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Canadian Intellectual Property Office

CA 2937600 C 2023/01/03

(11)(21) 2 937 600

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2015/02/04
(87) Date publication PCT/PCT Publication Date: 2015/08/13
(45) Date de délivrance/Issue Date: 2023/01/03
(85) Entrée phase nationale/National Entry: 2016/07/21
(86) N° demande PCT/PCT Application No.: EP 2015/052342
(87) N° publication PCT/PCT Publication No.: 2015/118031
(30) Priorité/Priority: 2014/02/06 (GB1402009.3)

(51) Cl.Int./Int.Cl. *C07K 16/28* (2006.01)

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(54) Titre : CONJUGUES ANTICORPS-MEDICAMENT ET IMMUNOTOXINES
(54) Title: ANTIBODY-DRUG CONJUGATES AND IMMUNOTOXINS

(57) Abrégé/Abstract:
The present invention relates to conjugates, in particular antibody- drug conjugates and iramunotoxins, having the formula (I): A-(L-D)p (I) or a pharmaceutically acceptable salts or solvates thereof, wherein : A is an antibody that selectively binds Endoglin; L is a linker; D is a drug comprising a cytolysin or a Nigrin-b A-chain; and p is 1 to 10, and to use of such conjugates in the therapeutic treatment of tumors. Methods of producing such conjugates and components for use in such methods are disclosed.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 August 2015 (13.08.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/118031 A3

(51) **International Patent Classification**:
C07K 16/28 (2006.01) ***A61K 47/48*** (2006.01)

(21) **International Application Number**:
PCT/EP2015/052342

(22) **International Filing Date**:
4 February 2015 (04.02.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
1402009.3 6 February 2014 (06.02.2014) GB

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

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

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) **Date of publication of the international search report**:
1 October 2015

WO 2015/118031 A3

(54) **Title**: ANTIBODY-DRUG CONJUGATES AND IMMUNOTOXINS

(57) **Abstract**: The present invention relates to conjugates, in particular antibody- drug conjugates and iramunotoxins, having the formula (I): A-(L-D)p (I) or a pharmaceutically acceptable salts or solvates thereof, wherein : A is an antibody that selectively binds Endoglin; L is a linker; D is a drug comprising a cytolysin or a Nigrin-b A-chain; and p is 1 to 10, and to use of such conjugates in the therapeutic treatment of tumors. Methods of producing such conjugates and components for use in such methods are disclosed.

Antibody-Drug Conjugates and Immunotoxins

Field of the invention

The present invention relates to antibody-drug conjugates (ADCs) and Immunotoxins that target Endoglin (ENG), and to their use in medicine, e.g. in the treatment of certain cancers.

Background to the invention

Malignant epithelial tumors are the main cancer-related cause of human death. These solid tumors frequently exhibit significant stromal reactions such as the so-called "desmoplastic stroma" or "reactive stroma", which represents 20-60% of total tumor mass and is characterized by the existence of large numbers of stromal cells and dense extracellular matrix (ECM). Recent studies have indicated the tumor-promoting roles of stromal cells, as exemplified by vascular cells, immune cells, fibroblasts, myofibroblasts, adipocytes and bone marrow-derived progenitors *(1-6)*. In particular, considerable numbers of cancer-associated fibroblasts (CAFs) are frequently observed within tumor-associated stroma of various human cancers, including breast, lung, colon, and pancreas carcinomas *(14,15)*. Interacting coordinately with the different components of the stroma, CAFs have the ability to promote neoangiogenesis and tumor growth; CAFs have also been shown as crucial for the development of aggressive tumors and tumor invasiveness during cancer progression *(16-25)*; CAFs facilitate the spreading and infiltration of tumor cells in distant organs, thus contributing to formation of metastases. Importantly, the relevance of stromal cells to the failure of systemic drug delivery to tumors and to the development of drug resistance has also been indicated *(7-11)*. The identification of cellular and molecular targets abrogating stromal-tumor cell interactions and thus attenuating tumorigenesis is currently one of the most important subjects in translational oncology. Indeed, targeting the peritumoral stroma is a fairly new strategy to treat metastatic tumors, which represent more than 90% of cancer patient mortality: only a few products have obtained therapeutic approval up to now, most of them being anti-angiogenic

drugs (Avastin®; *26*). Identifying and targeting other new molecules within the tumor microenvironment is then essential for increasing the efficacy of conventional therapies in combination with the stroma-based therapeutic approaches, and represent a powerful approach for cancer and metastasis treatment *(12, 13)*.

Monoclonal antibody (MAb) - based drugs represent a great promise in the fight against cancer. This is because they allow the treatment to be aimed at a molecular level in a precise and specific way. These advantages, together with their commercial appeal (short development times, restricted competence and being easily exportable to other cancer types once they have been approved), have pushed many pharmaceutical companies to invest heavily in the development of new antibody-based molecules, as well as in the in-licensing of new molecules or technologies from biotech companies.

However, despite the clinical success of therapeutic antibodies, naked MAbs targeting cell surface tumor antigens rarely present sufficient efficacy on their own. To increase the low activity of the MAbs, novel strategies are focusing on binding them to toxic molecules. Plant and bacterial toxins as well as small chemotherapeutic molecules can be good candidates, since they are very potent and active in very small quantities.

The field of immunotoxins (ITs) and Antibody-Drug conjugates (ADCs) for the treatment of cancer has recently experienced a growing development activity by pharmaceutical companies, due to the technological advances performed during the last years, aimed at solving the problems they initially presented about immunogenicity, undesirable toxicity, production, half-life and resistance.

Immunoconjugates are made of a human, humanized or chimeric recombinant antibody, covalently linked to a cytotoxic drug. The main goal of such a structure is joining the power of small cytotoxic (300 to 1000Da) and the high specificity of tumor-associated antigen targeted (TAA) MAbs.

The Ab must be very selective to reach the antigen, whose expression must be restricted in normal cells. The Ab also must be internalized efficiently into the cancerous cells.

The cytotoxic agent selected as the effector moiety must kill cells only after internalization and release into the cell cytoplasm. The most commonly used payloads in ADCs are DNA-harming drugs such as calicheamicins, duocarmicins, or microtubule-targeting compounds like auristatins and maitansinoids.

The Ab-cytotoxic linkers are designed to be stable systemically and to release the cytotoxic within the target cells.

TAAs are frequently cell membrane proteins that are overexpressed in diseased tissues or at least expressed sufficiently to facilitate the internalization-activated cytotoxicity. Ideally the antigen presents a restricted expression in normal tissues with a low or absent expression in vital organs. On top of this, the tumor antigen must be recognized selectively and with high affinity by an Ab.

Recent studies suggest that therapeutic agents designed to inhibit TGF-β signaling pathway at the tumor-stroma interphase could prevent cancer progression, improving prognosis and treatment. TGF-β co-receptor family is emerging as a target for cancer treatments acting on the tumor or on its neovasculature. Endoglin (ENG, CD105), an accessory protein of the type II TGF-β receptor complex, is part of this family and presents the following characteristics:

- Type I homodimer membrane protein
- Cell surface angiogenesis and neovascularisation-associated protein in many cancer types
- Overexpressed in tumor microvasculature cells, specifically in angiogenic areas of tumor
- No expression in normal tissue endothelium
- Breast metastasis-correlated plasma levels
- Internalization
- Optimal accessibility from bloodstream

Anti-ENG antibodies (e.g. scFvs) have been reported and their application in tumor stroma targeting strategies described. Specific binding, cell internalization and anti-tumoral effects of Doxorubicin-loaded, anti-ENG immunoliposomes have been reported *in vitro*, using ENG+ cells, and *in vivo* in mice. ENG-targeted conjugates of anti-ENG antibodies and ricin or native nigrin b have been evaluated in mouse tumor models (*27-36*).

Despite these advances, there remains an unmet need for further therapeutic strategies for the treatment of tumors, including epithelial tumors, and for components for use in such therapeutic strategies. The present invention addresses these and other needs.

Brief Description of the Invention

Broadly, the present invention relates to anti-ENG antibodies, conjugates thereof and optimised payloads for use in antibody conjugate strategies. In particular, the present inventors have found that anti-ENG antibodies as described herein exhibit highly specific binding and fast and efficient internalisation. Moreover, the present inventors have found that the A chain of Nigrin b can be isolated and produced in bacterial host cells, yet retains the ability to translocate into cells and exhibits cytotoxic activity in the absence of the Nigrin-b B-chain when conjugated to a monoclonal antibody. The Nigrin-b A-chain described herein and/or cytolysin derivatives are advantageously conjugated to anti-ENG antibodies for use in the treatment of tumors.

Accordingly, in a first aspect the present invention provides a conjugate having the formula I:

$$A-(L-D)_p \quad (I)$$

or a pharmaceutically acceptable salt or solvate thereof,
wherein:
A is an antibody that selectively binds Endoglin;
L is a linker;
D is a drug comprising a cytolysin or a Nigrin-b A-chain; and

p is 1 to 10.

In some cases in accordance with this and other aspects of the present invention A is a monoclonal antibody or binding fragment thereof that selectively binds to an extracellular region of human Endoglin. In particular cases A may comprise heavy chain complementarity determining regions 1-3 (CDRH1-3) and light chain complementarity determining regions 1-3 (CDRL1-3) having the following amino acid sequences:
(i) CDRH1: SEQ ID NO: 7 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 7;
(ii) CDRH2: SEQ ID NO: 8 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 8;
(iii) CDRH3: SEQ ID NO: 9 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 9;
(iv) CDRL1: SEQ ID NO: 10 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 10;
(v) CDRL2: SEQ ID NO: 11 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 11; and
(vi) CDRL3: SEQ ID NO: 12 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 12.

In certain cases, CDRH1-3 comprise the amino acid sequences of SEQ ID NOS: 7-9, respectively and CDRL1-3 comprise the amino acid sequences of SEQ ID NOS: 10-12, respectively.

In certain cases, A comprises a heavy chain variable region (VH) comprising an amino acid sequence having at least 90%, 95% or 99% sequence identity with the full-length sequence of SEQ ID NO: 5.

In certain cases, A comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO: 5.

In certain cases, A comprises a light chain variable region (VL) comprising an amino acid sequence having at least 90%, 95% or 99% sequence identity with the full-length sequence of SEQ ID NO: 6. In particular, A may comprise a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 6.

In certain cases, A comprises a heavy chain comprising an amino acid sequence having at least 90%, 95% or 99% sequence identity with the full-length sequence of SEQ ID NO: 3. In particular, A may comprise a heavy chain comprising the amino acid sequence of SEQ ID NO: 3.

In certain cases, A comprises a light chain comprising an amino acid sequence having at least 90%, 95% or 99% sequence identity with the full-length sequence of SEQ ID NO: 4. In particular, A may comprise a light chain comprising the amino acid sequence of SEQ ID NO: 4.

In certain cases, A may be a competitively binging anti-Endoglin antibody that is structurally different from the anti-Endoglin antibody molecules exemplified herein. For example, A may be an anti-Endoglin antibody molecule that competes with the anti-human Endoglin IgG1 antibody identified herein as "A5" for binding to immobilized recombinant human Endoglin. A5 has the heavy chain amino acid sequence of SEQ ID NO: 3 and the light chain amino acid sequence of SEQ ID NO: 4.

In certain cases, A is a monoclonal antibody or binding fragment thereof that selectively binds to an extracellular region of murine Endoglin. Conjugates that target murine Endoglin find particular use in pre-clinical testing, e.g., employing well-characterised murine models of various cancers. In particular, A may comprise heavy chain complementarity determining regions 1-3 (CDRH1-3) and light chain complementarity determining regions 1-3 (CDRL1-3) having the following amino acid sequences:
(i) CDRH1: SEQ ID NO: 19 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 19;

(ii) CDRH2: SEQ ID NO: 20 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 20;
(iii) CDRH3: SEQ ID NO: 21 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 21;
(iv) CDRL1: SEQ ID NO: 22 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 22;
(v) CDRL2: SEQ ID NO: 23 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 23; and
(vi) CDRL3: SEQ ID NO: 24 or a variant thereof having up to 1 or 2 amino acid substitutions compared with the sequence of SEQ ID NO: 24. For example, CDRH1-3 may comprise the amino acid sequences of SEQ ID NOS: 19-21, respectively and CDRL1-3 may comprise the amino acid sequences of SEQ ID NOS: 22-24, respectively.

In certain cases, A comprises a heavy chain variable region (VH) comprising an amino acid sequence having at least 90%, 95% or 99% sequence identity with the full-length sequence of SEQ ID NO: 17, e.g. A may comprise a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO: 17.

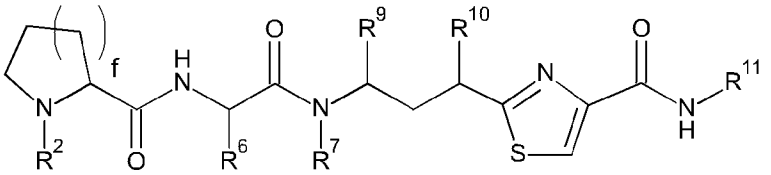
In certain cases, A comprises a light chain variable region (VL) comprising an amino acid sequence having at least 90%, 95% or 99% sequence identity with the full-length sequence of SEQ ID NO: 18, e.g., A may comprise a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 18.

In certain cases, A comprises a heavy chain comprising an amino acid sequence having at least 90%, 95% or 99% or even 100% sequence identity with the full-length sequence of SEQ ID NO: 15.

In certain cases, A comprises a light chain comprising an amino acid sequence having at least 90%, 95% or 99% or even 100% sequence identity with the full-length sequence of SEQ ID NO: 16.

In certain cases, A may be a competitively binding anti-Endoglin antibody that is structurally different from the anti-Endoglin antibody molecules exemplified herein. For example, A may be an anti-Endoglin antibody molecule that competes with the anti-murine Endoglin IgG1 antibody identified herein as "mE12" for binding to immobilized recombinant murine Endoglin. mE12 has the heavy chain amino acid sequence of SEQ ID NO: 15 and the light chain amino acid sequence of SEQ ID NO: 16.

In accordance with this and other aspects of the present invention, D may be a cytolysin. The cytolysin may, in some cases, be a compound disclosed in WO 2008/138561 A1 (compounds disclosed therein are also referred to as Tubulysine derivatives). The cytolysin may be synthesised as described in WO 2008/138561. In certain cases, the cytolysin may be as defined in Formula I or Formula IV of WO 2008/138561 A1. In certain cases, the cytolysin may be of formula IV:



(IV)

wherein:

R^2 (i) is directly or indirectly attached to linker L or (ii) is H or is C_1-C_4 alkyl;

R^6 is C_1-C_6 alkyl;

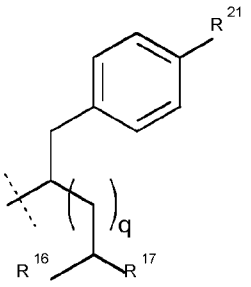
R^7 is C_1-C_6 alkyl, CH_2OR^{19} or CH_2OCOR^{20}, wherein R^{19} is alkyl, R^{20} is C_2-C_6-alkenyl, phenyl, or CH_2-phenyl;

R^9 is C_1-C_6 alkyl;

R^{10} is H, OH, O-alkyl or O-acetyl;

f is 1 or 2;

R^{11} has the following structure:



wherein

R^{21} is H, OH, halogen, NH_2, alkyloxy, phenyl, alkyl amino or dialkyl amino;

R^{16} is H or a C_1-C_6-alkyl group;

R^{17} (i) is directly or indirectly attached to linker L or (ii) is CO_2H, CO_2R^{18}, $CONHNH_2$, OH, NH_2, SH or a optionally substituted alkyl, cycloalkyl, heteroalkyl or heterocycloalkyl group, wherein R^{18} is an optionally substituted alkyl, heteroalkyl or hetercycloalkyl group; and

q is 0, 1, 2 or 3;

and wherein the term "optionally substituted" relates to groups, wherein one or several H atoms can be replaced by F, Cl, Br or I or OH, SH, NH_2, or NO_2; the term "optionally substituted" further relates to groups, which can be exclusively or additionally substituted with unsubstituted C_1-C_6 alkyl, C_2C_6 alkenyl, C_2-C_6 alkynyl, C_1-C_6 heteroalkyl, C_3-C_{10} cycloalkyl, C_2-C_9 heterocycloalkyl, C_6-C_{10} aryl, C_1-C_9 heteroaryl, C_7-C_{12} aralkyl or C_2-C_{11} heteroaralkyl groups.

In some cases R^2 is a bond to linker L.

In some cases R^{17} is C(O)X, CONHNHX, OX, NHX or SX, wherein X is a bond to linker L.

In some cases linker L may further comprise a spacer.

In some cases the spacer has a chain length of 2 to 30 atoms.

In some cases the spacer comprises or consists of an alkylene (i.e. divalent alkyl) or heteroalkylene (i.e. divalent heteroalkyl) group.

In some cases the spacer comprises or consists of an alkylene or oxyalkylene group.

In some cases the spacer comprises or consists of a group $-(CH_2)_n-$ or $-(OCH_2CH_2)_n-$, wherein $n \geq 1$.

In some cases the spacer comprises or consists of a group $-(OCH_2CH_2)_n-$, wherein $n \geq 1$. In particular, n may be 1 to 15, 1 to 10, 1 to 6, or 2 to 5. For example, n may be 3 or 4.

In some cases the space comprises between one and six ethylene glycol units, e.g. a triethylene glycol.

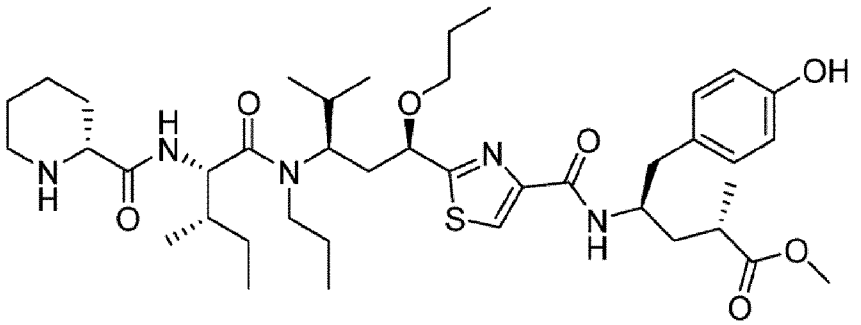
In some cases the spacer may be directly attached to group R^{17}, or may be attached to group R^{17} via a bridging group.

In some cases the spacer is attached to group R^{17} via a -C(O)X bridging group, wherein X is a bond to R^{17}.

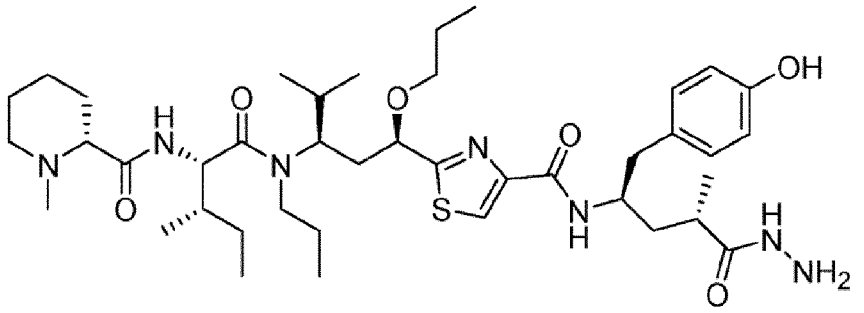
In some cases R^{17} is CONHNHX and the spacer is attached to group R^{17} via a -C(O)X bridging group, wherein X represents the bond between the spacer and R^{17}.

In some cases R^{17} is CONHNHX and the spacer is a $-(OCH_2CH_2)_n-$ attached to R^{17} via a -C(O)X bridging group, wherein n = 2, 3 or 4.

