(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 2015/118030 A2

(51) International Patent Classification:
A61K 47/48 (2006.01)

(21) International Application Number:
PCT/EP2015/052341

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

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1402006.9 6 February 2014 (06.02.2014) GB

(71) Applicant: **ONCOMATRYX BIOPHARMA, S.L.** [ES/ES]; Parque Tecnológico de Bizkaia, Edificio 801B - 2nd Floor, E-48160 Derio (ES).

(72) Inventors: **KONTERMANN, Roland**; c/o Oncomatryx Biopharma S.L., Parque Tecnológico de Bizkaia, Edificio 801B - 2nd Floor, E-48160 Derio (ES). **PFIZENMAIER, Klaus**; c/o Oncomatryx Biopharma S.L., Parque Tecnológico de Bizkaia, Edificio 801B - 2nd Floor, E-48160 Derio (ES). **FERRER, Cristina**; c/o Oncomatryx Biopharma S.L., Parque Tecnológico de Bizkaia, Edificio 801B - 2nd Floor, E-48160 Derio (ES). **FABRE, Myriam**; c/o Oncomatryx Biopharma S.L., Parque Tecnológico de Bizkaia, Edificio 801B - 2nd Floor, E-48160 Derio (ES). **SIMON, Laureano**; c/o Oncomatryx Biopharma S.L., Parque Tecnológico de Bizkaia, Edificio 801B - 2nd Floor, E-48160 Derio (ES).

(74) Agents: **CASLEY, Christopher** et al.; Mewburn Ellis LLP, 33 Gutter Lane, London, Greater London EC2V 8AS (GB).

(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:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

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

WO 2015/118030 A2

(54) Title: ANTIBODY-DRUG CONJUGATES AND IMMUNOTOXINS

(57) Abstract: The present invention relates to conjugates, in particular antibody-drug conjugates and immunotoxins, having the formula (I): A-(L-D)p or a pharmaceutically acceptable salts or solvates thereof, wherein A is an antibody that selectively binds FAP; 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 Fibroblast Activating Protein α (FAP), 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.

In many types of human cancer, fibroblast response is characterized by the induction of a cell surface protein, Fibroblast Activating Protein α (FAPα), a serine protease of 95kDa whose expression is highly restricted to developing organs, wound-healing and tissue remodeling.

FAP presents the following characteristics:

- Type II membrane glycoprotein with SER-protease activity (collagenase + DPP)
- 89% human-murine protein homology
- Tumor stroma-expressed in >90% carcinomas (breast, pancreas, lung, bladder and colon)
- Transitory and highly restricted expression in normal adult tissues during wound-healing and developing organs.
- FAP(+) fibroblasts located closed to tumor vasculature
- Very focal expression

- Internalization
- Implication in extracellular matrix remodeling, tumor growth and metastasis.

FAP expression has been recently found in Pancreas tumor cells as well as tumor-associated stromal fibroblasts. FAP expression was correlated with shorter patient survival and worse prognosis, suggesting a possible FAP-based autocrine/paracrine loop in this type of tumor *(32)*.

During the last 10 years, Kontermann and Pfizenmaier (IZI, University of Stuttgart, Germany) have developed anti-FAP MAb derivatives against both human and murine proteins *(27, 28)*. They have shown *in vitro* that anti-FAP scFv immunoliposomes bind specifically FAP+ cells and get internalized *(29)*. In a recent study they demonstrated the anti-tumoral effect of nanoparticles covered with lipids and anti-FAP scFvs and loaded with TNFα *(30)*.

Treatment with murine MAb FAP5-DM1 immunotoxin induced long lasting inhibition of tumor growth and full regression in pancreas and lung cancer xenograft models, without any intolerance-related effect *(31)*.

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-FAP antibodies, conjugates thereof and optimised payloads for use in antibody conjugate strategies. In particular, the present inventors have found that anti-FAP 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 *in vitro*

Ribosome Inactivating activity in the absence of the Nigrin-b B-chain and, only once conjugated to an antibody, exhibits both the ability to translocate into cells and the resulting cytotoxic activity without Nigrin-b B-chain.

The Nigrin-b A-chain described herein and/or cytolysin derivatives are advantageously conjugated to anti-FAP 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 FAP;
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 FAP. In some case, A may cross-react to both human and murine FAP. 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.

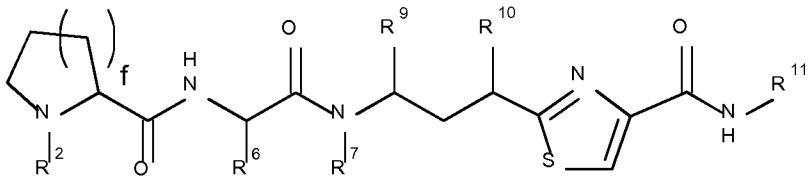
In certain cases, A comprises a heavy chain comprising an amino acid sequence having at least 90%, 95% or 99% sequence identity with the full-length sequence of SEQ ID NO: 3. In particular, A may comprise a heavy chain comprising the amino acid sequence of SEQ ID NO: 3.

