage ADCs with high DAR (>4) (Figure 19). The lower hydrophobicity may account for the observed lower macropinocytosis-dependent uptake of T-(Exa+Ber).

Example 31 – Clearance in immunocompetent mice

The impact of dual payload conjugation on the clearance of anti-HER2 ADC was further assessed in immunocompetent mice.

Female immunocompetent BALB/c mice aged 6 to 8 weeks were used in the experiment.

Test agents and concentrations used were as follows:

- ADC5. Concentration: 10 mg/kg
- ADC4. Concentration: 10 mg/kg
- Tratuzumab-N297A (unconjugated). Concentration: 10 mg/kg

Agents were administered intravenously (tail vein injection, slow IV) with Dose 1 at Day 0 and Dose 2 at Day 15. Blood was collected at 24 hours, 96 hours, 168 hours and 336 hours post-dose 1 and 24 hours, 96 hours, 168 hours, 336 hours and 504 hours post-dose 2. Blood was collected via cheek bleed in the absence of anti-coagulant and incubated at room temperature for 30 mins, following which the samples were centrifuged at 2000 g at 4°C for 15 mins. Serum was aliquoted carefully without disturbing the pellet.

HER2-capture ELISA was used to measure antibody concentration in mouse serum. Mouse serum samples from BALB/c dosed intravenously with the test agents were serially diluted (in PBS with 1% BSA) using a half log dilution series starting from 1:20 for 11 points. For the standard curve 2-fold serial dilution for 23 points starting from 5 µg/ml of either unconjugated or conjugated Tratuzumab was used.

384-well high binding plates (Corning, #3700) were coated with 1 µg/mL of Human HER2-His protein (Sino biologicals, #10004-H08H) overnight at 4°C. Subsequently, plates were washed and blocked with PBS with 1% BSA for 1 h at room temperature (RT). Plates were then washed 3x with PBS with 0.05% Tween20 (0.05% PBS-T), before being incubated with either serially diluted serum samples or the standards for 1 h at RT.

Plates were then washed thrice with 0.05% PBS-T before being incubated with HRP-Goat anti-Human secondary antibody (Abcam#ab97225) diluted at 1:7000 for 1 h at RT. Thereafter, plates were washed 4 times with 0.05% PBS-T and were developed with 1-step™ Turbo TMB-ELISA Substrate Solution (ThermoFisher, #34022) for 15 mins in the dark. The reaction was stopped with STOP solution (ThermoFisher, #SS04).

Absorbance was read at 450nm using a Victor Nivo 3T Multimode Plate Reader (PerkinElmer). Background was subtracted by subtracting the optical density (OD) of wells with only blocking buffer and no sample (negative control) from OD of the wells serially diluted test articles. The concentration of either unconjugated or conjugated Tratuzumab in mouse serum was then calculated by extrapolating values from the standard curve. Data were analysed using GraphPad PRISM.

Higher immunogenicity is associated with faster clearance and poor pharmacokinetics of ADCs. In the immunocompetent mice, no difference in clearance was observed between unconjugated or conjugated Tratuzumab (Figure 30), demonstrating that conjugation of the dual payload does not induce any unanticipated immunogenicity with no difference between the different click moieties (TMTHSI or DBCO).

Example 32 – *In vitro* analysis of the efficacy of DDR inhibitor and TOP1 inhibitor dual payload ADCs

The efficacy of dual payload ADCs on NCI-N87 gastric cell line was investigated using 2D IVP was analysed using the CellTiter-Glo® 2D Cell Viability Assay (Promega, #G9242).

Cells of the HER2-expressing cell line NCI-N87 (CRL-5822; gastric carcinoma) were seeded in 96-well white opaque plates in 175 µl of cell culture media, and incubated at 37°C, 5% CO_2 for 24 h. NCI-N87 were seeded at a density of 9000 cells/well.

The following agents were evaluated:

- ADC5
- ADC6
- ADC19

The agents were added to the cells in culture in aliquots of 25 µl, either alone or in combination, (from 0.33 µM, 10 points of 3-fold serial dilution).