In some cases D comprises a cytolysin having the following structure:



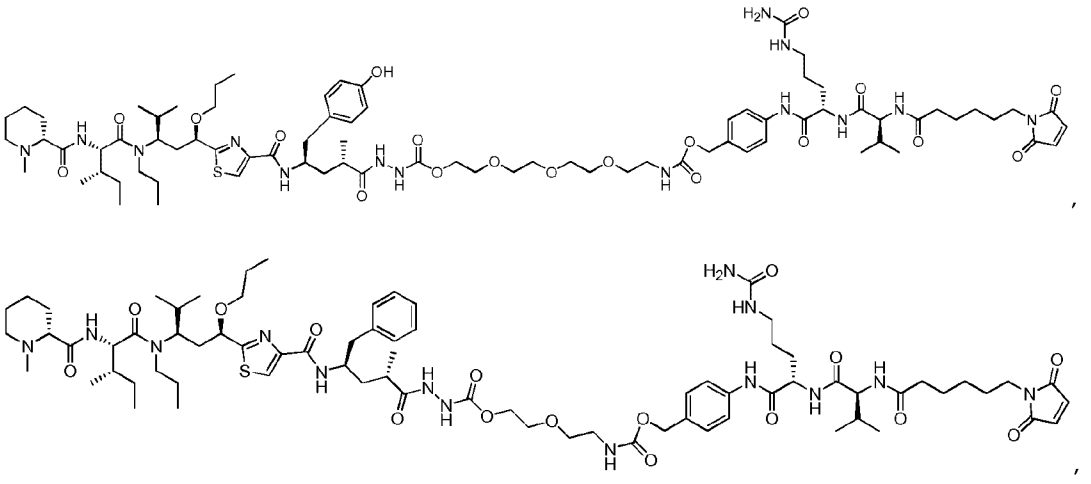
In some cases D comprises a cytolysin having the following structure:

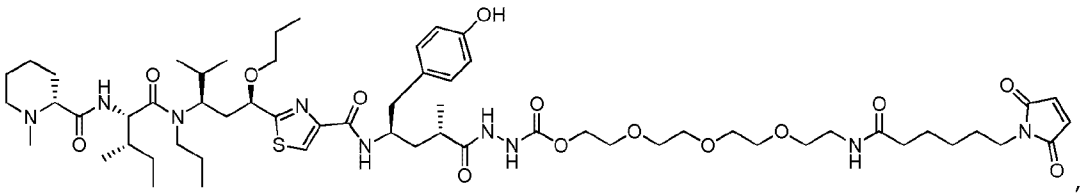


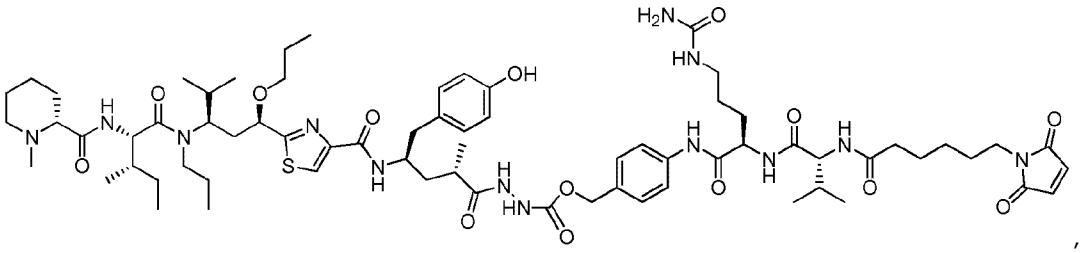
In certain cases L comprises an attachment group for attachment to A and protease cleavable portion. For example, L may comprise a valine-citrulline unit. In particular, L may comprise maleimidocaproyl-valine-citrulline-p-aminobenzylcarbamate.

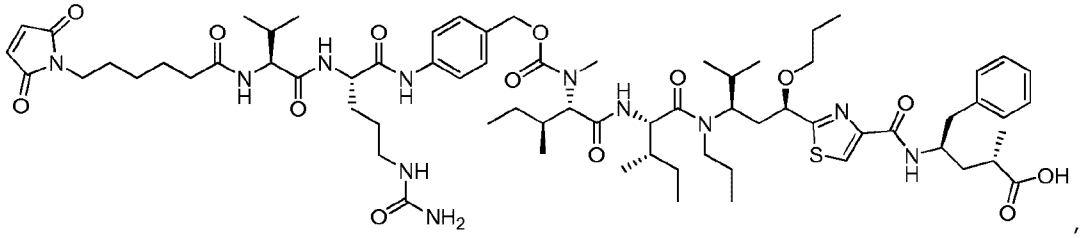
In some cases the double bond of the maleimide is reacted with a thiol group of a cysteine residue of the antibody A to form a sulphur-carbon bond in order to effect linkage of the linker L to the antibody A.

In some cases -L-D has a structure selected from the group consisting of:

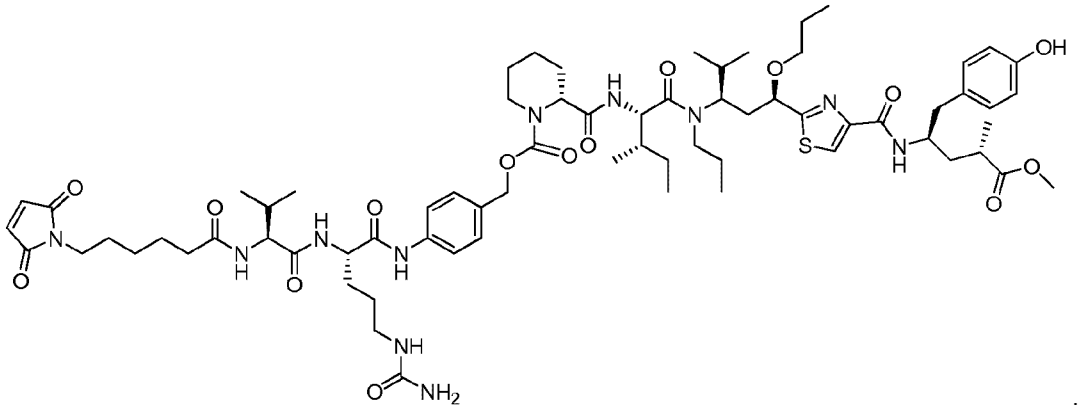




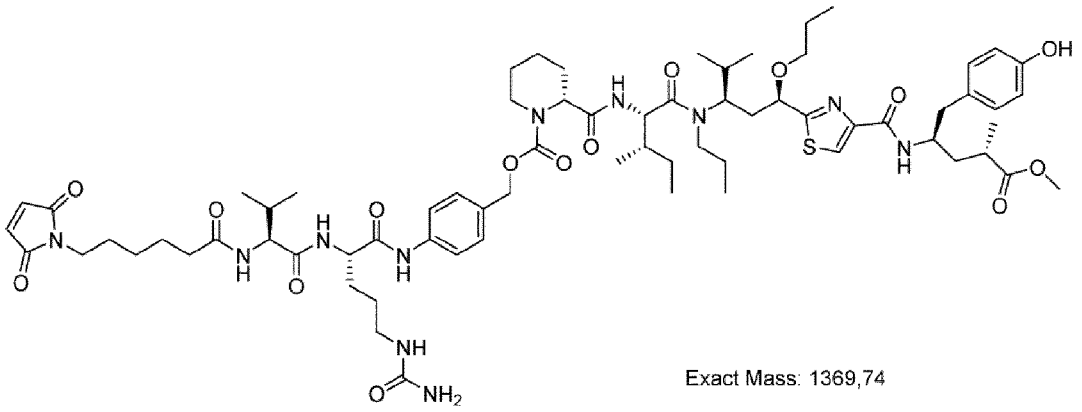




and



In certain cases -L-D may have the following structure:



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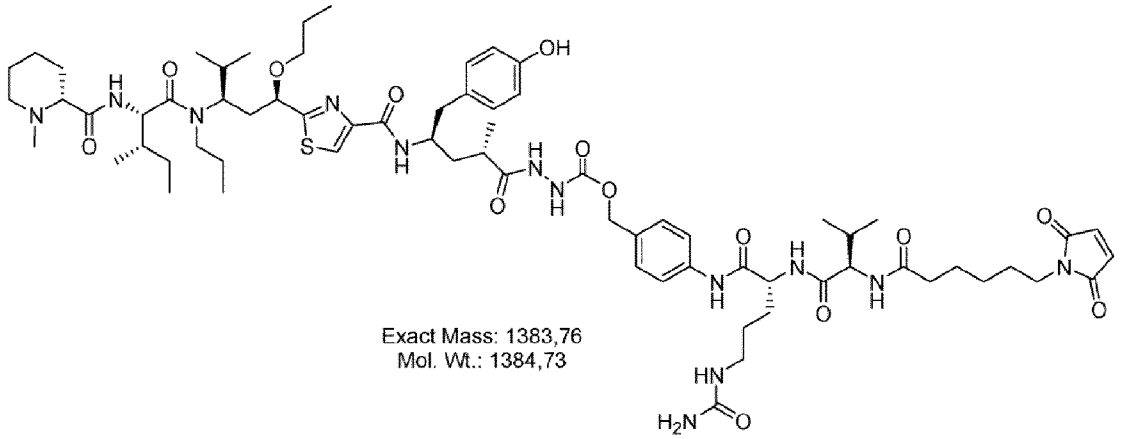


In accordance with this and other aspects of the present invention p may, in some cases, lie in the range 1 to 5, e.g. 1 to 4, or 1 to 3. In particular cases p may be 1 or 2. In particular, cases p may be 3 or 4.

In accordance with this and other aspects of the present invention D may be a Nigrin-b A-chain. Preferably, the Nigrin-b A-chain is in the absence of a Nigrin-b B-chain. The Nigrin-b A-chain may comprise or consist of the sequence of SEQ ID NO: 25.

In certain cases, the Nigrin-b A-chain may be or may have been recombinantly-produced, e.g. in a bacterial host cell. The present inventors have surprisingly found that Nigrin-b A-chain retains its activity (e.g. cytotoxic and/or ribosome inhibiting activity)

despite loss of or alteration of native glycosylation such as is the case when the Nigrin-b A-chain is produced recombinantly in a bacterial host cell.

When the conjugate of the present invention comprises a Nigrin-b A-chain as the toxic payload (i.e. D), L may simply be a disulphide bond between a sulphur atom on A and a sulphur atom on D. Therefore, L may comprise or consist of a bond, e.g. a disulphide bond.

In a second aspect the present invention provides a conjugate as defined in accordance with the first aspect of the invention for use in medicine.

In a third aspect the present invention provides a conjugate as defined in accordance with the first aspect of the invention for use in a method of treatment of a tumor in a mammalian subject. In certain cases the conjugate is for use in the treatment of a blood neoplasm. In other cases the conjugate is for use in the treatment of a solid tumor. In particular, the conjugate may be for use in the treatment of pancreatic cancer, Ewing sarcoma, breast cancer, melanoma, lung cancer, head & neck cancer, ovarian cancer, bladder cancer or colon cancer.

In some cases the conjugate is for simultaneous, sequential or separate administration with one or more other antitumor drugs. The one or more other antitumor drugs comprise a cytotoxic chemotherapeutic agent or an anti-angiogenic agent or an immunotherapeutic agent. In some cases the one or more other antitumor drugs comprise Gemcitabine, Abraxane bevacizumab, itraconazole, carboxyamidotriazole, an anti-PD-1 molecule or an anti-PD-L1 molecule (for example, nivolumab or pembrolizumab).

In a fourth aspect the present invention provides a method of treating a tumor in a mammalian subject, comprising administering a therapeutically effective amount of a conjugate as defined in accordance with the first aspect of the invention to the subject in

need thereof. In some cases the method may be for treating a blood neoplasm. In other cases the method may be for treating solid tumors. In particular, the method may be for treating pancreatic cancer, Ewing sarcoma, breast cancer, melanoma, lung cancer, head & neck cancer, ovarian cancer, bladder cancer or colon cancer.

In a fifth aspect the present invention provides use of a cytolysin in the preparation of an antibody-drug conjugate, wherein the antibody is an Endoglin-specific antibody, e.g., an Endoglin-specific antibody in accordance with the eighth aspect of the invention. In some cases the cytolysin may be as defined in accordance with the first aspect of the invention. In some case the use may be of a cytolysin in the preparation of an antibody-drug conjugate as defined in accordance with the first aspect of the invention.

In a sixth aspect the present invention provides an isolated Nigrin-b A-chain in the absence of the Nigrin-b B-chain. The amino acid sequence of the Nigrin-b A-chain may comprise or consist of the sequence of SEQ ID NO: 25.

In an seventh aspect the present invention provides use of an isolated Nigrin-b A-chain in accordance with the sixth aspect of the invention in the preparation of an immunotoxin. In some cases, the immunotoxin comprises a monoclonal antibody conjugated and/or bound to said isolated Nigrin-b A-chain. In some cases the immunotoxin comprises an antibody, such as a monoclonal antibody, e.g. a human monoclonal antibody, that selectively binds Endoglin. In some cases, the immunotoxin comprises an antibody in accordance with the eighth aspect of the invention.

In an eighth aspect the present invention provides a monoclonal antibody, e.g. a human monoclonal antibody, that:

(i) selectively binds human Endoglin and which comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 3 and a light chain comprising the amino acid sequence of SEQ ID NO: 4; or

(ii) selectively binds murine Endoglin and which comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 15 and a light chain comprising the amino acid sequence of SEQ ID NO: 16.

In a ninth aspect the present invention provides an antibody of the eighth aspect of the invention for use in medicine.

In a tenth aspect the present invention provides a conjugate of the first aspect of the invention or an antibody of the eighth aspect of the invention for use in the treatment of an inflammatory condition (e.g. rheumatoid arthritis) or an eye disease (e.g. diabetic retinopathy or macular degeneration, such as wet age related macular degeneration).

In an eleventh aspect the present invention provides a method of treating an inflammatory condition (e.g. rheumatoid arthritis) or an eye disease (e.g. diabetic retinopathy or macular degeneration, such as wet age related macular degeneration) in a mammalian subject, comprising administering a therapeutically effective amount of a conjugate of the first aspect of the invention or an antibody of the eighth aspect of the invention to the subject in need thereof.

In a twelfth aspect the present invention provides use of a monoclonal antibody in accordance with the eighth aspect of the invention in the preparation of an antibody-drug conjugate or an immunotoxin.

In a thirteenth aspect the present invention provides a host cell comprising a vector comprising a polynucleotide that encodes at least one polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NOS: 1-6, 13-18 and 25. In some cases the polynucleotide may comprise the nucleic acid sequence of SEQ ID NO: 26.

In a fourteenth aspect the present invention provides a process for the production of a conjugate in accordance with the first aspect of the invention, comprising:

(a) derivatising the antibody that selectively binds Endoglin to introduce at least one sulphydryl group; and

(b) reacting the derivatised antibody with an appropriate residue (e.g. a cysteine amino acid) on a Nigrin-b A-chain (absent a Nigrin-b B-chain) under conditions which permit the formation of a disulphide bond linkage between the antibody and the Nigrin-b A-chain thereby producing the conjugate. The process may further comprise a step (c) of purifying and/or isolating the conjugate.

In some cases step (a) may comprise reacting the antibody with 4-succynimidyloxycarbonyl-α-methyl-α-(2-pyridyl-dithio)toluene (SMPT), N-succynimidyl 3—(2-pyridyl-dithiopropionate) (SPDP) or methyl 4-mercaptobutyrimidate.

In a fifteenth aspect the present invention provides a process for the production of a conjugate in accordance with the first aspect of the invention, comprising:

(a) linking the antibody that selectively binds Endoglin to the linker via a thiol group; and

(b) linking the cytolysin to the linker via an appropriate group on the cytolysin molecule. In some cases, the cytolysin is linked to the linker via position R_2 or position R_{17}. Steps (a) and (b) can be performed in either order. In an optional further step (c), the process may comprise purifying and/or isolating the conjugate.

The present invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or is stated to be expressly avoided. These and further aspects and embodiments of the invention are described in further detail below and with reference to the accompanying examples and figures.

Brief Description of the figures

Figure 1 shows ELISA results of mE12-IgG for binding to recombinant mouse ENG (aa 27-581). Coated BSA was included as negative control. A concentration-dependent binding to mouse ENG was observed;

Figure 2 shows flow cytometry analysis of binding of mE12-IgG to **a**) B16 cells using 50 µg/ml antibody, **b**) B16 cells using 5 µg/ml antibody, and **c**) HT1080 cells included as negative control. Shaded area: cells alone, unshaded area: cells incubated with antibody;

Figure 3 shows **a**) ELISA of binding of A5 IgG1 to immobilized recombinant human ENG. **b**) Flow cytometry analysis of binding of A5-IgG to HT1080 cells;

Figure 4 shows MALDI-Tof profile of recombinant nigrin-b A-chain. Observed mass (Da): 28546.55; Expected mass (Da): 28546.09; Mass deviation: 0.5; Mass Accuracy: 16ppm.

Figure 5 shows ribosome inactivating protein (RIP) activity of recombinant Nigrin-b A-chain (recNgA) tested in rabbit reticulocyte cell-free lysates (RRL) versus native (WT) Nigrin-b (3a, 3b, 6c, 9c) represent different formulations of recNgA

Figure 6 shows cytotoxicity of recNgA tested on HT1080-FAP cell line through crystal violet viability assay (native Nigrin – diamonds; recombinant Nigrin-b A-chain – squares)

Figure 7 shows RIP activity of recNgA-conjugates in an RRL assay Native nigrin b: diamonds (♦); recNgA: squares(■); recNgA-conjugates HPS-124-37-1 (triangles: ▲) HPS-124-37-2 (crosses: **X**)

Figure 8 shows the general antibody conjugate structure for a cytolysin-conjugated antibody via a vcPABA linker. Attachment of the cytolysin may be via R_1 or R_4 (identified by arrows)

Figure 9 shows analysis of A5 anti-huENG IgG1 internalization by discrimination of cells (n=10-30) showing only membrane staining (PM), PM and intracellular staining, or only intracellular staining.

Figure 10 shows *in vitro* cytotoxic effect of cytolysin ADCs on (**A**) wild type (WT) and (**B**) antigen(AG)-expressing HT1080 cells. Cell proliferation arrest was evidenced through crystal violet staining after 72h incubation of each compound at a concentration range from 10^{-6} to 10^{-12}M. Parental TAM334 cytolysin was used as positive control for unspecific cytotoxicity.

Figure 11 shows tumor growth inhibition effect of recNgA and cytolysin immunoconjugates. (**A**) recNgA conjugates, administrated as single agent (OMTX505) or in combination with Gemcitabine (OMTX505:GEM); (**B**) TAM471 (OMTX705-471) versus TAM551 (OMTX705-551) conjugates; (**C**) TAM471 (OMTX705-471) and TAM553 (OMTX705-553) versus TAM558 (OMTX705-558) conjugates. Vehicle and GEM (Gemcitabine): negative and positive control groups.

Detailed description of the invention

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

Endoglin

As used "Endoglin" may be an Endoglin protein of any mammalian species. In some cases Endoglin is human Endoglin (also known as CD105, ENG or END), the amino acid sequence of which is disclosed at UniProt accession No. P17813 (Version 154, dated 13 November 2013) (SEQ ID NO: 27). In some cases, a molecule that binds Endoglin (e.g. an antibody molecule or a conjugate thereof) may bind to a region of the extracellular domain of Endoglin. The extracellular domain of human Endoglin comprises residues 26-561 of the full-length human Endoglin protein. In some cases Endoglin is murine Endoglin (also known as CD105, MJ7/18 antigen, ENG or END), the amino acid sequence of which is disclosed at UniProt accession No. Q63961 (Version 104, dated 13 November 2013) (SEQ ID NO: 28). The

extracellular domain of murine Endoglin comprises residues 27-581 of the full-length murine Endoglin protein.

Conjugate

As used herein "conjugate" includes the resultant structure formed by linking molecules and specifically includes antibody-drug conjugates (ADCs) and immunotoxins (ITs).

Selectively binds

The terms selectively binds and selective binding refer to binding of an antibody, or binding fragment thereof, to a predetermined molecule (e.g. an antigen) in a specific manner. For example, the antibody, or binding fragment thereof, may bind to Endoglin, e.g. an extracellular portion thereof, with an affinity of at least about $1x10^7M^{-1}$, and may bind to the predetermined molecule with an affinity that is at least two-fold greater (e.g. five-fold or ten-fold greater) than its affinity for binding to a molecule other than the predetermined molecule.

Antibody molecule

As used herein with reference to all aspects of the invention, the term "antibody" or "antibody molecule" includes any immunoglobulin whether natural or partly or wholly synthetically produced. The term "antibody" or "antibody molecule" includes monoclonal antibodies (mAb) and polyclonal antibodies (including polyclonal antisera). Antibodies may be intact or fragments derived from full antibodies (see below). Antibodies may be human antibodies, humanised antibodies or antibodies of non-human origin. "Monoclonal antibodies" are homogeneous, highly specific antibody populations directed against a single antigenic site or "determinant" of the target molecule. "Polyclonal antibodies" include heterogeneous antibody populations that are directed against different antigenic determinants of the target molecule. The term "antiserum" or "antisera" refers to blood serum containing antibodies obtained from immunized animals.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Thus reference to antibody herein, and with reference to the methods, arrays and kits of the invention, covers a full antibody and also covers any polypeptide or protein comprising an antibody binding fragment. Examples of binding fragments are (i) the Fab fragment consisting of V_L, V_H, C_L and C_H1 domains; (ii) the Fd fragment consisting of the V_H and C_H1 domains; (iii) the Fv fragment consisting of the V_L and V_H domains of a single antibody; (iv) the dAb fragment which consists of a V_H domain; (v) isolated CDR regions; (vi) $F(ab')_2$ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a V_H domain and a V_L domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site; (viii) bispecific single chain Fv dimers (WO 93/11161) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; 58). Fv, scFv or diabody molecules may be stabilised by the incorporation of disulphide bridges linking the VH and VL domains. Minibodies comprising a scFv joined to a CH3 domain may also be made.

In relation to a an antibody molecule, the term "selectively binds" may be used herein to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than its specific binding partner(s). The term is also applicable where e.g. an antigen-binding site is specific for a particular epitope that is carried by a number of antigens, in which case the specific binding member carrying the antigen-binding site will be able to bind to the various antigens carrying the epitope.

In some cases in accordance with the present invention the antibody may be a fully human antibody.

Cytotoxic chemotherapeutic agents

In some cases in accordance with any aspect of the present invention, the conjugate of the invention may administered with, or for administration with, (whether simultaneously, sequentially or separately) other antitumor drugs, including, but not limited to, a

cytotoxic chemotherapeutic agent or an anti-angiogenic agent or an immunotherapeutic agent.