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 binding anti-FAP antibody that is structurally different from the anti-FAP antibody molecules exemplified herein. For example, A may be an anti-FAP antibody

molecule that competes with the anti-FAP IgG1 antibody identified herein as "hu36" for binding to immobilized recombinant human FAP. hu36 has the heavy chain amino acid sequence of SEQ ID NO: 3 and the light chain amino acid sequence of SEQ ID NO: 4. The anti-FAP antibody may, in some case, bind to the same epitope as hu36. Methods for determining antibody binding competition and for epitope mapping are well known in the art, see for example "Epitope Mapping by Competition Assay" Ed Harlow and David Lane, Cold Spring Harb Protoc; 2006; doi:10.1101/pdb.prot4277.

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, the entire contents of which is expressly incorporated herein by reference (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;

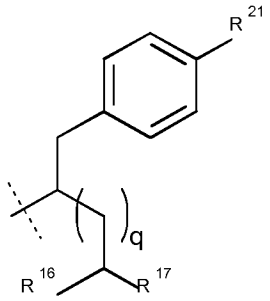
R^7 is C_1-C_6 alkyl, CH_2OR^{19} or CH_2OCOR^{20}, wherein R^{19} is alkyl, R^{20} is C_2-C_6-alkenyl, phenyl, or CH_2-phenyl;

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 spacer comprises between one and six ethylene glycol units, e.g. a triethylene glycol.

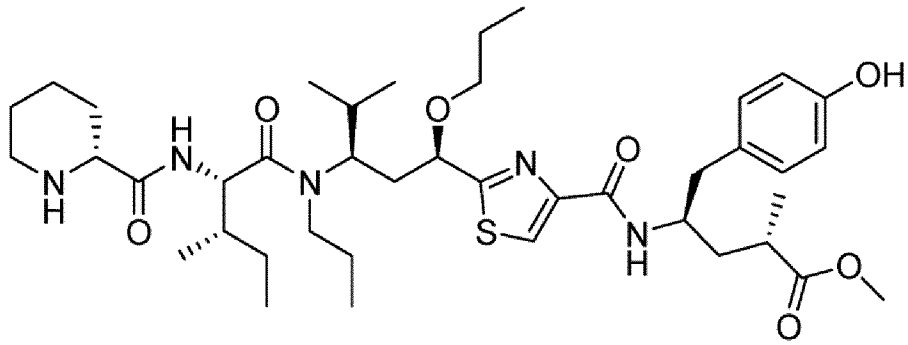
In some cases the spacer may be directly attached to group R^{17}, or may be attached to group R^{17} via a bridging group.

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}.

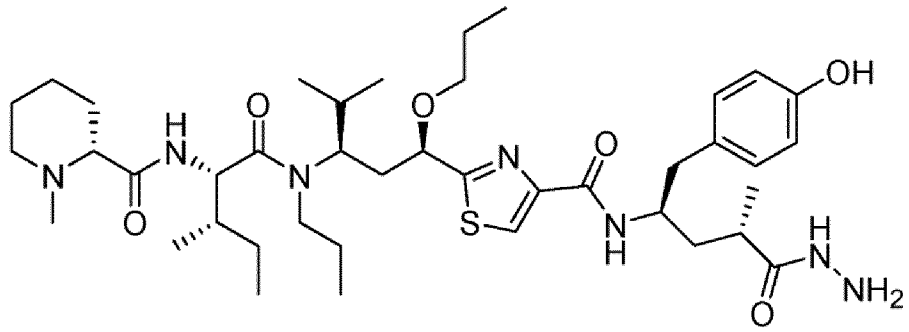
In some cases R^{17} is CONHNHX and the spacer is attached to group R^{17} via a -C(O)X bridging group, wherein X represents the bond between the spacer and R^{17}.

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:



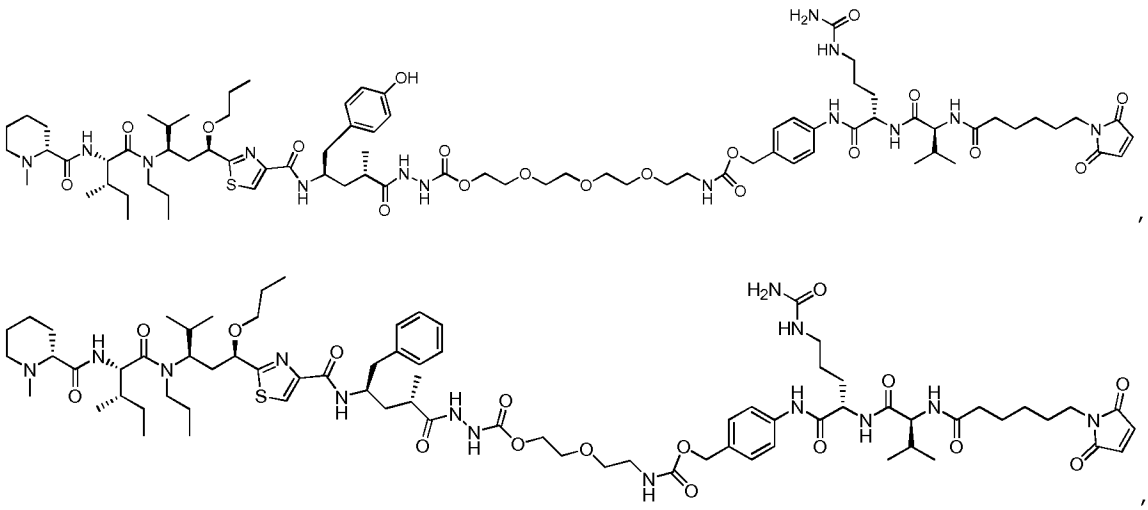
In some cases D comprises a cytolysin having the following structure:

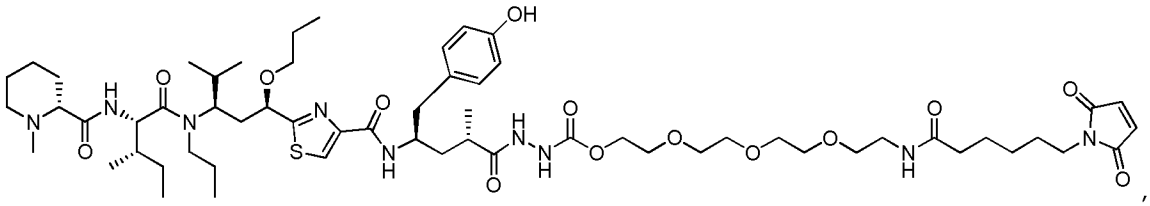


In certain cases L comprises an attachment group for attachment to A and protease cleavable portion. For example, L may comprise a valine-citrulline unit. In particular, L may comprise maleimidocaproyl-valine-citrulline-p-aminobenzylcarbamate.

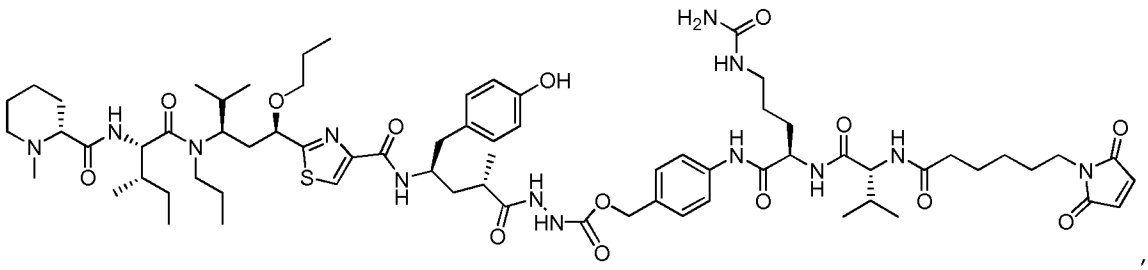
In some cases the double bond of the maleimide is reacted with a thiol group of a cysteine residue of the antibody A to form a sulphur-carbon bond in order to effect linkage of the linker L to the antibody A.