The cells were then incubated for 5 days at 37°C and 5% CO_2. Post-incubation, 50 µl of CellTiter-Glo reagent was added to the plates and incubated for 25 mins with gentle agitation at 600 rpm. Cell viability was measured via luminescence using Victor Nivo, PerkinElmer (2 replicates. Background luminescence was subtracted, based on luminescence detected from wells having media only (*i.e.* no cells). Precent inhibition was calculated using the formula 100- {(lum of cells treated with test article / lum of cells treated with buffer control) *100}, where lum= luminescence. The average and SEM was calculated by GraphPad PRISM 10. Graphs were plotted using GraphPad PRISM 10, fitting the data points to a four-parameter logistic model.

The results are shown in figure 31, and can be summarised as:

	ADC 19	**ADC 6**	**ADC 5**
IC_{50} (M)	1.613e-009	2.004e-009	2.069e-009

Claims:

1. An antigen-binding molecule that binds to HER2, comprising (i) a HER2-binding moiety, and (ii) at least one linker-payload moiety, wherein the antigen-binding molecule comprises (a) DNA damage response (DDR) inhibitor moiety, and (b) a DNA topoisomerase I (TOP1) inhibitor moiety.

2. The antigen-binding molecule according to claim 1, wherein the DDR inhibitor moiety is, or comprises, a DDR inhibitor selected from: an ATR inhibitor, a PARP inhibitor, an ATM inhibitor, a WEE1 inhibitor, a CHK1/2 inhibitor, a DNA-PK inhibitor, a PLK1 inhibitor, a Polθ inhibitor, a RAD51 inhibitor, a USP inhibitor, a PKMYT1 inhibitor, or an Aurora-A inhibitor.

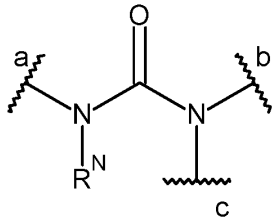
3. The antigen-binding molecule according to claim 1 or claim 2, wherein the DDR inhibitor moiety is, or comprises, a DDR inhibitor which is
(a) an ATR inhibitor, optionally wherein the DDR inhibitor moiety is, or comprises, berzosertib;
(b) a CHK1/2 inhibitor, optionally wherein the DDR inhibitor moiety is, or comprises, prexasertib;
(c)a WEE1 inhibitor, optionally wherein the DDR inhibitor moiety is, or comprises, adavosertib;
(d) an ATM inhibitor, optionally wherein the DDR inhibitor moiety is, or comprises, AZD0156; or
(e) a DNA-PK inhibitor, optionally wherein the DDR inhibitor moiety is, or comprises, nedisertib.

4. The antigen-binding molecule according to any one of claims 1 to 3, wherein the TOP1 inhibitor moiety is, or comprises, a TOP1 inhibitor selected from: camptothecin or a derivative thereof, exatecan, exatecan mesylate (DX-8951f), *N*-glycyl-exatecan, SN-38, DXd(1), DXd(2), irinotecan, etirinotecan, FL118, topotecan, gimatecan, belotecan, deruxtecan, belotecan, rubitecan, lurtotecan, diflomotecan, karenitecan, silatecan, namitecan, elomotecan, DRF-1042, delimotecan, NSC606985, chimmitecan, ZBH-1205, Genz-644282, non-CPT1, indotecan, indimitecan, AZ14170132, SHR9265, Ed-04, KL610023, A1.9, ZD06519, P1003, P1021, VIP126, ZBH-01 and LMP-744.

5. The antigen-binding molecule according to any one of claims 1 to 4, wherein the TOP1 inhibitor moiety is, or comprises, a TOP1 inhibitor selected from: exatecan, , belotecan, SN-38 and DXd.

6. The antigen-binding molecule according to any one of claims 1 to 5, wherein the antigen-binding molecule comprises a linker-payload moiety comprising (a) a DDR inhibitor moiety, and (b) a TOP1 inhibitor moiety.

7. The antigen-binding molecule according to claim 6, wherein the linker-payload moiety comprises:
(a) an amino group for conjugation to an antigen-binding moiety;
(b) at least one first payload comprising moiety clicked to a first click group, where the first payload comprising moiety comprises a DDR inhibitor moiety;
(c) at least one second payload comprising moiety clicked to a second click group, where the second payload comprising moiety comprises a TOP1 inhibitor moiety;
(d) the branching group:



wherein R^N is selected from H and -(C_{1-5} alkylene)-C(O)OH, where one CH_2 unit may be replaced by -O-,
a indicates where the amino group is linked to the branching group;
b indicates where the at least one first click group is linked to the branching group;
c indicates where the at least one second click group is linked to the branching group.