Cytotoxic chemotherapeutic agents are well known in the art and include anti-cancer agents such as:
Alkylating agents including nitrogen mustards such as mechlorethamine (HN2), cyclophosphamide, ifosfamide, melphalan (L-sarcolysin) and chlorambucil; 10 ethylenimines and methylmelamines such as hexamethylmelamine, thiotepa; alkyl sulphonates such as busulfan; nitrosoureas such as carmustine (BCNU), lomustine (CCNLJ), semustine (methyl-CCN-U) and streptozoein (streptozotocin); and triazenes such as decarbazine (DTIC; dimethyltriazenoimidazolecarboxamide);
Antimetabolites including folic acid analogues such as methotrexate (amethopterin); pyrimidine analogues such as fluorouracil (5-fluorouracil; 5-FU), floxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine arabinoside); and purine analogues and related inhibitors such as mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2'-deoxycofonnycin). Natural Products including vinca alkaloids such as vinblastine (VLB) and vincristine; epipodophyllotoxins such as etoposide and teniposide; antibiotics such as dactinomycin (actinomycin D), daunorabicin (daunomycin; rubidomycin), doxorubicin, bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin Q; enzymes such as L-asparaginase; and biological response modifiers such as interferon alphenomes. Miscellaneous agents including platinum coordination complexes such as cisplatin (cis-DDP) and carboplatin; anthracenedione such as mitoxantrone and antbracycline; substituted urea such as hydroxyurea; methyl hydrazine derivative such as procarbazine (N- methylhydrazine, MIH); and adrenocortical suppressant such as mitotane (o, p'-DDD) and aminoglutethimide; taxol and analogues/derivatives; and hormone agonists/antagonists such as flutamide and tamoxifen. A further preferred cytotoxic agent is Gemcitabine (Gemzar®). A further preferred cytotoxic agent is Paclitaxel bound to human serum albumin (Abraxane®).

Anti-angiogenic agents are well known in the art and include anti-cancer agents such as bevacizumab, itraconazole, and carboxyamidotriazole.

Immunotherapeutic agents are known in the art and include, for example, anti-programmed cell death protein 1 (PD-1) antibodies and anti-programmed death-ligand 1 (PD-L1) antibodies, including Nivolumab (MDX1106) and Pembrolizumab (MK-3475).

Pharmaceutical compositions

The conjugates of the present invention may be comprised in pharmaceutical compositions with a pharmaceutically acceptable excipient.

A pharmaceutically acceptable excipient may be a compound or a combination of compounds entering into a pharmaceutical composition which does not provoke secondary reactions and which allows, for example, facilitation of the administration of the conjugate, an increase in its lifespan and/or in its efficacy in the body or an increase in its solubility in solution. These pharmaceutically acceptable vehicles are well known and will be adapted by the person skilled in the art as a function of the mode of administration of the conjugate.

In some embodiments, conjugates of the present invention may be provided in a lyophilised form for reconstitution prior to administration. For example, lyophilised conjugates may be re-constituted in sterile water and mixed with saline prior to administration to an individual.

Conjugates of the present invention will usually be administered in the form of a pharmaceutical composition, which may comprise at least one component in addition to the conjugate. Thus pharmaceutical compositions may comprise, in addition to the conjugate, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere

with the efficacy of the conjugate. The precise nature of the carrier or other material will depend on the route of administration, which may be by bolus, infusion, injection or any other suitable route, as discussed below.

For intra-venous administration, e.g. by injection, the pharmaceutical composition comprising the conjugate may be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles, such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be employed as required including buffers such as phosphate, citrate and other organic acids; antioxidants, such as ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3'-pentanol; and m-cresol); low molecular weight polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers, such as polyvinylpyrrolidone; amino acids, such as glycine, glutamine, asparagines, histidine, arginine, or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose or dextrins; chelating agents, such as EDTA; sugars, such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions, such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants, such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Subject

The subject may be a human, a companion animal (e.g. a dog or cat), a laboratory animal (e.g. a mouse, rat, rabbit, pig or non-human primate), a domestic or farm animal (e.g. a pig, cow, horse or sheep). Preferably, the subject is a human. In some cases the subject may be a human diagnosed with or classified as being at risk

of developing a cancer, e.g., an epithelial tumor, a solid tumor or a blood neoplasm. In certain cases the subject may be a laboratory animal, e.g., a mouse model of a cancer. In certain cases the subject may be a mammal (e.g. a human) that has been diagnosed with or classified as being at risk of developing an inflammatory condition, such as osteoarthritis or rheumatoid arthritis (RA). In particular, the subject may be a human having osteoarthritis or RA. In certain cases the subject may be a mammal (e.g. a human) that has been diagnosed with or classified as being at risk of developing an eye disease, such as diabetic retinopathy or macular degeneration.

Cancer

The anti-ENG conjugates described herein find use in the treatment of a tumor in a mammalian subject. The tumor may be a solid tumor. In particular, the tumor may be a pancreatic cancer, breast cancer, melanoma, Ewing sarcoma, lung cancer, head & neck cancer, ovarian cancer, bladder cancer or colon cancer.

Inflammatory condition

In some cases in accordance with the present invention, the anti-ENG antibody or the antibody drug conjugate may be for use in the treatment of an inflammatory condition. ENG expression has been reported in osteoarthritis and rheumatoid arthritis. See, e.g., Szekanecz, Z. et al. Clinical Immunology and Immunopathology, 1995, 76, 187-194, and Leask A et al., Arthritis & Rheumatism, 2002, 46, 1857-1865. The present inventors believe that the anti-ENG antibodies described herein, and/or conjugates thereof described herein, are able to ameliorate osteoarthritis, rheumatoid arthritis and/or symptoms of osteoarthritis or rheumatoid arthritis.

Eye disease

In some cases in accordance with the present invention, the anti-ENG antibody or the antibody drug conjugate may be for use in the treatment of an eye disease (e.g. diabetic retinopathy or macular degeneration, such as age related macular degeneration). ENG expression has been reported in certain eye conditions, including macular degeneration and retinopathy. See, e.g., Tsutomu Yasukawa *et*

al., Curr Eye Res. 2000, 21, 952-961, and Abu El-Asrar AM *et al.*, Mediators Inflamm. 2012,2012:697489, and Malik RA *et al.*, *J Cell Mol Med.* 2005, 9:692-7. The present inventors believe that the anti-ENG antibodies described herein, and/or conjugates thereof described herein, are able to ameliorate eye diseases and/or symptoms thereof (including diabetic retinopathy or macular degeneration, such as age related macular degeneration).

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

Examples

Example 1 - Production of anti-ENG antibodies

Anti-ENG scFvs isolated from synthetic antibody phage libraries have been described previously (*29*). One scFv, directed against the extracellular region of human ENG, known as "A5" (*29*) and one scFv, directed against the extracellular region of murine ENG, known as "mE12" (*31*) were converted into full-length IgG for subsequent characterisation studies and for generation of immunotoxins and ADCs. All scFvs were produced in *E. coli* and purified by IMAC, IgGs were produced in mammalian cells (CHO) using the Lonza GS expression vectors pEE6.4 and pEE14.4 developed for antibody production. Features of the scFv starting material are summarized in Table 1.

Table 1: antibodies, specificities, subclass, and vectors used as starting material

Format	*Species*	*Antigen*	*Clone*	*Vl Subclass*	*Vector*	*Plasmid DNA #*
scFv	*human*	*human ENG*	*A5*	*kappa*	*pAB1*	*179*
scFv	*human*	*mouse ENG*	*mE12*	*lambda*	*pAB1*	*151.1*

All scFvs were bacterially produced in *E.coli* TG1 and purified from the periplasmic extracts of 1L cultures by IMAC.

Plasmids corresponding to full length IgG1 antibodies were generated and transfected into CHO cells for production of antibodies in Lonza's CHO expressing system with yields of approximately 1 mg/L of cell culture (lab scale). Antibodies were purified from cell culture supernatant by protein A chromatography. Purified proteins were characterized by SDS-PAGE and size exclusion chromatography. Bioactivity was analyzed by ELISA using recombinant ENG and detection of bound antibodies with HRP-conjugated anti-human IgG antibodies. Cell binding was analyzed by flow cytometry using ENG-expressing mouse B16 cell.

Results:

Plasmids generated (and sequenced):

A5 IgG1: pEE14.4 A5-IgG1 OCMTX003p (human anti-huENG IgG1)

mE12 IgG1: pEE14.4 mE12-IgG1 OCMTOO4p (human anti-muENG IgG1)

Example 2 - Characterisation of anti-ENG antibodies

The amino acid sequences of anti-human ENG IgG1 A5 (A5-IgG1) heavy chain (HC) and light chain (LC), respectively are shown below:

Anti-human Endoglin A5-IgG1-HC:

METDTLLLWVLLLWVPGSTG

EVQLLESGGGLVQPGGSLRLSCAASGFTFS**SYAMS**WVRQAPGKGLEWVS**AIYGSDGDTTY**ADSVKGRF

TISRDNSKNTLYLQMNSLRAEDTAVYYCAR**VFYTAGFDY**WGQGTLVTVSSASTKGPSVFPLAPSSKST

SGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN

HKPSNTKVDKKVEPKSCDKTHTCPPCPAP***PVA***GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE

VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK***G***LP***SS***IEKTISKAK

GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS

KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 1)

aa	*447*
MW of processed HC	*48,703*
Theoretical pI	*8.36*

Potential glycosylation site (double underlined): N297

Mutations leading to ADCC and CDC deficiency are shown in bold italics (see also WO 99/58572)
Signal sequence is shown boxed
VH domain is underlined; CDRH1-H3 are shown in bold and curved underlined.

Anti-human Endoglin A5-IgG1-LC:

METDTLLLWVLLLWVPGSTG
DIELTQSPSSLSASVGDRVTITC**RASQSISSSLN**WYQQKPGKAPKLLIY**AASSLQS**GVPSRFSGSGSG
TDFTLTISSLQPEDFATYYC**QQAPAKPPT**FGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC (SEQ ID NO: 2)

aa	*214*
MW of processed HC	*23,113*
theoretical pI	*7.76*

signal sequence is boxed
VL domain is underlined; CDRL1-L3 are shown in bold and curved underlined.

A5-IgG1-HC – without signal sequence:
EVQLLESGGGLVQPGGSLRLSCAASGFTFS**SYAMS**WVRQAPGKGLEWVS**AIYGSDGDTTY**ADSVKGRF
TISRDNSKNTLYLQMNSLRAEDTAVYYCAR**VFYTAGFDY**WGQGTLVTVSSASTKGPSVFPLAPSSKST
SGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN
HKPSNTKVDKKVEPKSCDKTHTCPPCPAP***PVA***GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK***G***LP***SS***IEKTISKAK
GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS
KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 3)

A5-IgG1-LC – without signal sequence:
DIELTQSPSSLSASVGDRVTITC**RASQSISSSLN**WYQQKPGKAPKLLIY**AASSLQS**GVPSRFSGSGSG
TDFTLTISSLQPEDFATYYC**QQAPAKPPT**FGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC (SEQ ID NO: 4)

A5-VH:

EVQLLESGGGLVQPGGSLRLSCAASGFTFS**SYAMS**WVRQAPGKGLEWVS**AIYGSDGDTTY**ADSVKGRF
TISRDNSKNTLYLQMNSLRAEDTAVYYCAR**VFYTAGFDY**WGQGTLVTVSS (SEQ ID NO: 5)

A5-VL:
DIELTQSPSSLSASVGDRVTITC**RASQSISSSLN**WYQQKPGKAPKLLIY**AASSLQS**GVPSRFSGSGSG
TDFTLTISSLQPEDFATYYC**QQAPAKPPT**FGQGTKLEIKR (SEQ ID NO: 6)

A5-CDRH1:
SYAMS (SEQ ID NO: 7)

A5-CDRH2:
AIYGSDGDTTY (SEQ ID NO: 8)

A5-CDRH3:
VFYTAGFDY (SEQ ID NO: 9)

A5-CDRL1:
RASQSISSSLN (SEQ ID NO: 10)

A5-CDRL2:
AASSLQS (SEQ ID NO: 11)

A5-CDRL3:
QQAPAKPPT (SEQ ID NO: 12)

Parameters of the full A5-IgG are as follows:

Total length of full-length IgG (aa):	*1,322*
Calculated molecular mass of full-length IgG:	*143,577*
Calculated extinction coefficient of full-length IgG:	*195,440*
Abs 0.1% (=1 g/l)	*1.361*
theoretical pI:	*8.36*
potential glycosylation site:	*N297*

Anti-murine Endoglin mE12-IgG1-HC:

METDTLLLWVLLLWVPGSTG
EVQLVESGGGVVQPGRSLRLSCAASGFTFS**SYGMH**WVRQAPGKGLVWVS**RINSDGSSTSYADSVKG**RF
TISRDNSKNTLYLQMNSLRAEDTAVYYCAR**ATGTWVMS**WGQGTLVTVSSASTKGPSVFPLAPSSKSTS

GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKKVEPKSCDKTHTCPPCPAP***PVA***GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK***G***LP***SS***IEKTISKAKG
QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 13)

aa *446*
MW of processed HC *48,574*
Calculated pI *8.88*
Potential glycosylation site (double underlined): *N297*
Mutations leading to ADCC and CDC deficiency are shown in bold italics
Signal sequence is shown boxed
VH domain is underlined

Anti-murine Endoglin mE12-IgG1-LC:

METDTLLLWVLLLWVPGSTG
SSELIQDPAVSVALGQTVRITC**QGDSLRSYYAS**WYQQKPGQAPVLVIY**GKNNRPS**GIPDRFSGSSSGN
TASLTITGAQAEDEADYYC**NSRDSSGTV**FGGGTKLTVLGQPKANPTVTLFPPSSEELQANKATLVCLI
SDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK
TVAPTECS (SEQ ID NO: 14)

aa *212*
MW (processed) *22,516*
Calculated pI *6.69*
Signal sequence is shown boxed
VL domain is underlined

mE12-IgG1-HC – without signal sequence:
EVQLVESGGGVVQPGRSLRLSCAASGFTFS**SYGMH**WVRQAPGKGLVWVS**RINSDGSSTSYADSVKG**RF
TISRDNSKNTLYLQMNSLRAEDTAVYYCAR**ATGTWVMS**WGQGTLVTVSSASTKGPSVFPLAPSSKSTS
GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKKVEPKSCDKTHTCPPCPAP***PVA***GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK***G***LP***SS***IEKTISKAKG
QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 15)

mE12-IgG1-LC – without signal sequence:
SSELIQDPAVSVALGQTVRITC**QGDSLRSYYAS**WYQQKPGQAPVLVIY**GKNNRPS**GIPDRFSGSSSGN
TASLTITGAQAEDEADYYC**NSRDSSGTV**FGGGTKLTVLGQPKANPTVTLFPPSSEELQANKATLVCLI
SDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK
TVAPTECS (SEQ ID NO: 16)

mE12-VH:
EVQLVESGGGVVQPGRSLRLSCAASGFTFS**SYGMH**WVRQAPGKGLVWVS**RINSDGSSTSYADSVKG**RF
TISRDNSKNTLYLQMNSLRAEDTAVYYCAR**ATGTWVMS**WGQGTLVTVSS (SEQ ID NO: 17)

mE12-VL:
SSELIQDPAVSVALGQTVRITC**QGDSLRSYYAS**WYQQKPGQAPVLVIY**GKNNRPS**GIPDRFSGSSSGN
TASLTITGAQAEDEADYYC**NSRDSSGTV**FGGGTKLTVLG (SEQ ID NO: 18)

mE12-CDRH1:
SYGMH (SEQ ID NO: 19)

mE12-CDRH2:
RINSDGSSTSYADSVKG (SEQ ID NO: 20)

mE12-CDRH3:
ATGTWVMS (SEQ ID NO: 21)

mE12-CDRL1:
QGDSLRSYYAS (SEQ ID NO: 22)

mE12-CDRL2:
GKNNRPS (SEQ ID NO: 23)

mE12-CDRL3:
NSRDSSGTV (SEQ ID NO: 24)

Purified anti-muENG mE12 and anti-huENG A5 antibodies were analyzed in ELISA for binding to recombinant ENG and using flow cytometry analysis (FACS). Affinity results are shown for mE12 anti-muENG (Figures 1 and 2) and A5 anti-huENG IgG1s (Figure 3), and are summarized in Table 2. Figure 1 shows concentration-dependent

binding of mE12-IgG to recombinant mouse ENG (aa 27-581), but not to negative control (BSA). Figure 2 shows flow cytometry analysis of binding of mE12-IgG to a) B16 cells using 50 µg/ml antibody, b) B16 cells using 5 µg/ml antibody, and c) HT1080 cells included as negative control. Figure 3 demonstrates concentration-dependent binding of A5-IgG to human ENG by a) ELISA and b) flow cytometry analysis.

For scale-up the antibody constructs were cloned in GS double vectors (pEE14.4). The DNA plasmids were transformed, amplified, and transiently transfected into CHOK1SV cells for expression evaluation at a volume of 200 ml. In a second step the antibodies were transiently expressed in 5-10 L large scale cultures. Clarified culture supernatant was purified using one-step Protein A chromatography. Product quality analysis through SE-HPLC, SDS-PAGE and LAL was carried out using purified material at a concentration of 1 mg/ml, alongside an in-house human antibody as a control sample.

The purified protein samples were filtered through a 0.2 µm filter and analysed by SE-HPLC chromatograms. The mE12-IgG was purified to >98.8%. The A5-IgG was purified to >90%. The endotoxin levels were < 0.5 EU/mg.
All purified proteins were analyzed by SDS-PAGE in reducing and non-reducing conditions (data not shown).

Purified proteins A5-IgG and mE12-IgG were characterized by SDS-PAGE and size exclusion chromatography. Bioactivity was analyzed by ELISA, using recombinant mouse/human ENG and detection of bound antibodies with HRP-conjugated anti-human IgG antibodies. Cell binding was analyzed by flow cytometry, using ENG-positive HT1080 and ENG expressing mouse eEnd2. Melting points were determined by dynamic light scattering using a zetasizer nano. Affinities were determined by QCM using an Attana A100. Internalization study was performed by indirect immunofluorescence confocal microscopy on permeabilized cells, detecting bound and internalized antibodies with a FITC-labeled secondary antibody.

The full-length IgG1 purified antibodies were successfully produced at both lab scale and large scale, for the generation of immunoconjugates. A summary of antibody properties is shown in Table 2. The antibodies retained their specificity, as shown by ELISA and flow cytometry experiments. Affinities, as determined by QCM, were comparable with that of parental antibodies. QCM measurements indicated the contribution of avidity effects to high-affinity binding. Thermal stability differed between the different IgGs (64-72°C).

Preliminary internalization studies indicate rapid cellular internalization for A5-IgG against cells expressing human ENG. Indeed within 30min, almost 90% of the A5-IgG is found within HT1080 cells.

Table 2: Summary of antibody properties

antibody	***A5-IgG1***	***mE12-IgG1***
antigen	*human endoglin*	*mouse endoglin*
isotype	*γ1* / κ*	*γ1* / λ*
IgG type	*human*	*human*
plasmid	*OCMTX003p*	*OCMTX004p*
purity (SEC)	*minor aggregates*	√
Tm (DLS)	*72 °C*	*64 °C*
EC_{50} ELISA	*0.3 nM (rhENG)*	*60 nM (rmENG)*
EC_{50} FACS	*0.4 nM (HT1080)*	*89 nM (eEnd2)*
binding constants K_D (QCM)	*rhENG: K_D1 = 260 nM K_D2 = 2.5 nM*	*n.d.*
internalization	*HT1080 30-60min*	*n.d.*

y1 = deficient for ADCC and CDC (see Amour et al., 1999; Richter et al., 2013).*

Figure 9 shows internalization of anti-huENG A5 IgG1.

Example 3 - Nigrin-b A-chain

In order to avoid side effects of free toxin that could be released in the bloodstream and to reduce potential immunogenicity of the RIP toxin, as extensively described with ricin, the enzymatic domain of Nigrin b, the A chain, was cloned and expressed in bacteria. The present inventors hypothesized that, if the A chain produced in bacteria was able to retain its activity, it would not be able to enter the cells, unless conjugated to a vehicle molecule, such as an antibody.

Production

Nigrin-b A-chain was synthetized taking into account codon optimization for bacterial expression and the synthetized gene was cloned in two different vectors, Nigrin_pET30b-3 and Nigrin_pET33b-1 (+/- His tag) for expression in two different *E. coli* strains, *E. coli* BLR(DE3) and *E. coli* HMS174(DE3). Different culture media were used to check different expression conditions. Process purification was established using Capto Q chromatography and SP Sepharose High Performance. Purified recombinant Nigrin-b A-chain (recNgA) was formulated at 5mg/ml in PBS 1X pH7.4, DTT 0.5 mM, glycerol 10%. Endotoxin levels were <1EU/mg of Nigrin and the purity >99% in monomeric form.

Eldman N-terminal sequencing revealed that N-terminal end of recNgA corresponded to the expected sequence.