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

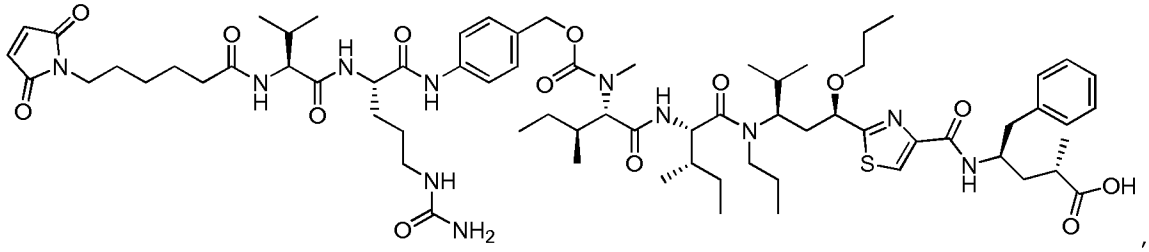




,

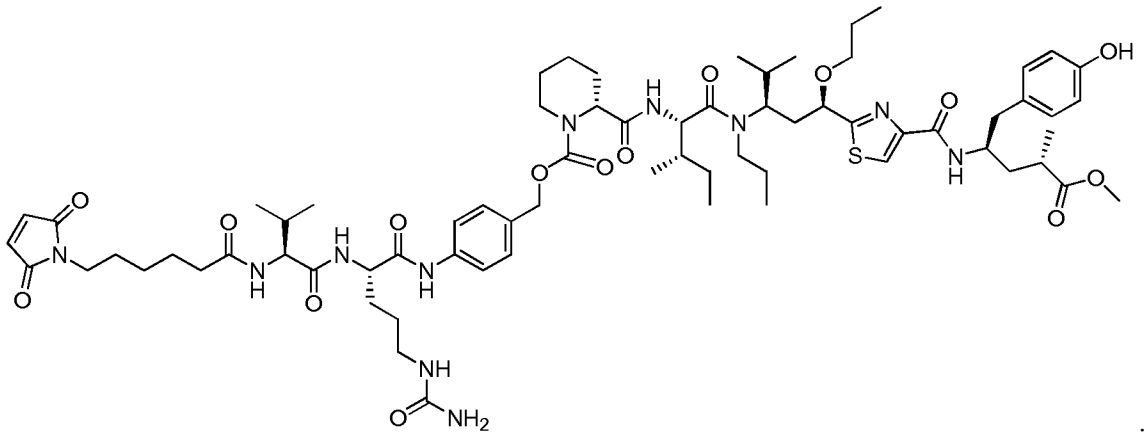


,



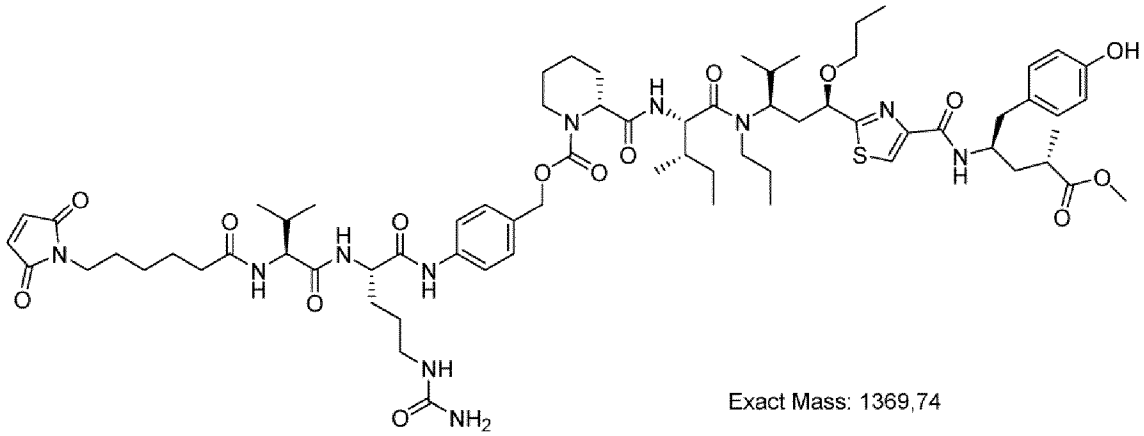
,

and

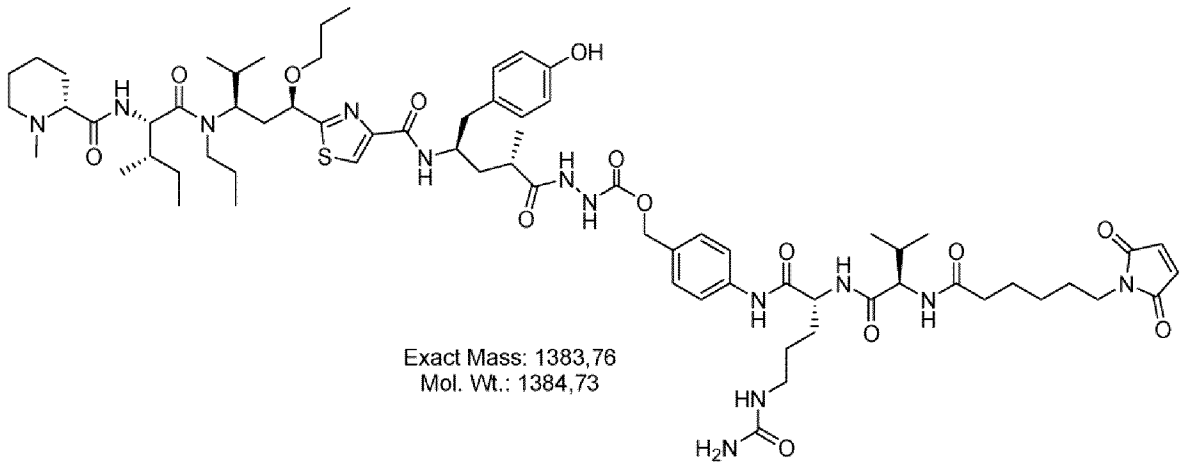


.

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



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



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: 13.

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 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 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 certain 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, breast cancer, melanoma, lung cancer, head & neck cancer, ovarian cancer, bladder cancer or colon cancer.

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 solid tumor. In particular, the method may be for treating pancreatic cancer, 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 FAP-specific antibody, e.g., an FAP-specific antibody in accordance with the eighth 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 a conjugate of the first aspect of the invention for use in the treatment of an inflammatory condition (e.g. rheumatoid arthritis).