8. The antigen-binding molecule according to any one of claims 1 to 7, wherein the HER2-binding moiety comprises:

(i) a heavy chain variable (VH) region incorporating the following CDRs:

HC-CDR1 having the amino acid sequence of SEQ ID NO:15

HC-CDR2 having the amino acid sequence of SEQ ID NO:16

HC-CDR3 having the amino acid sequence of SEQ ID NO:17; and

(ii) a light chain variable (VL) region incorporating the following CDRs:

LC-CDR1 having the amino acid sequence of SEQ ID NO:23

LC-CDR2 having the amino acid sequence of SEQ ID NO:24

LC-CDR3 having the amino acid sequence of SEQ ID NO:25.

9. The antigen-binding molecule according to any one of claims 1 to 8, wherein the antigen-binding moiety that binds to HER2 comprises:

a VH region comprising an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:14; and

a VL region comprising an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:22.

10. The antigen-binding molecule according to any one of claims 1 to 9, wherein the antigen-binding moiety that binds to HER2 comprises:

a polypeptide comprising, or consisting of, an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:12; and

a polypeptide comprising, or consisting of, an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:13.

11. A composition comprising an antigen-binding molecule according to any one of claims 1 to 10, and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant.

12. An antigen-binding molecule according to any one of claims 1 to 10, or a composition according to claim 11, for use in a method of medical treatment or prophylaxis, or in a method of diagnosis or prognosis.

13. An antigen-binding molecule according to any one of claims 1 to 10, or a composition according to claim 11, for use in treating or preventing a cancer.

14. Use of an antigen-binding molecule according to any one of claims 1 to 10, or a composition according to claim 11, in the manufacture of a medicament for treating or preventing a cancer.

15. A method of treating or preventing a cancer, comprising administering to a subject a therapeutically- or prophylactically-effective amount of an antigen-binding molecule according to any one of claims 1 to 10, or a composition according to claim 11.

16. The antigen-binding molecule or composition for use according to claim 13, the use according to claim 14, or the method according to claim 15, wherein the cancer is selected from: a cancer comprising cells expressing/overexpressing an EGFR family member, a cancer comprising cells expressing/overexpressing HER2, a cancer comprising cells that do not overexpress an EGFR family member, a cancer comprising cells that do not overexpress HER2, a HER2-low cancer, a HR-positive cancer, a solid tumor, bladder cancer, breast cancer, HER2-positive breast cancer, metastatic HER2-positive breast cancer, HER2-low breast cancer, unresectable or metastatic HER2-low breast cancer, HR-positive breast cancer, triple-negative breast cancer, cervical cancer, gastric cancer, HER2-positive gastric cancer, locally-advanced or metastatic HER2-positive gastric cancer, cholangiocarcinoma, colorectal cancer, esophageal esophagogastric junction cancer, gallbladder cancer, gastric adenocarcinoma, gastroesophageal junction adenocarcinoma, HER2-positive gastric gastroesophageal junction adenocarcinoma, locally-advanced or metastatic HER2-positive gastric gastroesophageal junction adenocarcinoma, gastrointestinal stromal tumor, glioblastoma multiforme, glioma, head and neck carcinoma, hepatocellular carcinoma, intestinal cancer, kidney cancer, lung cancer, non-small cell lung cancer, unresectable or metastatic non-small cell lung cancer comprising activating mutation to *ERBB2*, small cell lung cancer, melanoma, neuroendocrine tumor, oligodendroglioma, ovarian cancer, pancreatic adenocarcinoma, penile cancer, pituitary cancer, prostate cancer, sarcoma, solitary fibrous tumor, testicular cancer, thymic cancer, thyroid cancer and uterine cancer.

17. The antigen-binding molecule or composition for use according to claim 13 or claim 16, the use according to claim 14 or claim 16, or the method according to claim 15 or claim 16, wherein the cancer is refractory or relapsed to treatment with a DNA damage repair inhibitor, and/or wherein the cancer is refractory or relapsed to treatment with a DNA topoisomerase I inhibitor.

18. Use of an antigen-binding molecule according to any one of claims 1 to 10, or a composition according to claim 11, to deplete or increase killing of cells expressing HER2.

19. An *in vitro* complex, optionally isolated, comprising an antigen-binding molecule according to any one of claims 1 to 10 bound to HER2.