Recombinant Nigrin-b A-chain amino acid sequence:
MIDYPSVSFNLDGAKSATYRDFLSNLRKTVATGTYEVNGLPVLRRESEVQVKSRFVLVPLTNYNGNTV
TLAVDVTNLYVVAFSGNANSYFFKDATEVQKSNLFVGTKQNTLSFTGNYDNLETAANTRRESIELGPS
PLDGAITSLYHGDSVARSLLVVIQMVSEAARFRYIEQEVRRSLQQATSFTPNALMLSMENNWSSMSLE
IQQAGNNVSPFFGTVQLLNYDHTHRLVDNFEELYKITGIAILLFRCSSPSND (SEQ ID NO: 25)

The recombinant Nigrin-b A-chain has the following characteristics:
Number of amino acids: 256
Molecular weight: 28546.0
Theoretical pI: 5.45

The nucleotide sequence encoding recombinant Nigrin-b A-chain is as follows:
atagactatc cctccgtctc cttcaacttg gatggagcca agtcggctac atacagggac
ttcctcagca acctgcgaaa aacagtggca actggcacct atgaagtaaa cggtttacca
gtactgaggc gcgaaagtga agtacaggtc aagagtcggt tcgttctcgt ccctctcacc
aattacaatg gaaacaccgt cacgttggca gtagatgtga ccaaccttta cgtggtggct
tttagtggaa atgcaaactc ctacttttttc aaggacgcta cggaagttca aaagagtaat
ttattcgttg gcaccaagca aaatacgtta tccttcacgg gtaattatga caaccttgag
actgcggcga atactaggag ggagtctatc gaactgggac ccagtccgct agatggagcc
attacaagtt tgtatcatgg tgatagcgta gcccgatctc tccttgtggt aattcagatg
gtctcggaag cggcaaggtt cagatacatt gagcaagaag tgcgccgaag cctacagcag
gctacaagct tcacaccaaa tgctttgatg ctgagcatgg agaacaactg gtcgtctatg
tccttggaga tccagcaggc gggaaataat gtatcaccct tctttgggac cgttcagctt
ctaaattacg atcacactca ccgcctagtt gacaactttg aggaactcta taagattacg
gggatagcaa ttcttctctt ccgttgctcc tcaccaagca atgat (SEQ ID NO: 26)

Materials

- Nigrin_pET30b-3 genetic construct.
- *Escherichia coli* (Migula) Castellani and Palmers BLR(DE3)
-Culture media: auto induced medium (AIM)
-Extraction culture buffer: Glycine/NaOH 10mM, Leupeptine 1µg/ml, Pepstatine 1µg/ml, pH 9.5.
-Extraction supernatant buffer Tris-HCl 50 mM, NaCl 200 mM, $MgCl_2$ 2mM, leupeptine 1µgml^{-1}, pepstatine 1 µgml^{-1}, lysozyme 0.1 mgml^{-1}, pH8.0.
-Dialysis solution: Citric acid/NaOH 25mM pH5.0. -Capto Q FPLC: Equilibration buffer A: Glycine/NaOH 50 mM pH9.5. Elution buffer B: Glycine/NaOH 50mM pH9.5, NaCl 1 M.
- Pooled fractions from Capto Q step (+ 80 ml extraction).
-SP Sepharose HP FPLC: Equilibration buffer A: Citric acid 25 mM pH4.0.Elution buffer B: Citric acid 25 mM pH4.0, NaCl 1 M.

Methods

E. coli BLR(DE3) holding expression Nigrin_pET30b-3 cultivated in 1L format of Auto Inducible Medium (AIM) with 30 μgml^{-1} Kanamycin. Protein expression was triggered by lactose activation and glucose depletion after about 3-4 hours of growth. Then, the temperature was lowered to 20°C for an overnight duration.

For extraction, each cell pellet was initially resuspended in 80ml of extraction buffer per liter of culture, and 3 cycles of 7 minutes disintegration at 1100-110 Bar were performed after 30 minutes of incubation at 8°C under shaking. Then the extract underwent 60 minutes centrifugation at 15,900g, 8°C. The supernatant was the purification's starting material.

Capto Q FPLC: 160ml of extracted product from 8l culture were loaded into 160ml Capto Q and equilibrated using 4CV of equilibration buffer and washed with 15CV of equilibration buffer. Elution was carried in three steps: 15CV at 1.5mS/cm (7.6%B); 20CV at 23.8 mS/cm (18.9%B); 20CV 100%B.

Dialysis was performed at the following conditions: 650ml of the product were dialyzed in 4x5Lbathsin in citric acid/NaOH 25 mM pH5.0, cut-off 6-8000Da. Dialysis factor ~3500, <24h. After dialysis, a 30 minutes centrifugation at 20,500g and 8°C allowed to separate soluble from insoluble fractions. SDS-PAGE was performed on the total and soluble fractions both pre and post dialysis (10µl loaded on SDS-PAGE). The eluent was dialysed into PBS pH7.4 and filtered φ=0.22µm using 2x20cm^2 EKV filters.

SP Sepharose HP: 610ml of dialyzed pool of Capto Q in Citric acid 25 mM pH5.0 were loaded into 240ml SP Sepharose High Performance with 4CV of equilibration buffer and washed with 15CV of equilibration buffer and eluted at 25Cv gradient to 20% B; 4CV step of 100% B.

Pooled fractions from SP Sepharose HP step were dialysed in PBS pH7.4, DTT 0.5mM (5x4L baths, pooled fractions of 950 mL at 0.97 mg/ml). Cut off was 6-8000 Da, dialysis factor was ~3130, time >24h. Afterwards a 30 min centrifugation at 20,55g and 8°C allowed to separate soluble from insoluble fractions. 10% glycerol was added afterwards.

Finally the eluent was dialysed into PBS pH7.4 (5 baths ~3100) and filtered φ=0.2µm, then the recNg b A batch was snap frozen at -80°C. A SEC in Semi-Preparative S200 Superdex was later carried out.

Size exclusion chromatography and mass spectrometry analysis demonstrated monomeric and purification status of the obtained recombinant nigrin-b A-chain (recNgA) (Figure 4).

Stability studies were performed to evaluate pH and temperature effect on nigrin-b A-chain protein itself and its activity. recNgA is stable at pH ranging from 5 to 9, and in presence or not of glycerol (from 10 to 45%) (data not shown).

Activity

The ribosome-inactivating protein (RIP) activity of recombinant Nigrin-b A-chain was tested in rabbit reticulocyte cell-free lysates: IC_{50} value obtained was similar to native nigrin-b and within 2.5 to 25 pM range (see Figure 5). Thus, the A chain from Nigrin-b, expressed as a recombinant protein in bacteria, maintains its enzymatic activity, supporting that glycosylation is not required for RIP activity of Nigrin-b A-chain.

RecNgA retains its activity in rabbit reticulocyte cell-free lysates if stored frozen at (-80°C) and below 3 freeze-thaw cycles (not shown).

The cytotoxic activity of recNgA was tested on cell cultures through crystal violet-based viability assay. recNgA, lacking the B chain to translocate within cells, presents a 100 to 1000 less toxic activity than native Nigrin-b, as shown in Figure 6. Native nigrin b showed an $IC_{50} \approx 2x10^{-8}M$ (similar to previous published data see 37), while recNgA showed an $IC_{50} \approx 2x10^{-6}M$.

Previously published studies showed that native Nigrin b presents higher RIP activity than Ricin in RRL assay, while it is much less toxic (30-10,000 time, approximately) in cells or *in vivo* (see IC_{50} and LD_{50} values in Table 3).

Upon removing of B chain, Ricin A chain loses activity in both RRL assay and cytotoxicity assay. Unexpectedly, Nigrin b A chain, generated for the first time in this present invention, only loses activity in cell cytotoxicity assay, while it was even increased in RRL assay with respect to native Nigrin b. These data were suggesting that, in the case of Ricin, removing B chain was affecting not only binding and translocation of A chain, but also its RIP activity, while this was not the case for Nigrin b A chain that retains and even increases its RRL activity with respect to its native counterpart. As a result, Nigrin b A chain is 50 times more active than Ricin A chain in RRL.
Consequently, upon conjugation, Nigrin b A chain conjugates present higher cytotoxic activity (IC_{50} within pM range) than Ricin A chain conjugates (nM range)(not shown).

Table 3: *In vitro* and *in vivo* activity data for Ricin and Nigrin b (native and A chain).

	Rabbit Lysate IC_{50} (pM)	**HeLa Cells IC_{50} (pM)**	**Mouse LD_{50} (μgkg^{-1})**
Nigrin b	30	27,600.00 (20-2300nM; dpt cell line)	12,000.00
Nigrin b A chain	6.5	750, 000.00 (HT1080-FAP) 300, 000.00 (HT1080)	ND
Ricin	100	0.67	3.00
Ricin A chain	300	260,000.00 (T cells)	ND

(Inventors' own data Nigrin b A chain; see also Ferreras J.M. et al., Toxins, 3:420, 2011; Svinth M. et al., BBRC, 249: 637, 1998)

Example 4 - Conjugation of Nigrin-b A-chain to anti-ENG antibodies

For immunoconjugates containing RIPs to exhibit maximal cytotoxicity the RIP must be released from the targeting vehicle in fully active form, which requires avoiding steric hindrance (38). The disulfide bond is the only type of linkage that fit this criterium (39, 40). This bond allows conjugation using reagents for the introduction of free sulfhydryl groups such as N-succynimidyl 3(2-pyridyl-dithiopropionate) (SPDP) and 4-succynimidyloxycarbonyl -α-methyl-α (2-pyridyl-dithio)toluene (SMPT). Immunotoxins consisting of mAbs covalently bound to toxins by hindered disulfide linkers, often labeled as second generation immunotoxins, are stable, long lived and display potent cytotoxicity to target cells (41).

SPDP has already been used in the making of immunotoxins (ITs) containing nigrin b (36, 42). Moreover SMPT protects the disulfide bond from attack by thiolate anions, improving *in vivo* stability of the linkage (43, 44).

Material

-Recombinant nigrin b A chain in PBS, pH7.4, 10%glycerol, 0,5mM DTT, 4.92gl^{-1}, stored at 5°C.
-5,5'-dithio-bis-(2-nitrobenzoic acid)
- GE PD MiniTrap G-10 desalting columns.
-0.2 µm 28 mm sterile Minisart filters.
-Sciclone ALH 3000 workstation.
- Sarstedt Microtest Plate 96-Well Flat Bottom, ref n° 82.1581.

Methods

Dithiothreitol (DTT, Cleland´s reagent) is a redox agent that will be used to free the thiol groups present in the protein sample. Once said groups have been freed and so are available for reacting 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman reagent) will be added. Ellman reagent disulphide bridge will be cleaved and the 2 resulting thio-nitrobenzoate molecules (TNB) will attach to the protein at the thiol group sites. To titrate the TNBs absorbance values will be taken at λ=412nm, a wavelength at which DTT is not absorbed, rendering the concentration of thiol groups. The proportion of these with the concentration of the protein taken from its absorbance at

λ=280 will yield the number of free thiol groups per protein molecule.

Direct thiol titration was performed as follows:
204 µl recNg b A were dissolved in 796µl 20 mM phosphate 250 mM NaCl 1 mM EDTA pH 7.0 (assay buffer) (1.0033gl^{-1}=final concentration). Ellman reagent was dissolved in phosphate 0.2 M at 3gl^{-1}. For both buffers monobasic and dibasic sodium phosphate were added in a 1.61 to 1 mass proportion. PH was adjusted at room temperature and buffers were filtered.100ml Ellman buffer and 500ml assay buffer were prepared. Ellman reagent was completely resuspended rather than weighed.
The recNgA sample was incubated in the presence of 4.8 mM DTT at room temperature for 30 min. The recNgbA sample was then purified in the column and the first 10 ml of the eluent aliquoted (V=0.5ml). The A_{280} of the aliquots was taken and the two most concentrated mixed. A_{280} was taken again. 10 µl of 3 gl^{-1} DTNB were added and A_{412} measured after 2 min (n=1), using Ellman diluted in assay buffer in the same concentration as a blank (n_b=3). Readings belonged to the 0.1-3 AU linear range. Protein solutions were pipetted right beneath the meniscus after vortexing. 100 µl were pipetted per well.
The results of this study show that the thiol group belonging to recNgA´s single cysteine residue is free and available for reaction, not being blocked by its tertiary structure. This will allow recNgbA to be conjugated using a linker that requires a hindered inter-chain disulfide bond.

It is well established that immunoconjugates which contain ribosome-inactivating proteins exhibit maximal cytotoxicity only when the toxin molecule is released from the targeting vehicle in a fully active form. The separation of the RIP molecule from the carrier is required to avoid steric hindrance and to allow an effective translocation of the toxin into the cytoplasm (*38*). At present, the disulfide bond is the only type of linkage which appears to fit these criteria (*40*).

The coupling of two different protein macromolecules, that results in heterodimer formation, requires that each protein is modified prior to mixing them to react. In the case of the A chains of type 2 RIPs, the modification is limited to the reductive cleavage of the native cysteine residue that links the active (A) and the binding (B) chains of the molecule.

For IgG molecules, this is not possible because cysteine residues are involved in maintaining the tertiary and/or quaternary structure of the protein, so that it is not possible to reduce them without loss of the specific protein functions. Moreover, presumably some of the cysteine residues are not sterically accessible, as it was demonstrated by the 10 thiols groups per immunoglobulin that had to be generated for an optimal conjugation to an activated RIP (*45*).

For these reasons, in most IgG molecules, thiol groups are chemically inserted using hetero-bifunctional reagents, and several methods have been developed in order to generate hetero-conjugates avoiding or reducing to a minimum the formation of homopolymers. In most cases, the reagents used to introduce thiol groups react with amino groups, forming amide or amidine bonds. Amino groups are reactive, abundant and, in a limited way for most proteins, expendable. That is, a limited number of amino groups can be modified without diminishing the biological activity of the protein (40).

The most commonly used reagents for the introduction of free sulphydryl groups are N-succynimidyl 3—(2-pyridyl-dithiopropionate) (SPDP) and 4-succynimidyloxycarbonyl-α-methyl-α-(2-pyridyl-dithio)toluene (SMPT), that introduce 2-pyridyl disulphide groups into the protein by reacting with amino groups to form neutral amides, and methyl 4-mercaptobutyrimidate (2-iminothiolane.Traut's reagent) that introduces mercaptobutyrimidoyl groups, reacting to form charged amidines, thus preserving the positive charge of the derivatized amino acid (40;*44*).

SPDP and SMPT introduce hindered disulphide bond, while 2-iminothiolane -SH must be protected by reacting it with 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent).
The reaction with Ellman's reagent is also used for the quick measurement of protein sulphydryl groups (*45, 46*).

SMPT has a methyl group and a benzene ring attached to the carbon atom adjacent to disulphide bond that protects it from attack by thiolate anions, thus improving the *in vivo* stability of the linkage (*43, 44*).

Based on these data, IgG proteins can be modified with SMPT, which do not significantly affect the antigen binding property of the molecules in the following conditions, even if they change the charge of the protein in the reaction site.

In one study the present inventors investigated conjugating IgG1s with recNgA, using 2 different recNgA:mAb molar ratio of 2.5 and 3.5, after derivatization using an SMPT:mAb molar ratio of 6, following conjugation protocols (see 40). Purification was performed by Size Exclusion chromatography on Sephacryl S200 (see *41*).

Under the described conditions, the immunotoxin is predominantly a mixture of antibody linked to one or two toxin molecules, with the presence of high molecular weight components (IgG linked to several RIP proteins), as well as free and polymeric RIPs (dimeric in the case of recNgA) and free antibody. Thus, a careful purification is thought to be desirable to obtain a pure product.

In vitro activity testing

Activity testing on conjugates prepared as described above was performed though evaluation of RIP activity in rabbit reticulocyte cell-free lysate (RRL) assay. Results are presented in Figure 7.

IC_{50} values obtained for the native Nigrin-b or recNgA were in the 2.5pM range and those for conjugates were similar and within 1-0.5pM range, even higher than native Nigrin-b positive control, showing

that antibody conjugation did not affect the enzymatic activity of recNgA.

Example 5 - Conjugation of cytolysins to anti-ENG antibodies

The cytolysins employed for conjugation studies were chosen from the general structure shown above (formula IV). These structures exhibit activity against different cancer cell lines (nM to pM range).

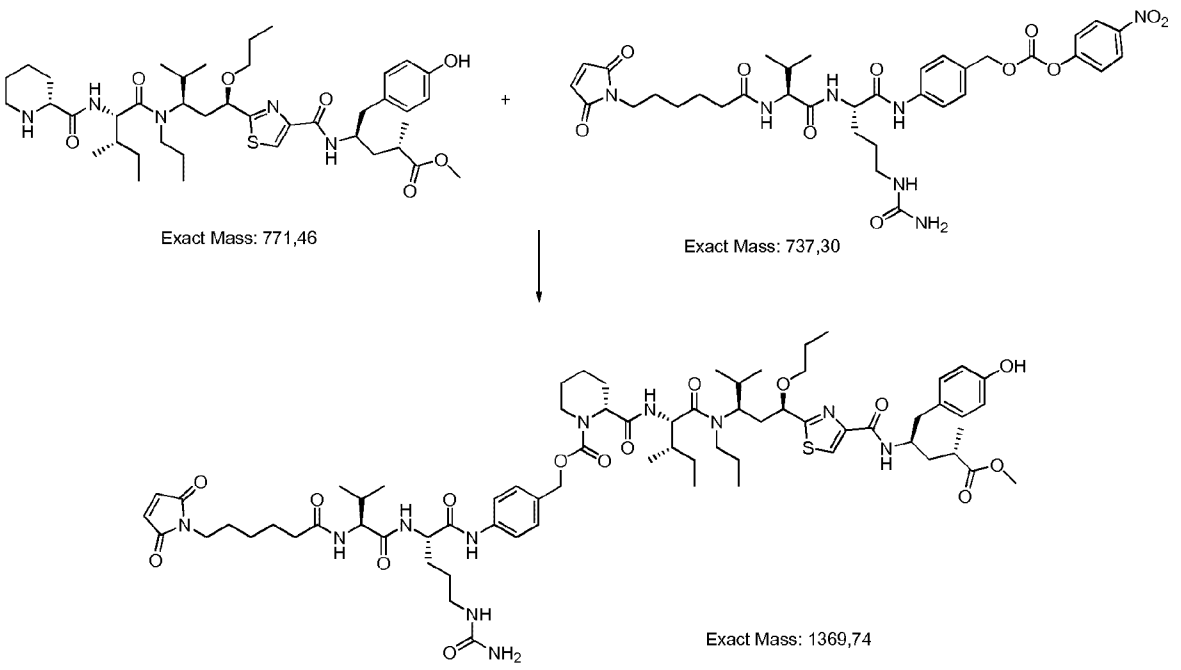
Various linker systems can be used and attached to either R^2 or R^{17} position of the molecule.

The general outline of the cytolysin conjugates, including the vcPABA linker and anti-ENG antibody, is shown in Figure 8 (in the structure depicted in Figure 8, the attachment site of the cytolysin to the vcPABA linker is at position R_1 or R_4 - the R_1 and R_4 numbering system used in Figure 11 differs from the R group numbering system used, e.g., in the claims; it is intended that R_1 of Figure 11 corresponds to R^2 in the claims and that R_4 of Figure 11 corresponds to R^{17} of the claims).

The vcPABA (valine-citrulline-PABC) protease-cleavable linker has been previously used in the ADC molecule Brentuximab Vedotine, developed by Seattle Genetics and Takeda, and recently approved by the FDA and EMEA as Adcetris® (2011, and Nov. 2012, respectively). In this ADC the vcPABA has been coupled at its free NH2 to maleimide caproyl for thiol-based conjugation on mAb (cAC10 anti-CD30 antibody). On the other side, vcPABA has been conjugated through its COOH to the Auristatin cytotoxic drug from Seattle Genetics (MMAE). (see 48)

The present inventors have used this linker (maleimide caproyl-vcPABA) to conjugate anti-ENG antibodies through thiol-based reaction with the maleimide caproyl, and on the other end, to the cytolysin cytotoxic molecules through its cyclic piperidine with vcPABA (R_1 or R_4 positions of the cytolysin shown in Figure 8).

Synthesis of Maleimido-val-cit-PABOCO-Tubulysin/Cytolysin-TAM461:

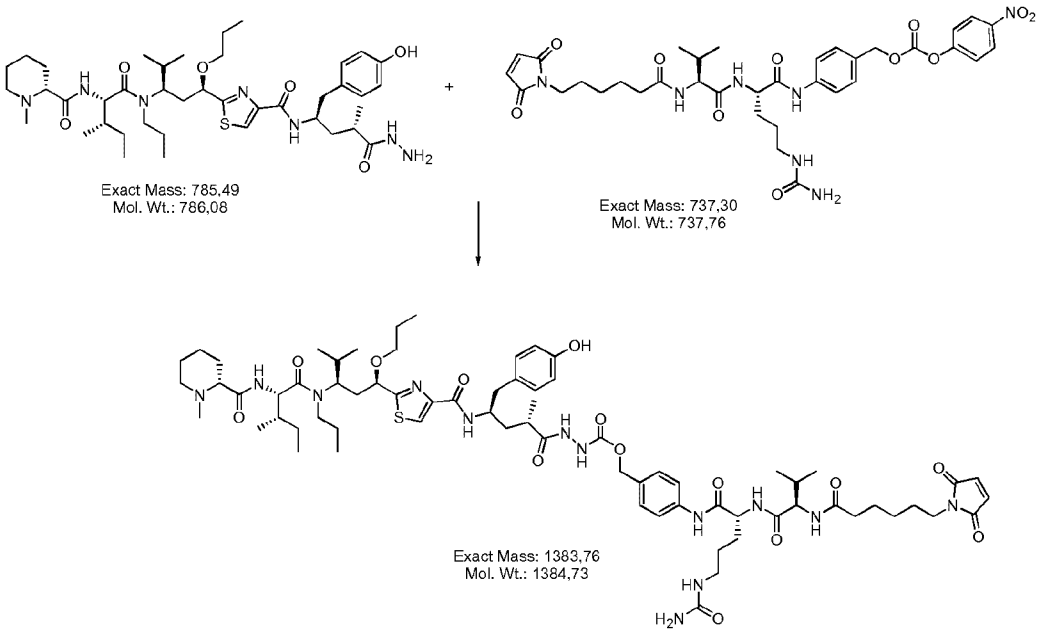


TAM467

TAM461 (Tubulysin/Cytolysin):	30.0 mg (0.041 mmol)
DMF:	3 mL
TAM465 (Linker):	35 mg (0.045 mmol)
HOBt:	1.4 mg
DIPEA:	10 µL

TAM461 and **TAM465** were dissolved in anhydrous DMF under dry conditions and the resulting solution was treated with HOBt and DIPEA. The reaction was stirred at RT for 18h. The reaction mixture was concentrated and the resulting oil was purified by column chromatography using 2-6% methanol: DCM to give 35 mg (64%) of **TAM467** as a white solid. ESI-MS: m/z = 1371 [M+H].