In a seventh aspect the present invention provides a method of treating an inflammatory condition (e.g. rheumatoid arthritis) in a mammalian subject, comprising administering a therapeutically effective amount of a conjugate of the first aspect of the invention to the subject in need thereof.

In an eighth 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: 13.

In a ninth aspect the present invention provides use of an isolated Nigrin-b A-chain in accordance with the eighth 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 the absence of the Nigrin-b B-chain). In some cases the immunotoxin comprises an antibody, such as a monoclonal antibody, e.g. a human monoclonal antibody, that selectively binds FAP. In some cases, the immunotoxin comprises an antibody in accordance with the tenth aspect of the invention.

In a tenth aspect the present invention provides a monoclonal antibody, e.g. a human monoclonal antibody, that selectively binds FAP 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.

In an eleventh aspect the present invention provides the antibody of the tenth aspect of the invention for use in medicine. The antibody may be for use in the treatment of an inflammatory condition (e.g. rheumatoid arthritis).

In a twelfth aspect the present invention provides use of a monoclonal antibody in accordance with the tenth 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 and 13. In some cases the polynucleotide may comprise the nucleic acid sequence of SEQ ID NO: 14.

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 FAP 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 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 FAP 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 characterization of humanized scFv hu33 and hu36. **A**) SDS-PAGE analysis of purified scFv fragments. Coomassie staining. R-reducing, NR – non reducing. **B**) Flow cytometry analysis of binding of hu36 (humanized) and mo36 (chimeric) to HT1080-huFAP cells. Bound antibodies were detected with an anti-His-tag antibody (n=2). **C**) ELISA of binding of hu36 scFv and mo36 scFv to immobilized recombinant human FAP (coated at 100ng/ml). Bound antibodies were detected with an HRP-conjugated anti-Myc-tag antibody.

Figure 2 shows **A**) ELISA of anti-FAP mo36-IgG1 (circles) and hu36-IgG1 (squares) for binding to recombinant human FAP (rhFAP) or

control protein (BSA) (triangles and inverted triangles, respectively). 50 ng protein were coated per well. Bound antibodies were detected with HRP-conjugated anti-human IgG-Fc. **B**) Flow cytometry analysis of anti-FAP mo36-IgG1 (triangles and stars) and hu36-IgG1 (squares and circles) for binding to HT1080-FAP. Bound proteins were detected with a PE-labeled anti-hu IgG-Fc antibody.

Figure 3 shows flow cytometry analysis of binding of hu36-IgG1 to stably transfected HT1080 to express **A**) human FAP (HT1080-huFAP) and **B**) mouse FAP (HT1080-moFAP). Bound antibodies were detected with a PE-labeled anti-human Fc antibody.

Figure 4 shows confocal microscopy of HT1080-FAP cells, incubated with hu36-IgG1 for various times (0, 30 and 60 mins) and stained with FITC-labelled anti-IgG antibody, WGA-TRed (membrane staining), and DAPI (nucleus). The right-hand panels show a merged image of the three stains.

Figure 5 shows analysis of internalization of hu36-IgG1 by discrimination of cells (n=10-30) showing only membrane staining (PM; open bars), PM and intracellular staining (shaded bars), or only intracellular staining (filled bars). A clear time-dependent internalization is evidenced.

Figure 6 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 7 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 8 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 9 shows RIP activity of anti-FAP hu36-IgG1-recNgbA immunotoxin conjugates (HSP131-001; crosses) in an RRL assay compared to native (WT) nigrin (triangles) and recombinant Nigrin-b A-chain (recNgA; squares).

Figure 10 shows cytotoxic activity of anti-FAP hu36-IgG1-recNgbA immunotoxin conjugates (HSP131-001; triangles), unconjugated (naked) anti-FAP hu36-IgG1 (squares) and recombinant Nigrin-b A-chain (recNgA; diamonds) on **A**) HT1080-WT cell line; and **B**) HT1080-FAP cell line. Fold-change in proliferation is plotted against antibody/immunotoxin concentration.

Figure 11 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 12 shows immunodetection of anti-FAP hu36 tumour sections of patient-derived xenograft (PDX) mice (pancreatic tumour). Specific Dose- and Time- dependent staining of stroma is observed in subcutaneous tumors from PDX mouse model for pancreas cancer (Panc185) - Single dose (1 & 5 mg/kg) of anti-hu/moFAP hu36 IgG1 was administrated intraperitoneally in PDX mice Panc-185; immunodetection was performed with an anti-human IgG1 secondary antibody- 20x scale pictures are shown. Control-48h: Mice administrated with Vehicle and tumors excised after 48h.

Figure 13 shows animal weight monitored after treatment with anti-FAP:recNgA immunotoxin at different doses (2.5, 1, 0.5, 0.25, 0.1 mg/kg) administrated once a week. Significant weight loss and toxicity was observed in Group 1 and 2 (2.5 and 1 mg/kg, respectively), similarly to treatment with 5mg/kg (not shown); 0.5mg/kg was the highest tolerated dose when applied as single agent.