Synthesis of Maleimido-val-cit-PABOCO-Tubulysin/Cytolysin-TAM470:



TAM471

TAM470 (Tubulysin/Cytolysin):	0.07 mmol
DMF:	5 mL
TAM466 (Linker):	50 mg (0.065 mmol)
HOBt:	2.4 mg
DIPEA:	18 µL

TAM470 and **TAM466** were dissolved in anhydrous DMF under dry conditions and the resulting solution was treated with HOBt and DIPEA. The reaction was stirred at RT for 18h and then analysed with TLC, indicating completion of reaction, The reaction mixture was concentrated and the resulting oil was purified with column chromatography using 4-12% methanol: DCM to give 56mg of **TAM471** (yield: 62%). ESI-MS: 1384.6 [M+1].

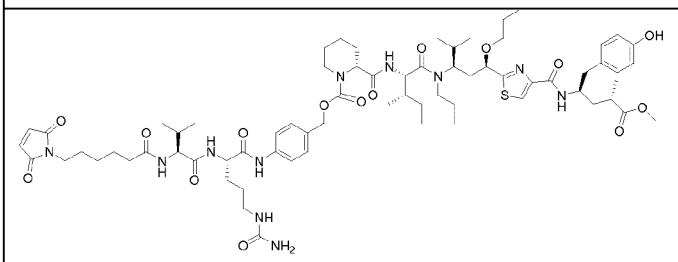
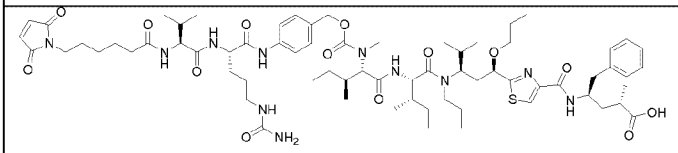
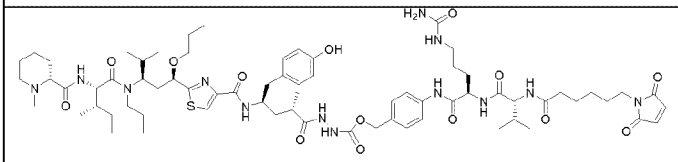
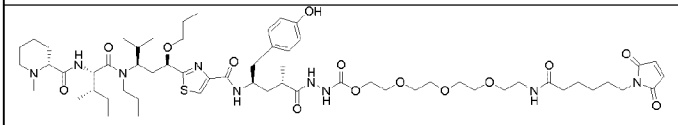
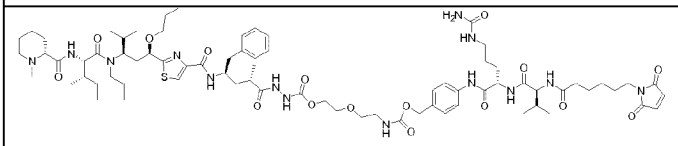
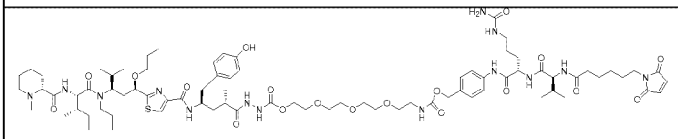
In vitro activity testing is performed. Functional activity is evaluated through microtubule inhibition assay, while cytotoxic activity is determined through crystal violet viability assay.

Generation of cytolysin-linker derivatives

Different cytolysin-linker derivatives were synthesized according to the general structure presented in Figure 11, where vcPABA linker was added either in position R1 (TAM467, TAM551) or R4 (TAM471, TAM553, TAM558), alone or with ethylene-glycol spacer (EG; n=1 to

3), or substituted by ethylene glycol groups (n=3) (TAM552). The respective chemical structures are presented in Table 4.

Table 4: Chemical structure of cytolysin-linker derivatives

Product	Code	Mol. Wt.
	TAM467	1370.7
	TAM551	1356.7
	TAM471	1384.7
	TAM552	1198.5
	TAM553	1499.8
	TAM558	1603.9

Microtubule inhibition activity and cytotoxic activity of each new derivative were evaluated through tubulin polymerization inhibition assay (TPI; Tubulin Polymerization assay kit; Cytoskeleton, Cat. #BK011P), and cell proliferation arrest on HT1080 cells (CPA; crystal violet). IC50 were calculated and results are presented in Table 5.

Table 5: Microtubule inhibition activity and Cell Cytotoxicity activity of cytolysin-linker derivatives. ***(ND: Not determined)***

Compound	**IC50 (TPI assay; µM)**	**IC50 (CPA assay; nM)**
TAM467 (Linker in R1)	150	230-420
TAM551 (Linker in R1)	ND	90
TAM471 (Linker in R4; vcPABA)	14	17-42
TAM552 (Linker in R4; no vcPABA; 3EG)	1.9	10
TAM553 (Linker in R4; vcPABA; 1EG)	6	98
TAM558 (Linker in R4; vcPABA; 3EG)	1.9	98
TAM334 (parental cytolysin; no linker)	2	0.3-0.6
Tubulysin A	ND	0.04-0.2
Tubulysin A + linker	ND	5-20
MMAE (Seattle Genetics)	ND	0.1-0.6
DM1-DM4 (Immunogen)	ND	0.01-0.1

In vitro activity of parental cytolysin TAM334 is within the same range of other payloads currently used for the generation of antibody-drug conjugates such as auristatins (MMAE) or maytansinoids (DM1-DM4). As expected and previously described for other compounds from the Tubulysin A family, upon addition of linker, cell cytotoxic activity of cytolysins was decreased with respect to the parental compound TAM334. In addition, TAM467 derivative was presenting significantly lowest activity in both assays. All the derivatives were used in conjugation to generate ADC molecules and were evaluated comparatively both *in vitro* and *in vivo* to select the most active cytolysin-linker derivative.

Conjugation and Chemical characterization of ADCs

Each of the newly generated derivatives was conjugated to monoclonal IgG1 human antibodies following a non-site-specific conjugation method on cysteine residues. To this aim, one batch of antibody was reduced and reacted with each of the derivatives. Different TCEP

ratios were tested to reach optimal DAR of 3-4, less than 10% of free antibody and drug. Optimal conjugation conditions were as followed: TCEP=2.5 and 3.57 Thiol levels Ellmann's. Conjugates were then purified on G25 Sephadex and analysed through Size Exclusion Chromatography (SEC) to determine their purity, as well as Hydrophobic Interaction Chromatography (HIC) and Polymeric liquid reversed-phase chromatography (PLRP) to determine DAR, content of free antibody and distribution profile of different ADC species (0-8 drugs/mAb). Content of free drug was evaluated by UV detection method at 280nm. Results of chemical analysis were determined (not shown) and biochemical characteristics of ADCs are shown in Table 6.

Table 6: Summary of chemical characteristics of the different ADC molecules

Lot	Drug	mAb Conc.	HIC free mAb	DAR	SEC purity 280 nm	Free Drug	Volume
HPS157-039-001	TAM471	1.195 mg/mL	10.1%	3.38	92%	0%	~5.8 mL (6.931 mg)
HPS157-039-002	TAM551	1.332 mg/mL	22.4%	3.08	74%	0%	~5.8 mL (7.726 mg)
HPS157-039-003	TAM552	1.319 mg/mL	5.1%	3.84	97%	0%	~5.8 mL (7.650 mg)
HPS157-039-004	TAM553	1.305 mg/mL	7.0%	4.10	84%	0%	~5.8 mL (7.569 mg)
HPS157-039-005	TAM558	1.332 mg/mL	5.8%	3.92	93%	0%	~5.8 mL (7.726 mg)

The various drugs produced different levels of aggregation. Specifically ADC HPS157-039-002 (TAM551) showed highest level of aggregation already at DAR=3.08, leaving 22.4% of unconjugated antibody. A preliminary conjugation with TAM467 also showed high level of aggregation: at DAR 3.27, SEC purity was already only 67% with 16% of free drug (data not shown). These data were suggesting that vcPABA linker in position R1 was not optimal for this type of cytolysin molecules.

In vitro evaluation of cytolysin conjugates Cytolysin ADC molecules were evaluated comparatively in vitro through proliferation arrest assay (crystal violet staining). Results are presented in Figure 10 and IC_{50} values in Table 7.

Table 7: IC_{50} values obtained in Proliferation Arrest Assay (nM)

Compound	HT1080-WT	HT1080-AG(+)
TAM334	1.04	0.77
ADC-471 (HPS-157-039-001)	5.6	10.33
ADC-551 (HPS-157-039-002)	964	552
ADC-553 (HPS-157-039-004)	90	108
ADC-558 (HPS-157-039-005)	555	0.96

Location of vcPABA linker alone in R1 position (ADC-551) generated conjugates with much less cytotoxic activity in vitro with respect to R4 position (ADC-471) (Figure 10; Table 7). In addition, increasing the number of ethylene-glycol groups as spacer to vcPABA linker in R4 position (ADC-471 (n=0) versus ADC-553 (n=1) and ADC-558 (n=3)) was shown to increase antigen-specific cytotoxic activity *in vitro* (Figure 10). Indeed, while ADC-471 and ADC-553 showed low and no antigen-specific cytotoxic activity (10nM and 100nM IC_{50} range, respectively) with no difference between wild type (WT) and antigen (AG) expressing HT1080 cells, ADC-558 presented a 1 nM range specific cytotoxic activity with a specificity ratio of 500 between AG and WT HT1080 cells.

Example 6 - Evaluation of in vivo anti-tumoral effect of conjugates

Both types of immunoconjugates, recNgA- and cytolysin- conjugates, were evaluated for their anti-tumoral effect in vivo in a patient-derived xenograft mouse model for pancreas cancer (PAXF-736), previously selected for antigen expression.

Dose range studies were performed to define the maximum tolerated dose to be used in efficacy studies (not shown). For recNgA immunoconjugates, a highest tolerated dose of 0.5mg/kg was found, while cytolysin conjugates, independently of the derivative used, were administrated at doses from 2.5mg/kg up to 20mg/kg, without any weight loss or toxic effect.

Immunoconjugates were then administrated once a week intraperitoneally over 5 weeks. Tumor volume and body weight were measured every 2-3 days. Vehicle-treated and Gemcitabine-treated (150mg/kg) PDX mice were used as negative and positive control groups, respectively. Results are shown in Figure 11.

The recNgA immunoconjugates (OMTX505) presented a high *in vivo* anti-tumoral efficacy (60%) at a dose of 0.5 mg/kg in PDX murine models of pancreas cancer (Figure 11A). When combined with Gemcitabine, it even showed 100% tumor growth inhibition and tumor regression.

According to the *in vitro* results (see Figure 10 & Table 7), location of vcPABA linker alone in R1 position (OMTX705-551) generated conjugates with no anti-tumoral activity in vivo (Figure 11B).

Supporting the *in vitro* data, increasing the number of ethylene-glycol groups as spacer to vcPABA linker in R4 position (OMTX705-471 (n=0) versus OMTX705-553 (n=1) and OMTX705-558 (n=3)) was shown to increase anti-tumoral effect *in vivo* (Figure 11C). OMTX705-471 and OMTX705-553 did not show any anti-tumoral effect *in vivo*, while OMTX705-558 presented a 40% tumor growth inhibition effect at 2.5mg/kg dose in PDX mouse model for pancreas cancer.

From these data, recNgA and TAM558 molecules were selected as best payloads for anti-ENG conjugates.

Example 7 - Ewing sarcoma models

Tumor cell plasticity enables certain types of highly malignant tumor cells to dedifferentiate and engage a plastic multipotent embryonic-like phenotype, which enables them to 'adapt' during tumor progression and escape conventional therapeutic strategies. A recent study demonstrated that ENG expression correlates with tumor cell plasticity in Ewing sarcoma, and it is significantly associated with worse survival of Ewing sarcoma patients. Ewing sarcoma with reduced ENG levels showed reduced tumor growth *in vivo*. This study thus

delineates an important role of ENG in tumor cell plasticity and progression of aggressive tumors *(51)*.

The present inventors hypothesize the therapeutic potential of anti-ENG monoclonal antibodies, ITs and ADCs, in the treatment of Ewing Sarcoma.

14 cell line models of Ewing sarcoma have been developed for *in vitro* studies, and their corresponding xenograft models, for the screening and characterization of therapeutic molecules for the treatment of Ewing Sarcoma. ENG expression has been confirmed in all the 14 cell lines, and all the human Ewing sarcoma patient samples (n=10) that have been examined.

The specific embodiments described herein are offered by way of example, not by way of limitation. Any sub-titles herein are included for convenience only, and are not to be construed as limiting the disclosure in any way.

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Claims:

1. An antibody-drug conjugate having the formula I:

$$A-(L-D)_p \quad (I)$$

or a pharmaceutically acceptable salt or solvate thereof,

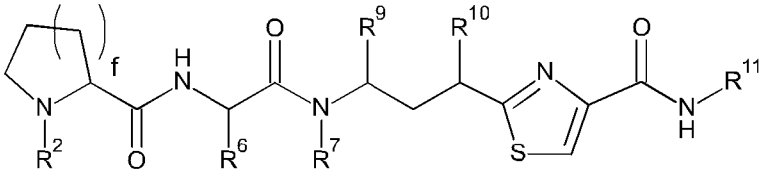
wherein:

A is an antibody that selectively binds Endoglin;

L is a linker;

p is 1 to 10; and

D is a drug comprising a cytolysin of formula IV:



(IV)

wherein:

R^2 is H or C_1-C_4 alkyl;

R^6 is C_1-C_6 alkyl;

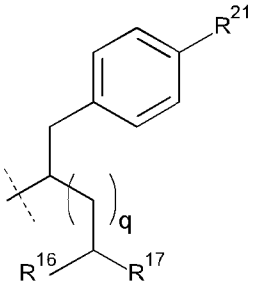
R^7 is C_1-C_6 alkyl, CH_2OR^{19} or CH_2OCOR^{20}, wherein R^{19} is alkyl, R^{20} is C_2-C_6-alkenyl, phenyl, or CH_2-phenyl;

R^9 is C_1-C_6 alkyl;

R^{10} is H, OH, O-alkyl or O-acetyl;

f is 1 or 2;

R^{11} has the following structure:



wherein

R^{21} is H, OH, halogen, NH_2, alkyloxy, phenyl, alkyl amino or dialkyl amino;

R^{16} is H or a C_1-C_6-alkyl group;

R^{17} is directly or indirectly attached to linker L; and

q is 0, 1, 2 or 3.

2. The antibody-drug conjugate of claim 1, wherein A comprises heavy chain complementarity determining regions 1-3 (CDRH1-3) and light chain complementarity determining regions 1-3 (CDRL1-3) having the following amino acid sequences:
(i) CDRH1: SEQ ID NO: 7;
(ii) CDRH2: SEQ ID NO: 8;
(iii) CDRH3: SEQ ID NO: 9;
(iv) CDRL1: SEQ ID NO: 10;
(v) CDRL2: SEQ ID NO: 11; and
(vi) CDRL3: SEQ ID NO: 12.

3. The antibody-drug conjugate of claim 2, wherein A comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO: 5 and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 6.

4. The antibody-drug conjugate of claim 2, wherein A comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 3 and a light chain comprising the amino acid sequence of SEQ ID NO: 4.

5. The antibody-drug conjugate of any one of claims 1 to 4, wherein R^{17} is C(O)X, CONHNHX, OX, NHX or SX, wherein X is a bond to linker L.

6. The antibody-drug conjugate of any one of claims 1 to 5, wherein linker L further comprises a spacer.

7. The antibody-drug conjugate of claim 6, wherein the spacer has a chain length of 2 to 30 atoms.

8. The antibody-drug conjugate of claim 6 or claim 7, wherein the spacer comprises or consists of a group $-(CH_2)_n-$ or $-(OCH_2CH_2)_n-$, wherein n = 1 to 10.

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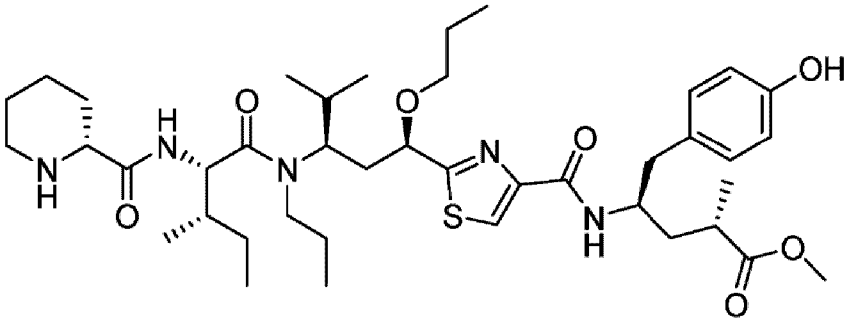
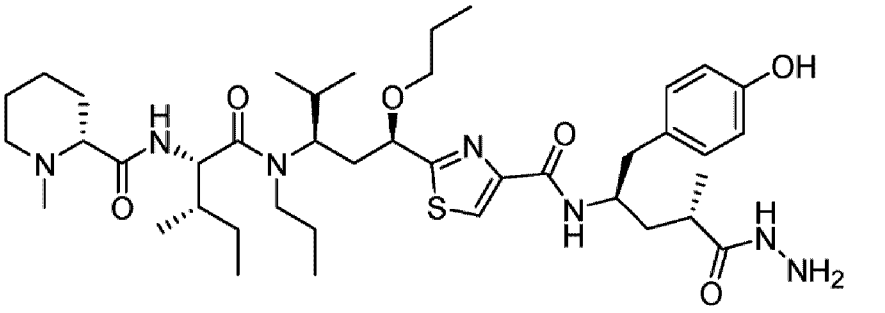
9. The antibody-drug conjugate of any one of claims 6 to 8, wherein the spacer is directly attached to group R^{17}, or is attached to group R^{17} via a bridging group.

10. The antibody-drug conjugate of claim 9, wherein the spacer is attached to group R^{17} via a -C(O)X bridging group, wherein X is a bond to R^{17}.

11. The antibody-drug conjugate of claim 1, wherein D comprises a cytolysin having the following structure:



or



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12. The antibody-drug conjugate of any one of claims 1 to 11, wherein L comprises an attachment group for attachment to A and protease cleavable portion.

13. The antibody-drug conjugate of claim 12, wherein L comprises maleimidocaproyl-valine-citrulline-p-aminobenzylcarbamate.

14. The antibody-drug conjugate of any one of claims 1 to 13, wherein -L-D has a structure selected from the group consisting of:

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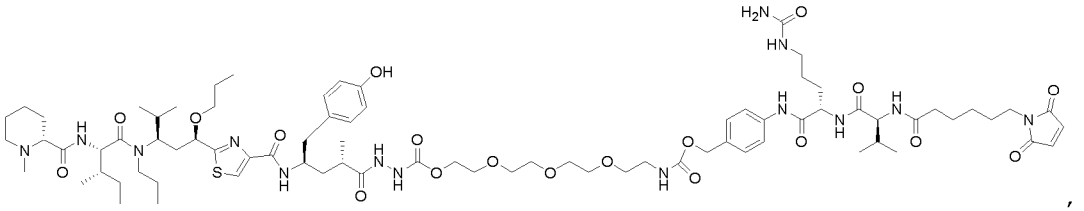

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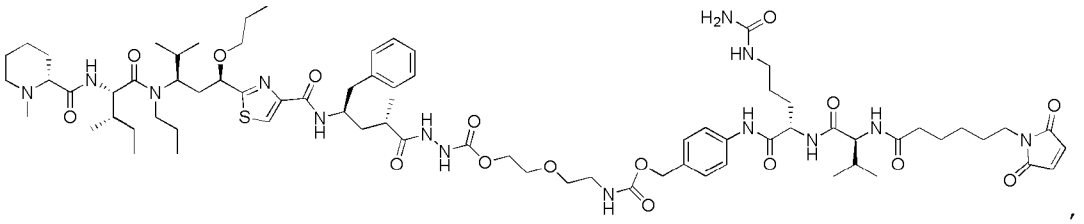

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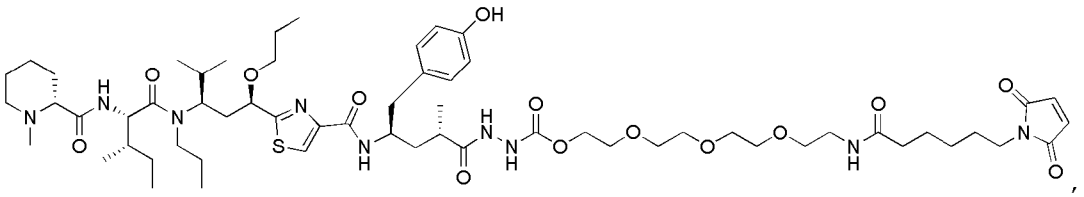

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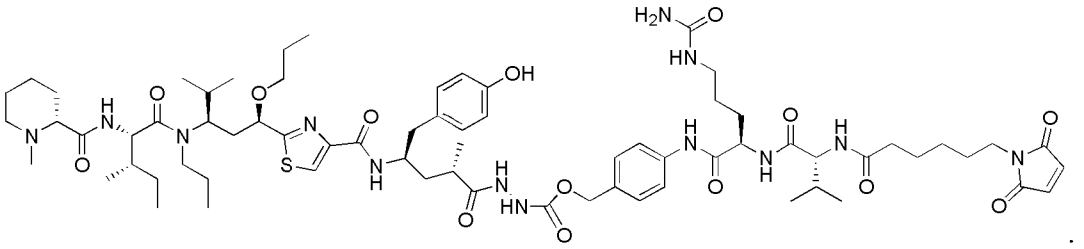
and



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15. Use of an antibody-drug conjugate as defined in any one of claims 1 to 14 to treat a tumor in a mammalian subject.