Figure 14 shows (**A**) Relative Body weight and (**B**) Tumor volume measured from patient-derived xenograft mice (PAXF 736) untreated

(Vehicle; 10ml/kg/day; once a week), treated with Gemcitabine (GEM; 150 mg/kg; once a week), or antiFAP:recNgA immunotoxin (OMTX505; 0.5/0.25mg/kg; once a week), or both (OMTX505 (0.25mg/kg):GEM(150mg/kg)), for 4 weeks (treatment days 1, 8, 15, 22, 29).

Figure 15 shows ELISA and FACS analysis of ADC471 binding to FAP target. (**A**) ELISA detection of ADC-471 binding to huFAP fusion protein compared to naked anti-hu/mo FAP hu36 antibody; EC50 values are indicated for HPS124-3 ADC-471 molecule with DAR=3.48; (**B**) & (**C**): FACS analysis of binding on HT1080-huFAP, HT1080-wt and HEK293 cells of HPS131-143-1 (ADC-471; DAR 4), HPS131-124-1 (ADC-467; DAR 1.2) and HPS131-124-3 (ADC-471; DAR 3.48) ADCs. EC_{50} values are indicated for this latter (B).

Figure 16 shows Time-lapse immunofluorecence analysis of internalization capacity of anti-FAP hu36:cytolysin ADC (ADC-471; HPS131-124-3) on living HT1080-FAP cells. *Left panel*: Incubation with naked anti-hu/moFAP hu36 (FITC-AB; green); *Right panel*: Incubation with ADC-471 (FITC-ADC; green). Time 0, 30, 60, 90min (upper panels): HT1080-FAP cells. Time 30min (lower panels): HT1080-wild type cells.

Figure 17 shows *in vitro* cytotoxic effect of anti-hu/moFAP hu36: cytolysin ADCs on (**A**) HT1080-wt and (**B**) FAP(+) 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 18 shows tumor growth inhibition effect of anti-hu/moFAP hu36:cytolysin ADC candidates. (**A**) ADC471 versus ADC551; (**B**) ADC471 and ADC553 (OMTX705-553) versus ADC558 (OMTX705-558). 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.

FAP

As used herein "Fibroblast activation protein", "fibroblast activating protein", "FAP" and "FAPα" are used interchangeably. The FAP may be an FAP of any mammalian species. In some cases FAP is human FAP (also known as Seprase, 170 kDa melanoma membrane-bound gelatinase, fibroblast activation protein alpha or integral membrane serine protease), the amino acid sequence of which is disclosed at UniProt accession No. Q12884 (Version 140, dated 11 December 2013) (SEQ ID NO: 15). In some cases, a molecule that binds FAP (e.g. an antibody molecule or a conjugate thereof) may bind to a region of the extracellular domain of FAP. The extracellular domain of human FAP comprises residues 26-760 of the full-length human FAP protein. In some cases FAP is murine FAP (also known as fibroblast activation protein alpha or integral membrane serine protease), the amino acid sequence of which is disclosed at UniProt accession No. P97321 (Version 117, dated 11 December 2013) (SEQ ID NO: 16). The extracellular domain of murine FAP comprises residues 26-761 of the full-length murine FAP 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 FAP, e.g. an extracellular portion thereof, with an affinity of at least about $1x10^{7}M^{-1}$, 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) one or more 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. 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 rheumatoid arthritis (RA). In particular, the subject may be a human having RA.

Cancer

The anti-FAP 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, 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-FAP antibody or the antibody drug conjugate may be for use in the

treatment of an inflammatory condition. FAP expression has been reported in fibroblast-like synoviocytes (FLSs) in rheumatoid arthritis (RA) patients (see, e.g., *Bauer et al., Arthritis Res. Therp.* (2006):8(6); R171). The present inventors believe that the anti-FAP antibodies described herein, and/or conjugates thereof described herein, are able to ameliorate RA and/or symptoms of RA.

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-FAP antibodies

Anti-FAP scFvs selected by phage display from an immunized $FAP^{-/-}$ knock-out mouse have been described previously (*28*). Two scFvs, "MO36" and "MO33", cross-reactive for human and murine FAP (*28*) were converted into full-length IgG for subsequent characterisation studies and for generation of immunotoxins and ADCs. These scFv (scFv33 and scFv36) were used to generate chimeric antibodies, fusing heavy and light chain constant domains to VH and VL, respectively. In addition, both were humanized by CDR grafting and tested for binding to FAP-expressing cells and recombinant FAP in comparison to the parental scFv. From this comparison, the best binder was used to generate full-length IgG. 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 scFvs 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	mouse	hu/mo FAP	mo33	lambda	pAB1	376
scFv	mouse	hu/mo FAP	mo36	kappa	pAB1	277
scFv	humanized	hu/mo FAP	hu33	lambda	pAB1	1214
scFv	humanized	hu/mo FAP	hu36	kappa	pAB1	1215