16. Use according to claim 15, further comprising the simultaneous, sequential or separate use of one or more other antitumor drugs.

17. Use according to claim 16, wherein said one or more other antitumor drugs comprise a cytotoxic chemotherapeutic agent or an anti-angiogenic agent or an immunotherapeutic agent.

18. Use according to claim 17, wherein said one or more other antitumor drugs comprise Gemcitabine, Abraxane, bevacizumab,

itraconazole, carboxyamidotriazole, an anti-PD-1 molecule or an anti-PD-L1 molecule.

19. Use according to claim 18, wherein said anti-PD-1 molecule or anti-PD-L1 molecule comprises nivolumab or pembrolizumab.

20. Use according to any one of claims 15 to 19, wherein the tumor is a blood neoplasm, pancreatic cancer, Ewing sarcoma, breast cancer, melanoma, lung cancer, head and neck cancer, ovarian cancer, bladder cancer or colon cancer.

21. Use of the antibody-drug conjugate of any one of claims 1 to 14 to treat an inflammatory condition or an eye disease.

22. Use according to claim 21, wherein:

said inflammatory condition is rheumatoid arthritis; and/or

said eye disease is diabetic retinopathy or macular degeneration.

23. Use of an antibody-drug conjugate as defined in any one of claims 1 to 14 in the manufacture of a medicament to treat a tumor in a mammalian subject.

24. Use according to claim 23, wherein the tumor is a blood neoplasm, pancreatic cancer, Ewing sarcoma, breast cancer, melanoma, lung cancer, head and neck cancer, ovarian cancer, bladder cancer or colon cancer.

25. Use of the antibody-drug conjugate of any one of claims 1 to 14 in the manufacture of a medicament to treat an inflammatory condition or an eye disease.

26. Use according to claim 25, wherein:

said inflammatory condition is rheumatoid arthritis; and/or

said eye disease is diabetic retinopathy or macular degeneration.

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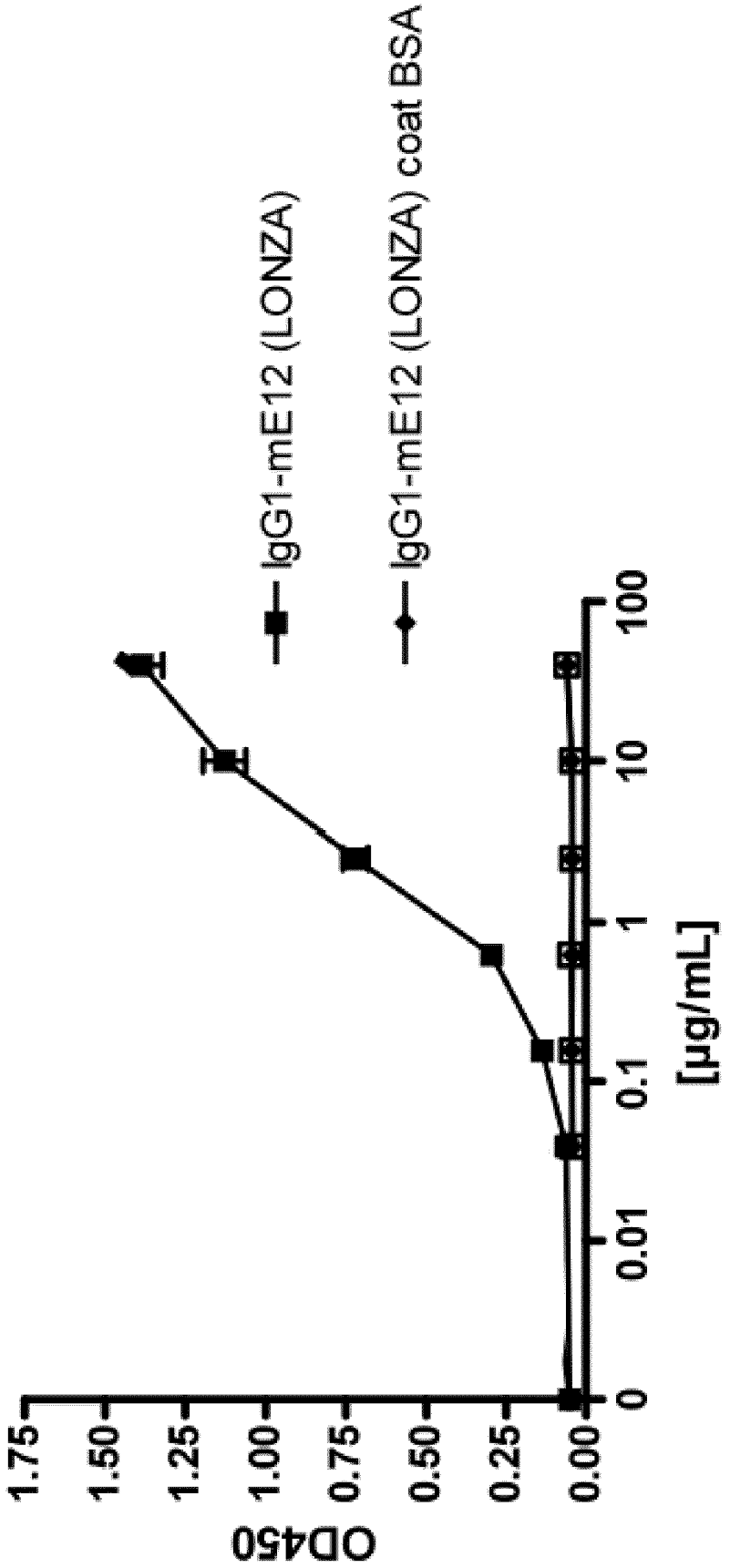
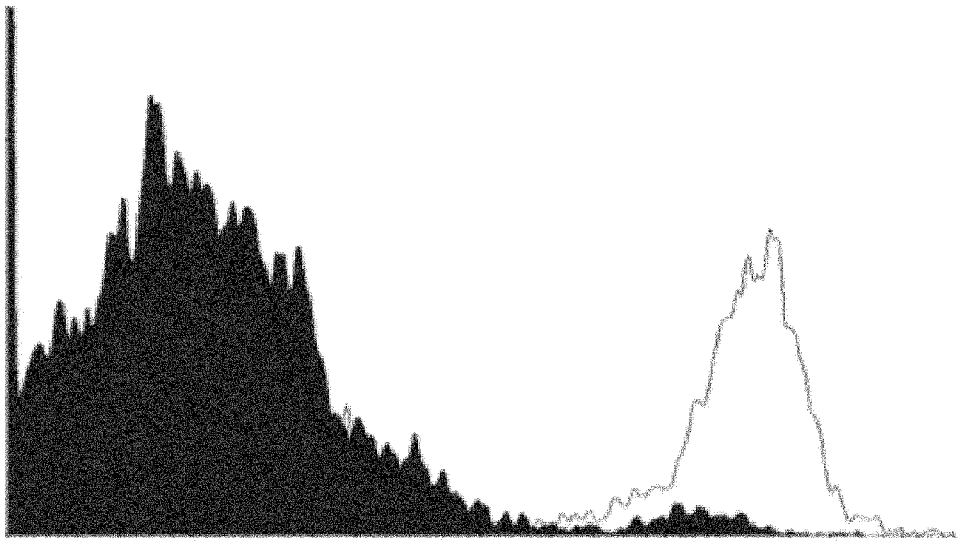

IgG1-mE12 (LONZA)
IgG1-mE12 (LONZA) coat BSA
OD450
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
0.01
0.1
1
10
100
[µg/mL]

Figure 1

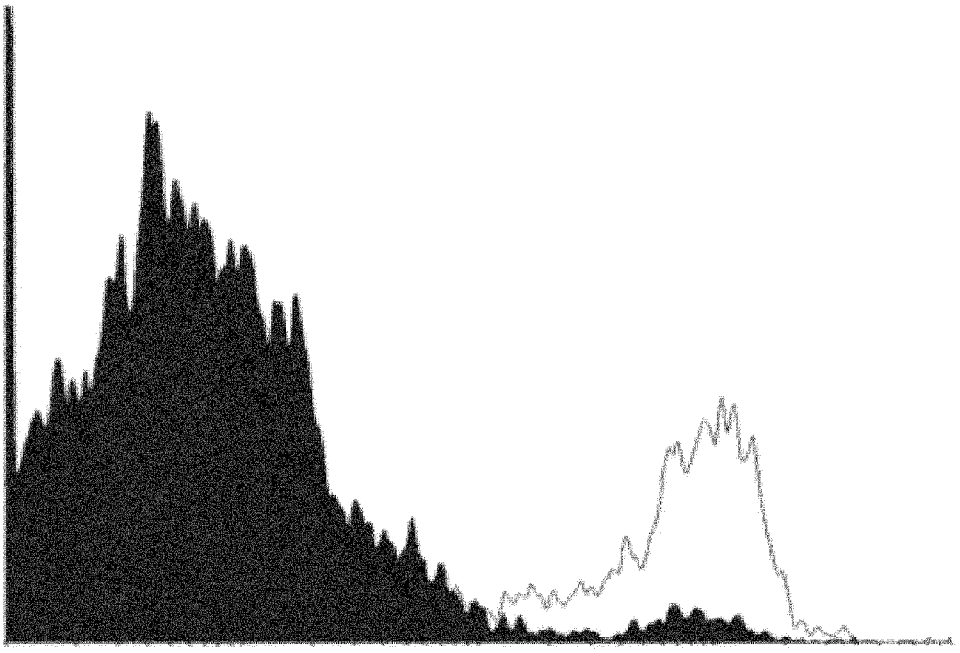
a)



B16 + IgG1 mE12 (50 µg/ml)

Figure 2A

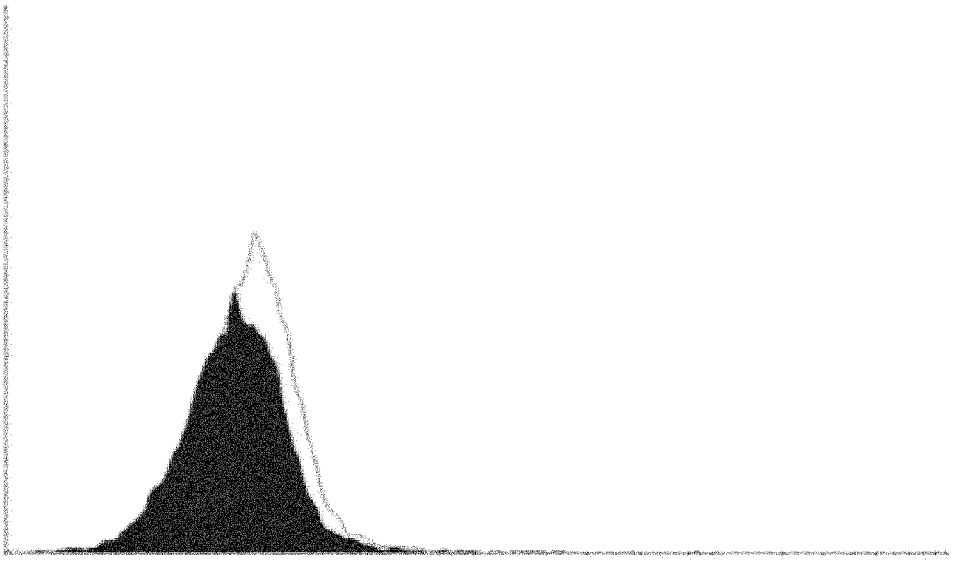
b)



B16 + IgG1 mE12 (5 µg/ml)

Figure 2B

c)



H1080 + IgG1 mE12 (5 µg/ml)

Figure 2C

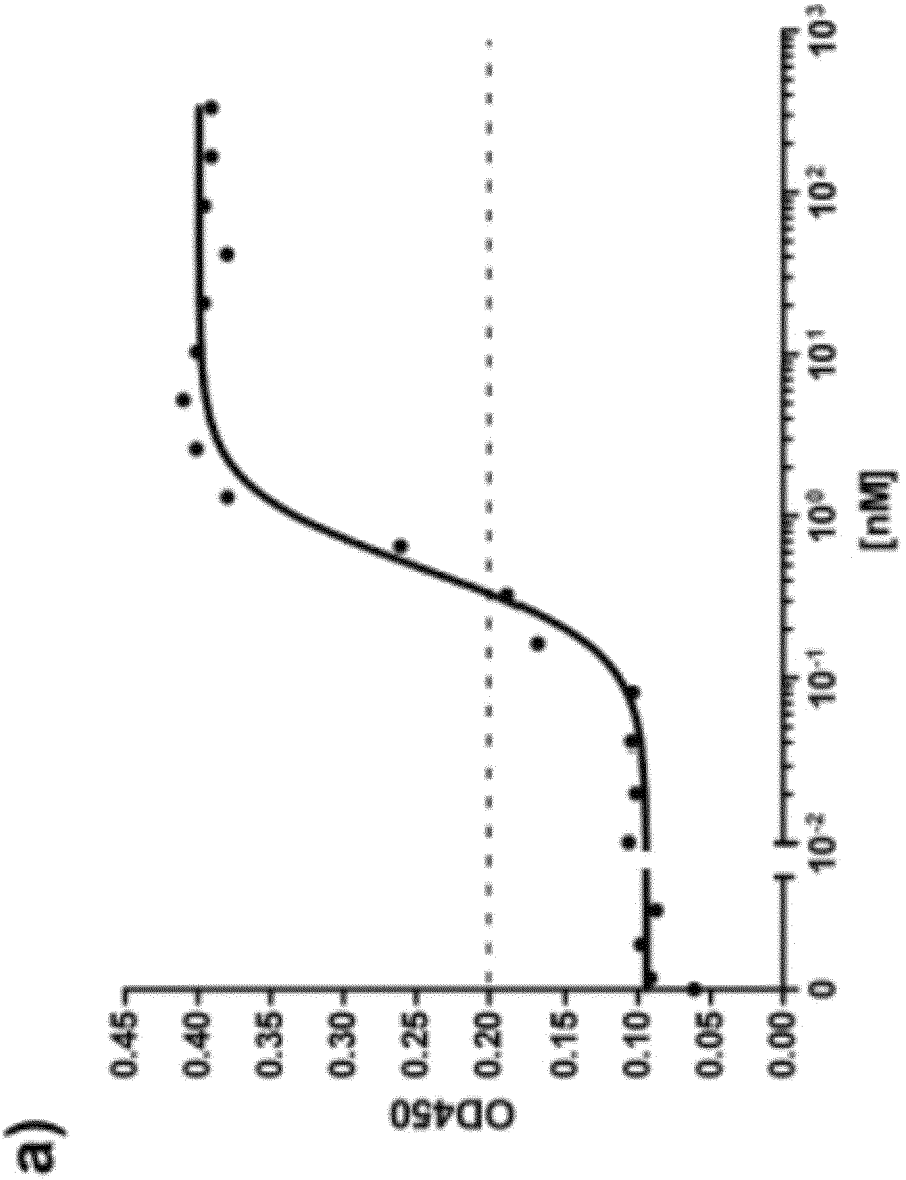

a)
OD450
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
[nM]

Figure 3A

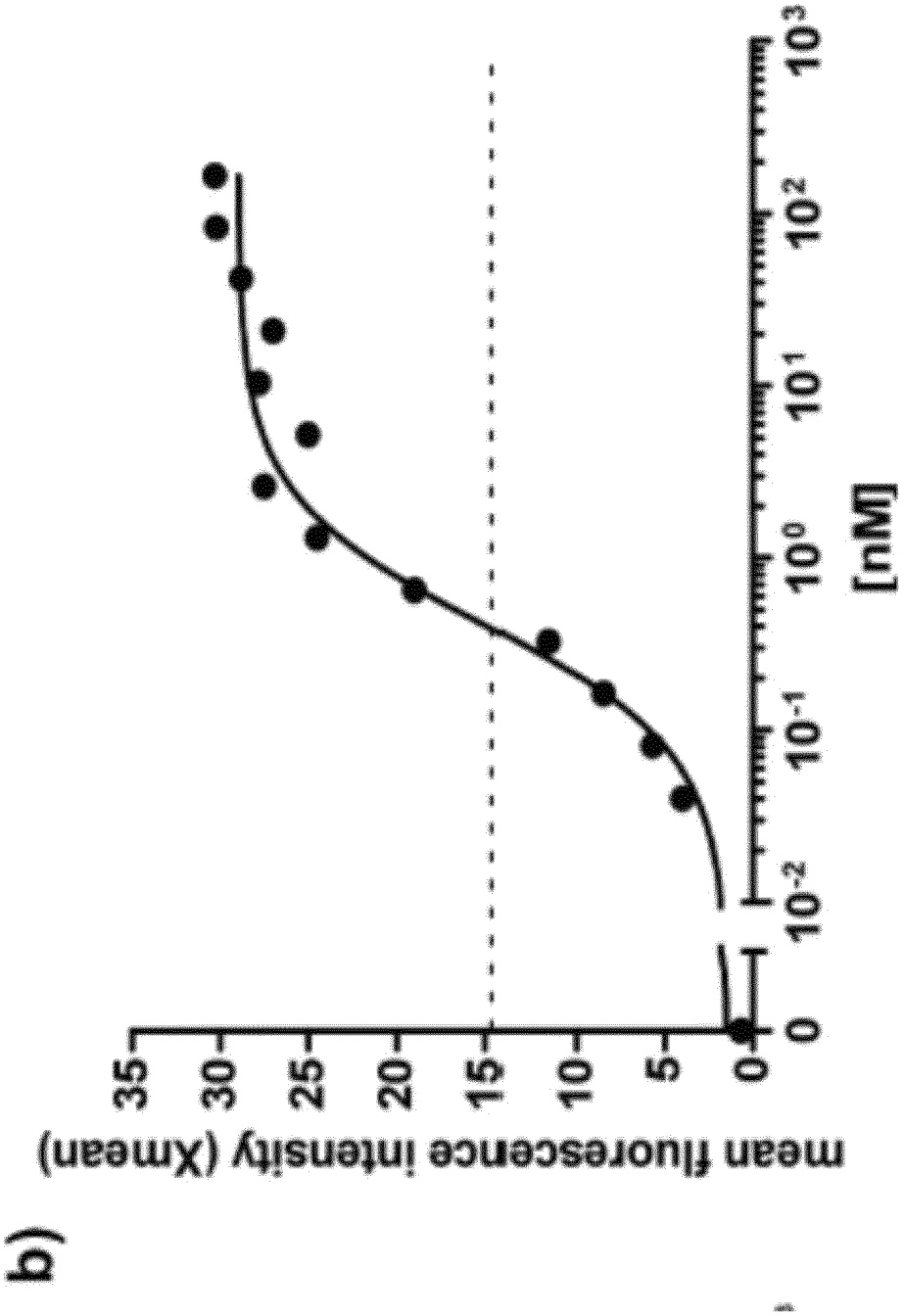

b)
mean fluorescence intensity (Xmean)
35
30
25
20
15
10
5
0
0
10-2
10-1
100
101
102
103
[nM]