All scFvs were bacterially produced in *E.coli* TG1 and purified from the periplasmic extracts of 1L cultures by IMAC. Both humanized antibodies (scFv hu33 and hu36) were purified in soluble form with yields of approximately 0.6mg/L culture. In SDS-PAGE the proteins migrated with the expected size of approximately 30kDa (Figure 1A). Purity was estimated to be >90%. In flow cytometry experiments using HT1080 cells expressing human FAP (stable transfectants), a similar binding was observed for scFv hu36 and mo36 scFv, which was also produced in bacteria (not shown). EC_{50} values were in the low nanomolar range. Some differences were observed at higher concentrations (Figure 1B). scFv hu33 showed no binding or only marginal binding in these experiments. Further development therefore focused on hu36. Binding of hu36 scFv was also observed by ELISA with recombinant human FAP (extracellular region aa 26-760; R&D systems), although binding was somewhat weaker than that seen for mo36 scFv (Figure 1C).

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 FAP and detection of bound antibodies with HRP-conjugated anti-human IgG antibodies. Cell binding was analyzed by flow cytometry using HT1080-FAP cell line.

Results:

Plasmids generated (and sequenced):

mo36 IgG1: pEE14.4 mo36-IgG1 OCMTX001p (chimeric anti-FAP IgG1)

hu36 IgG1: pEE14.4 hu36-IgG1 OCMTX002p (humanized anti-FAP IgG1)

Example 2 - Characterisation of anti-FAP antibodies

The amino acid sequences of humanized anti-FAP IgG1 hu36 (hu36-IgG1) heavy chain (HC) and light chain (LC), respectively are shown below:

Anti-FAP hu36-IgG1-HC:

METDTLLLWVLLLWVPGSTG

QVQLVQSGAEVKKPGASVKVSCKASGYTFT**ENIIH**WVRQAPGQGLEWMG**WFHPGSGSIKYNEKFKD**RV
TMTADTSTSTVYMELSSLRSEDTAVYYCAR**HGGTGRGAMDY**WGQGTLVTVSSASTKGPSVFPLAPSSK
STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAP***PVA***GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK***G***LP***SS***IEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 1)

aa	*449*
MW of processed HC	*49,069*
Theoretical pI	*8.69*

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-FAP hu36-IgG1-LC:

METDTLLLWVLLLWVPGSTG
DIQMTQSPSSLSASVGDRVTITC**RASKSVSTSAYSYMH**WYQQKPGKAPKLLIY**LASNLES**GVPSRFSG
SGSGTDFTLTISSLQPEDFATYYC**QHSRELPYT**FGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASV
VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC (SEQ ID NO: 2)

aa *218*
MW of processed HC *23,919*
theoretical pI *7.77*
signal sequence is boxed
VL domain is underlined; CDRL1-L3 are shown in bold and curved underlined.

hu36-IgG1-HC – without signal sequence:
QVQLVQSGAEVKKPGASVKVSCKASGYTFT**ENIIH**WVRQAPGQGLEWMG**WFHPGSGSIKYNEKFKD**RV
TMTADTSTSTVYMELSSLRSEDTAVYYCAR**HGGTGRGAMDY**WGQGTLVTVSSASTKGPSVFPLAPSSK
STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAP***PVA***GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK***G***LP***SS***IEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 3)

hu36-IgG1-LC – without signal sequence:
DIQMTQSPSSLSASVGDRVTITC**RASKSVSTSAYSYMH**WYQQKPGKAPKLLIY**LASNLES**GVPSRFSG
SGSGTDFTLTISSLQPEDFATYYC**QHSRELPYT**FGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASV
VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC (SEQ ID NO: 4)

hu36-VH:
QVQLVQSGAEVKKPGASVKVSCKASGYTFT**ENIIH**WVRQAPGQGLEWMG**WFHPGSGSIKYNEKFKD**RV
TMTADTSTSTVYMELSSLRSEDTAVYYCAR**HGGTGRGAMDY**WGQGTLVTVSS (SEQ ID NO: 5)

hu36-VL:

DIQMTQSPSSLSASVGDRVTITC**RASKSVSTSAYSYMH**WYQQKPGKAPKLLIY**LASNLES**GVPSRFSG SGSGTDFTLTISSLQPEDFATYYC**QHSRELPYT**FGQGTKLEIKR (SEQ ID NO: 6)

hu36-CDRH1:
ENIIH (SEQ ID NO: 7)

hu36-CDRH2:
WFHPGSGSIKYNEKFKD (SEQ ID NO: 8)

hu36-CDRH3:
HGGTGRGAMDY (SEQ ID NO: 9)

hu36-CDRL1:
RASKSVSTSAYSYMH (SEQ ID NO: 10)

hu36-CDRL2:
LASNLES (SEQ ID NO: 11)

hu36-CDRL3:
QHSRELPYT (SEQ ID NO: 12)