Figure 3B

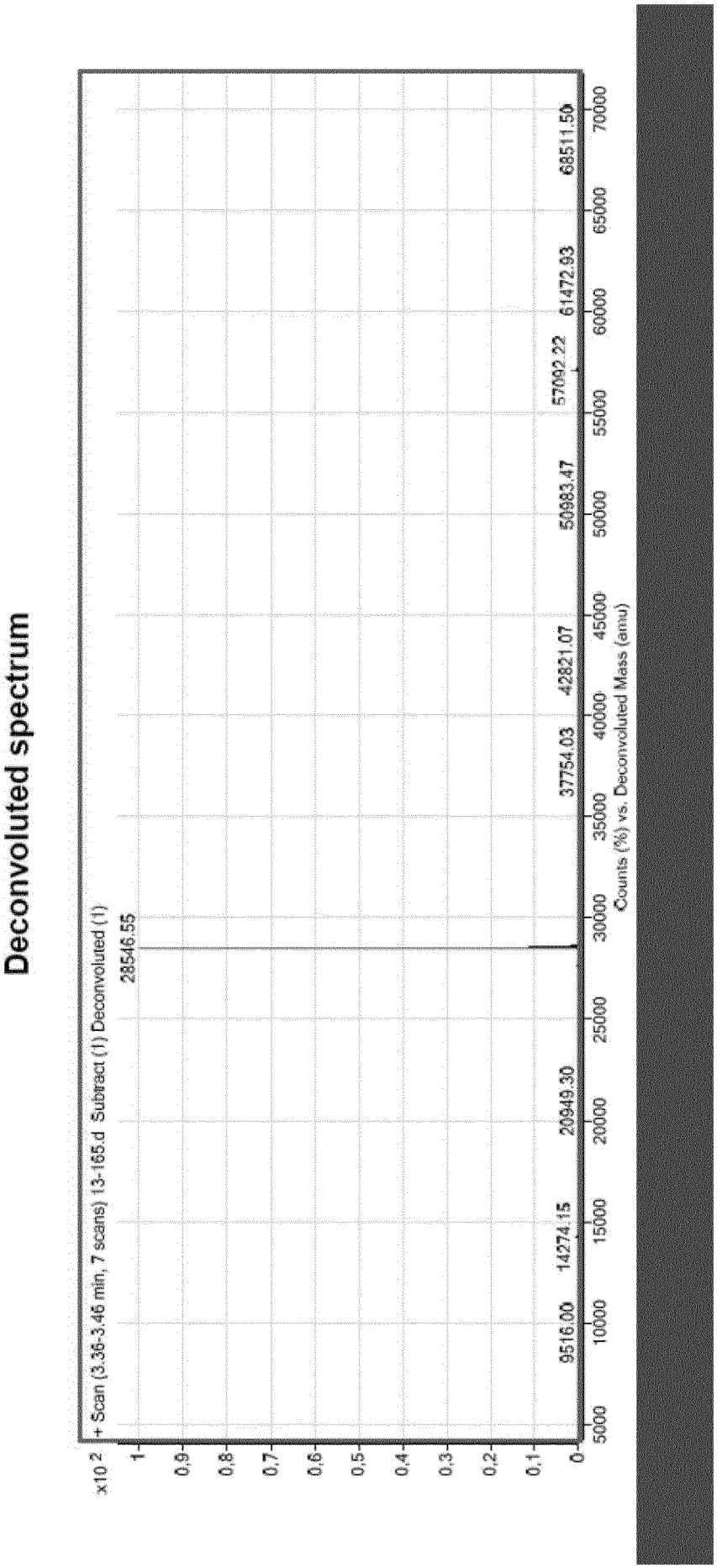

Deconvoluted spectrum
+ Scan (3.36-3.46 min, 7 scans) 13-165.d Subtract (1) Deconvoluted (1)
x10 2
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
28546.55
9516.00
14274.15
20949.30
37754.03
42821.07
50983.47
57092.22
61472.93
68511.50
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000
55000
60000
65000
70000
Counts (%) vs. Deconvoluted Mass (amu)

Figure 4

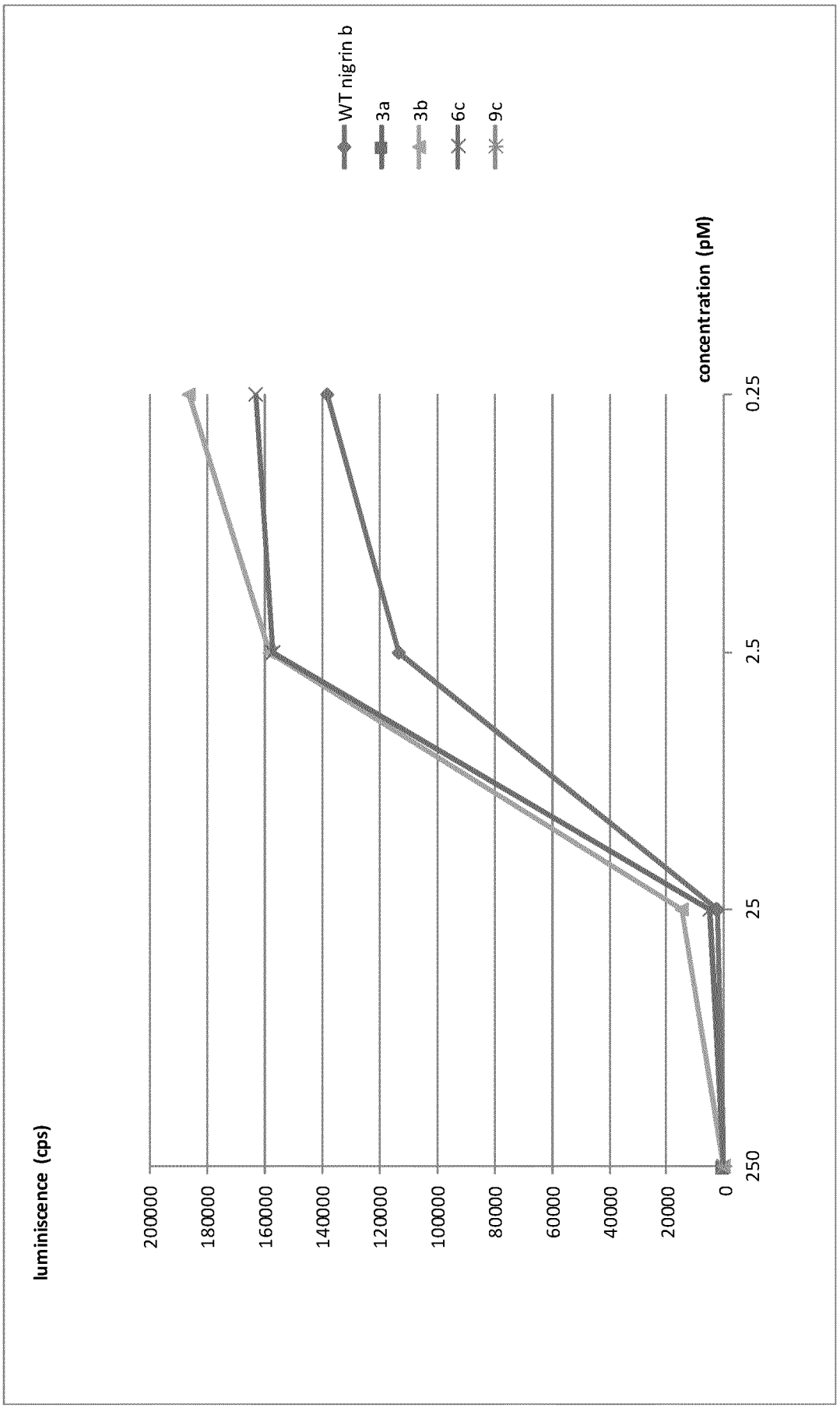

WT nigrin b
3a
3b
6c
9c
luminiscence (cps)
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
concentration (pM)
250
25
2.5
0.25

Figure 5

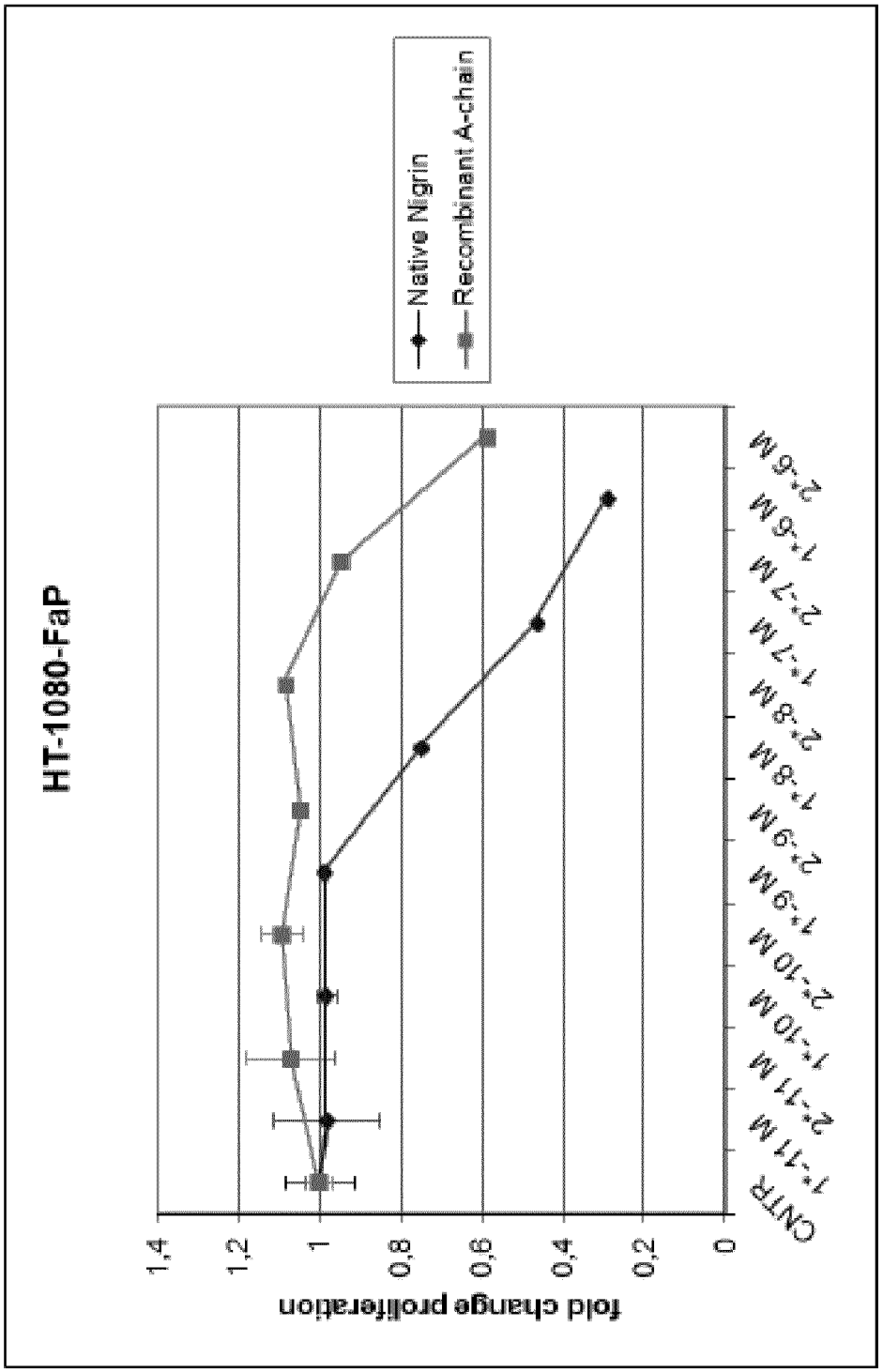

HT-1080-FaP
Native Nigrin
Recombinant A-chain
fold change proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CNTR
1-11 M
2-11 M
1-10 M
2-10 M
1-9 M
2-9 M
1-8 M
2-8 M
1-7 M
2-7 M
1-6 M
2-6 M

Figure 6

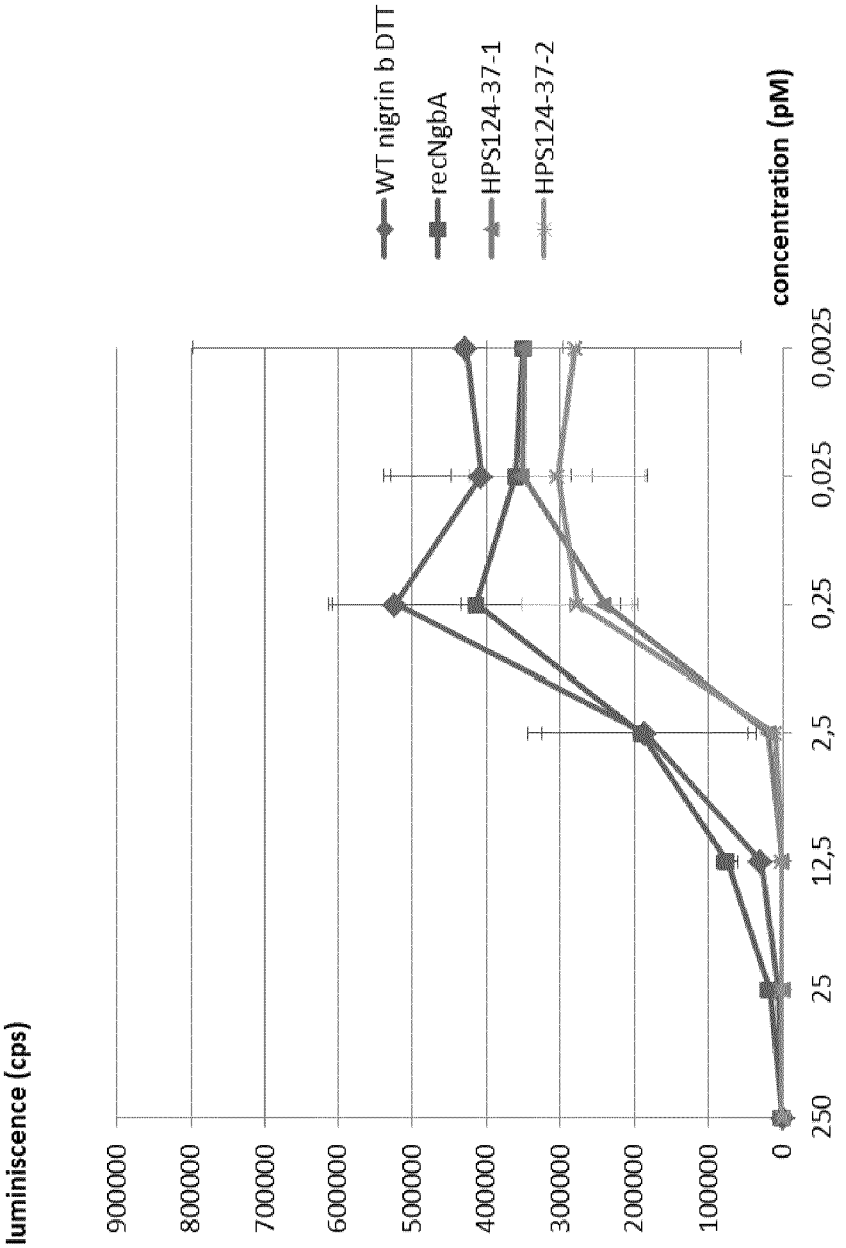

luminiscence (cps)
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
250
25
12,5
2,5
0,25
0,025
0,0025
concentration (pM)
WT nigrin b DTT
recNgbA
HPS124-37-1
HPS124-37-2

Figure 7

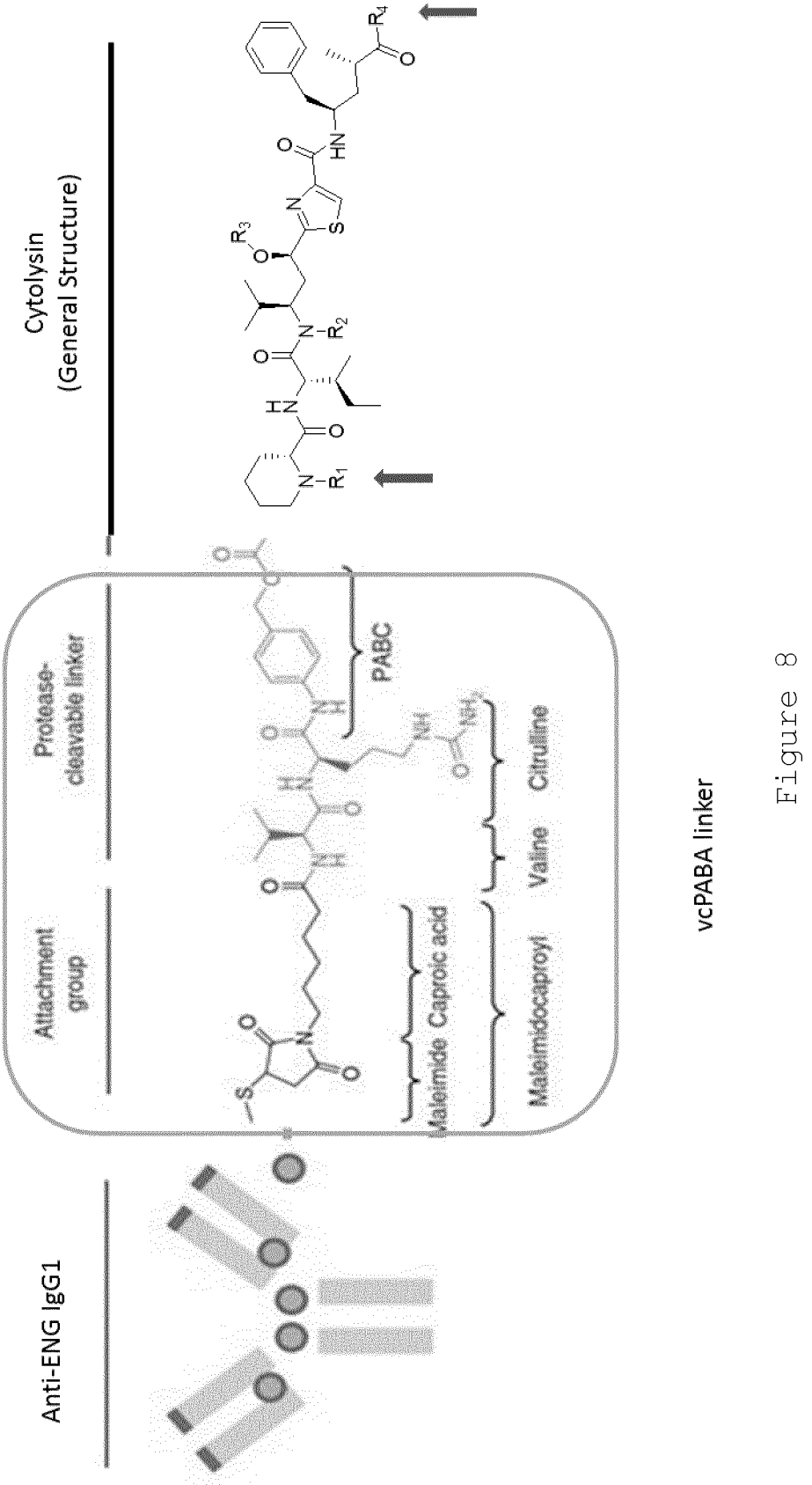

Anti-ENG IgG1
Attachment group
Protease-cleavable linker
Cytolysin (General Structure)
Maleimide
Caproic acid
Maleimidocaproyl
Valine
Citrulline
PABC
vcPABA linker

Figure 8

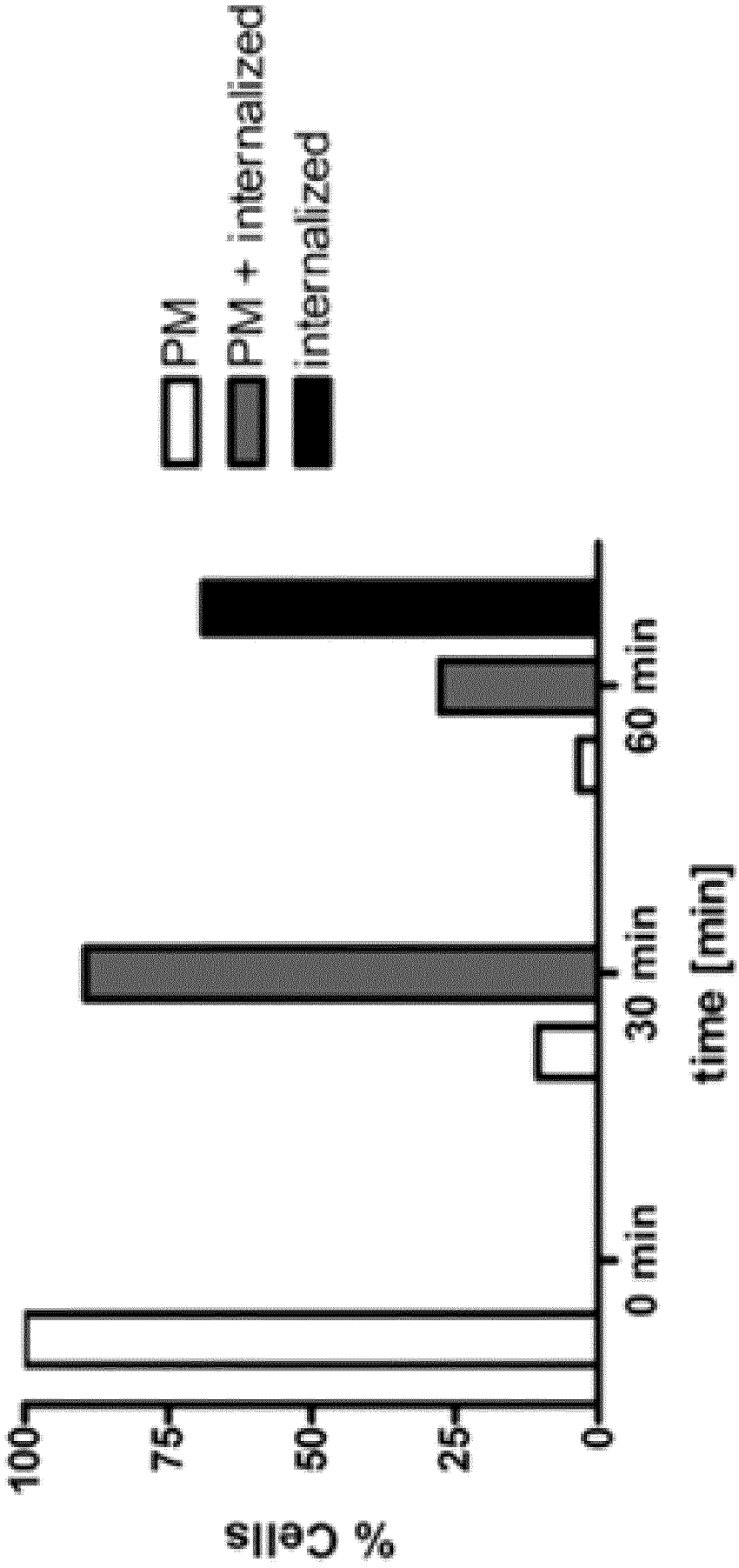

PM
PM + internalized
internalized
% Cells
100
75
50
25
0
0 min
30 min
60 min
time [min]

Figure 9

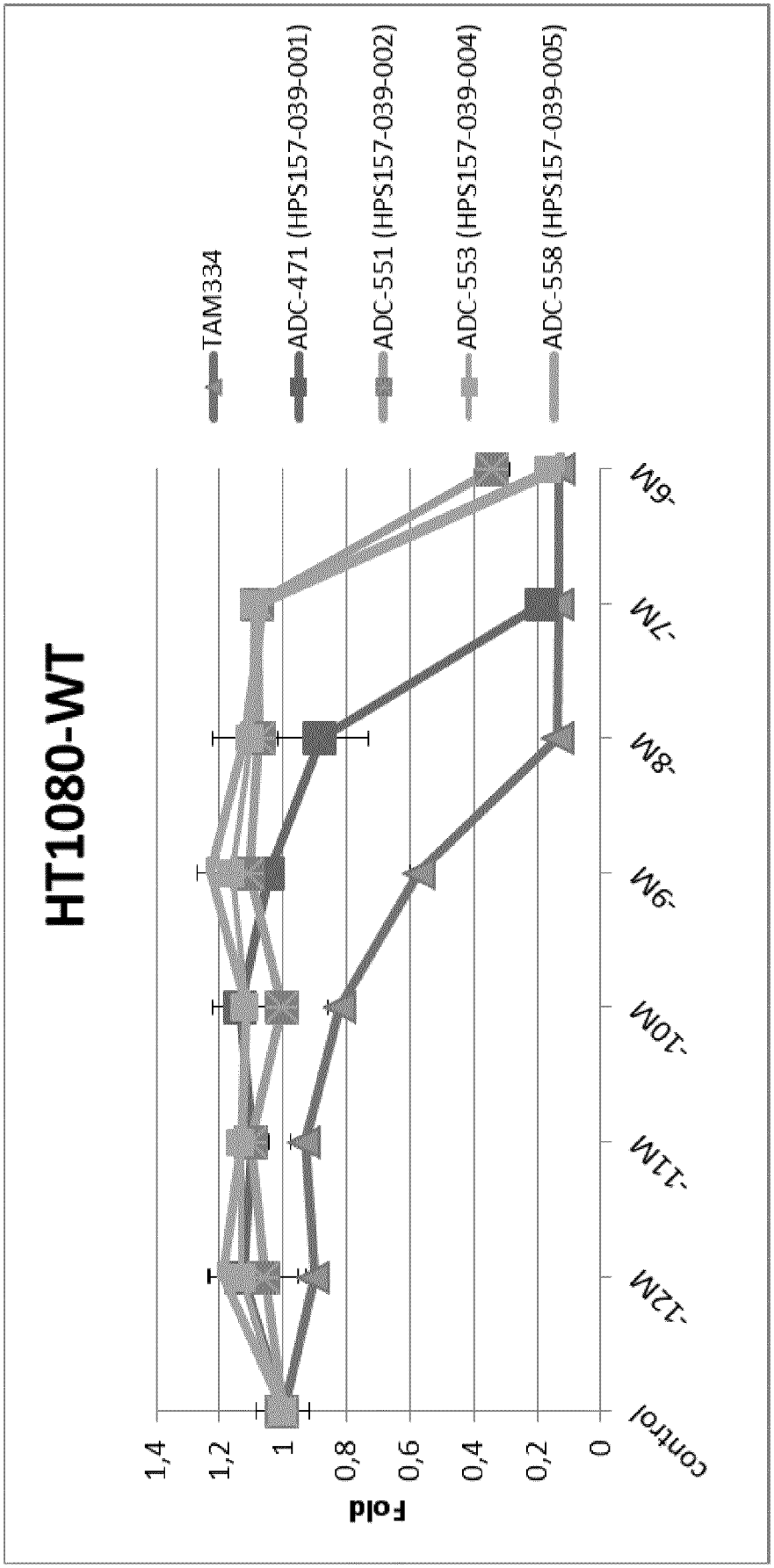

HT1080-WT
TAM334
ADC-471 (HPS157-039-001)
ADC-551 (HPS157-039-002)
ADC-553 (HPS157-039-004)
ADC-558 (HPS157-039-005)
Fold
1,4
1,2
1
0,8
0,6
0,4
0,2
0
control
-12M
-11M
-10M
-9M
-8M
-7M
-6M

Figure 10A

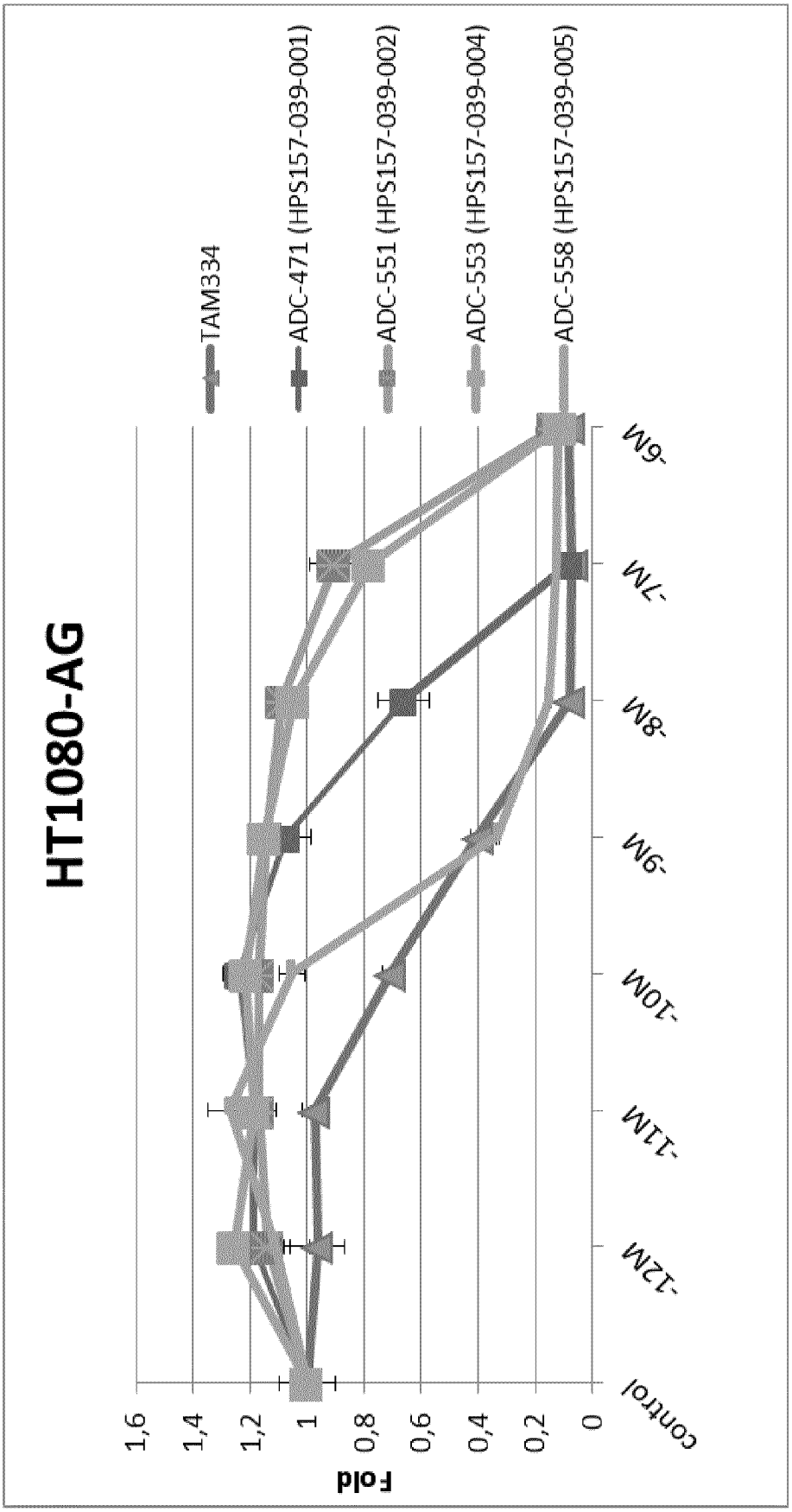

HT1080-AG
TAM334
ADC-471 (HPS157-039-001)
ADC-551 (HPS157-039-002)
ADC-553 (HPS157-039-004)
ADC-558 (HPS157-039-005)
Fold
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
control
-12M
-11M
-10M
-9M
-8M
-7M
-6M

Figure 10B

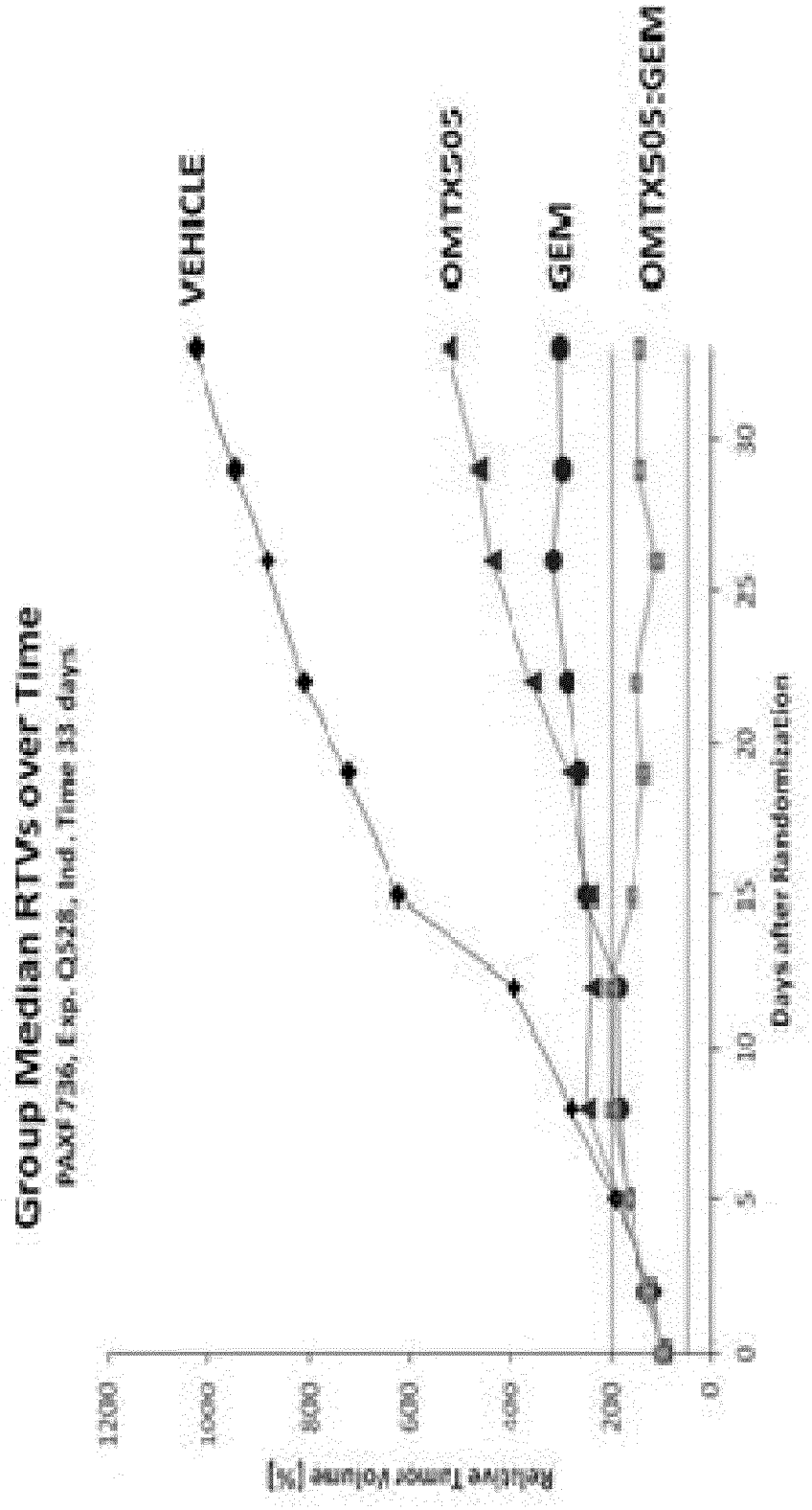

Group Median RTVs over Time
PAXI 736, Exp. 0328, Ind. Time 33 days
Relative Tumor Volume [%]
Days after Randomization
VEHICLE
OMTX505
GEM
OMTX505+GEM
0
5
10
15
20
25
30
2000
4000
6000
8000
10000
12000

Figure 11A

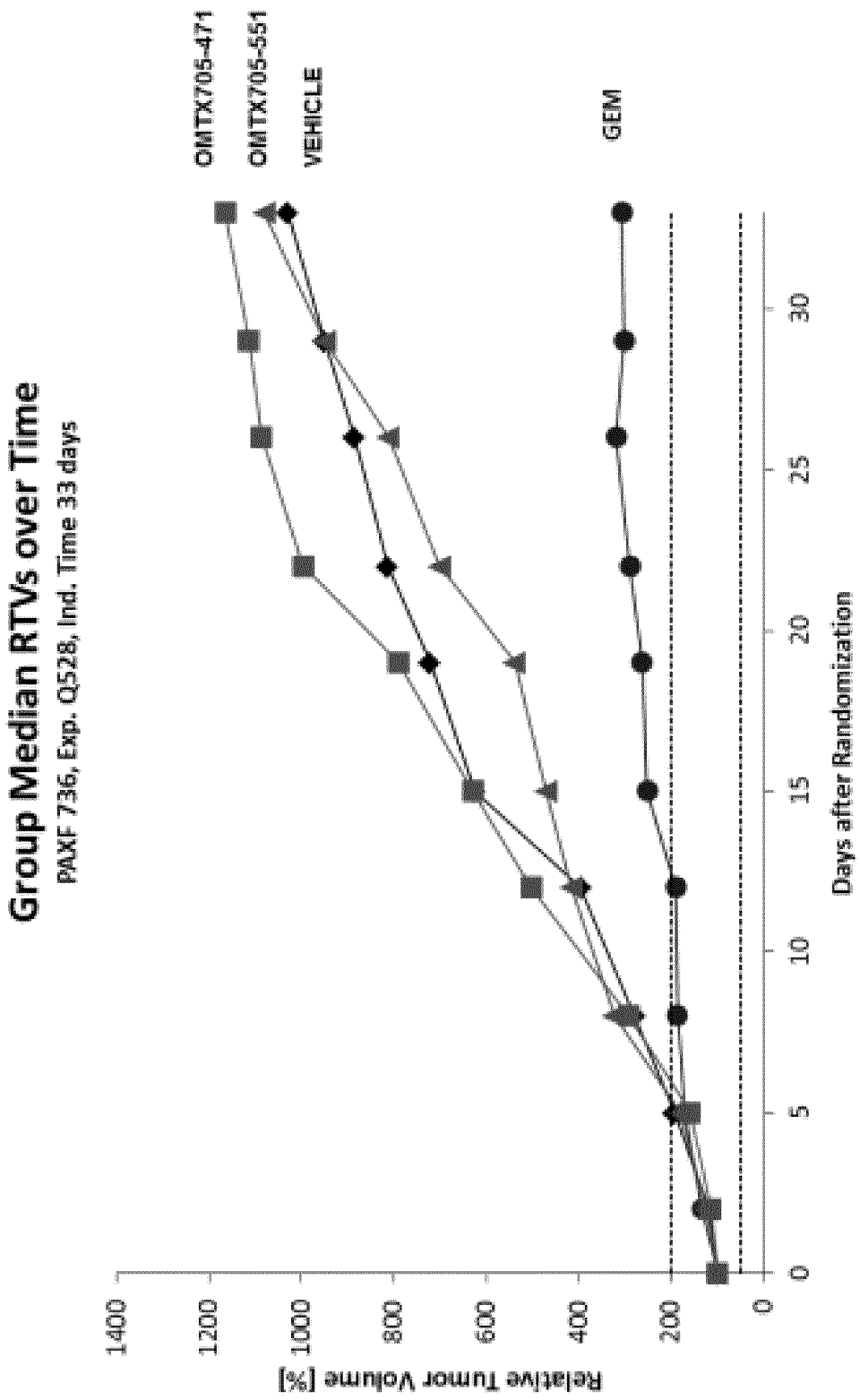

Group Median RTVs over Time
PAXF 736, Exp. Q528, Ind. Time 33 days
OMTX705-471
OMTX705-551
VEHICLE
GEM
Relative Tumor Volume [%]
Days after Randomization
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
30

Figure 11B

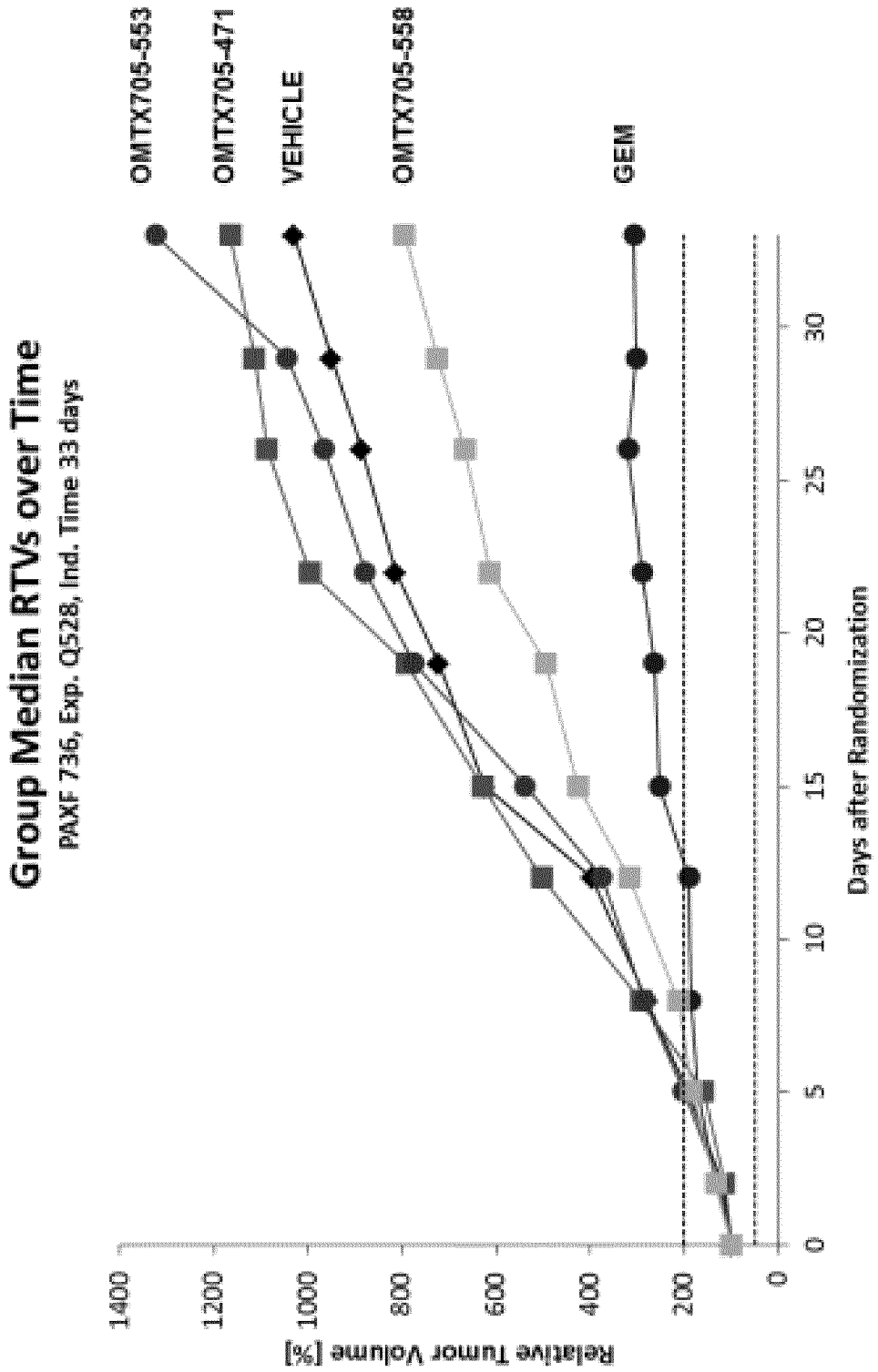

Group Median RTVs over Time
PAXF 736, Exp. Q528, Ind. Time 33 days
OMTX705-553
OMTX705-471
VEHICLE
OMTX705-558
GEM
Relative Tumor Volume [%]
Days after Randomization
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
30

Figure 11C