Parameters of the full hu36-IgG are as follows:

Total length of full-length IgG (aa):	*1,334*
Calculated molecular mass of full-length IgG:	*145,922*
Calculated extinction coefficient of full-length IgG:	*209,420*
Abs 0.1% (=1 g/l)	*1.435*
theoretical pI:	*8.60*
potential glycosylation site:	*N297*

Purified chimeric and human anti-FAP antibodies mo36 and hu36 were analyzed in ELISA for binding to recombinant FAP. Both anti-FAP antibodies showed specific and strong binding to recombinant FAP with similar EC_{50} values (around 5 nM) (Figure 2A). Furthermore, both antibodies showed binding to HTP1080-FAP expressing human FAP on their cell surface (Figure 2B). The humanized IgG gave stronger signals compared with the chimeric IgG, however, with similar EC_{50} values. The humanized hu36 anti-FAP antibody was able to cross-react

to both human and murine FAP as shown by FACS analysis (Figures 3A and 3B). Hu36-IgG1 bound in a concentration-dependent manner to both cell lines with subnanomolar EC50 values (0.33 and 0.25nM).

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 antibodies were purified to >98.8%. 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 hu36-IgG and mu36-IgG were characterized by SDS-PAGE and size exclusion chromatography. Bioactivity was analyzed by ELISA, using recombinant FAP and detection of bound antibodies with HRP-conjugated anti-human IgG antibodies. Cell binding was analyzed by flow cytometry, using HT1080-FAP cell line. 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. The antibodies bound FAP-expressing cells with subnanomolar EC_{50} values. 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 (77-80°C).

Rapid internalisation was shown for hu36-IgG1 (humanized anti-FAP antibody) on HT1080-FAP cells (see Figures 4 and 5).

Table 2: Summary of antibody properties

antibody	***mo36-IgG1***	***hu36-IgG1***
antigen	*hu and mo FAP*	*hu and mo FAP*
isotype	*γ1* / κ*	*γ1* / κ*
IgG type	*chimeric*	*humanized*
plasmid	*OCMTX001p*	*OCMTX002p*
purity (SEC)	*minor aggregates*	√
Tm (DLS)	*77 °C*	*80 °C*
EC_{50} ELISA	*3 nM (rhFAP)*	*3 nM (rhFAP)*
EC_{50} FACS	*0.5 nM (HT1080-huFAP)*	*0.3 nM (HT1080-huFAP)* *0.2 nM (HT1080-moFAP)*
binding to primary tumor fibroblasts	*n.d.*	*+*
binding constants K_D (QCM)	*rhFAP:* *K_D1 = 112 nM* *K_D2 = 0.6 nM*	*rhFAP:* *K_D1 = 218 nM* *K_D2 = 0.4 nM*
internalization	*n.d.*	*HT1080-FAP* *30-60 min*

γ1* = deficient for ADCC and CDC (see Amour et al., 1999; Richter et al., 2013).

Anti-FAP IgG1 in vivo binding.

Anti-FAP IgG1 hu36 was administrated intraperitoneally to patient-derived xenograft mice for pancreas cancer at a single dose of 1 and 5mg/kg. Tumors were excised after 12, 24, and 48 h administration, formalin-fixed and paraffin-embedded. Immunodetection of anti-FAP hu36 was performed with an anti-human IgG secondary antibody. Figure 12 shows the specific dose- and time-dependent staining of stroma, only in tumor samples from treated mice.

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: 13)

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: 14)

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 $\mu g ml^{-1}$ 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 6).

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 7). 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 8. Native nigrin b showed

an $IC_{50}\approx 2x10^{-8}M$ (similar to previous published data; see *33*), 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 the 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) (data not shown).

Table 3: *In vitro* and *in vivo* activity data for Ricin and Nigrin (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-FAP 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 *(34)*). The disulfide bond is the only type of linkage that fit this criterium (*35, 36*). 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 *(37)*.

SPDP has already been used in the making of immunotoxins (ITs) containing nigrin b (*38, 39*). Moreover SMPT protects the disulfide bond from attack by thiolate anions, improving *in vivo* stability of the linkage (*40, 41*).

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 *(34)*). At present, the disulfide bond is the only type of linkage which appears to fit these criteria (*36*).

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 (*42*).

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 (*36*).
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 (*36, 41*).

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 (*43, 44*).

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 (*40, 41*).

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 the present study the inventors investigated conjugating humanized anti-FAP-IgG1s with recNgA, using 2 different recNgA:mAb molar ratios of 2.5 and 3.5, after derivatization using an SMPT:mAb molar ratio of 6, following conjugation protocols (*36*). Purification

was performed by Size Exclusion chromatography on Sephacryl S200 (*37*).

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.

Biochemical characterization

Anti-FAP hu36-IgG1-recNgA immunotoxin conjugates were produced and characterized as follows:

Conjugate HPS131-001-1

Concentration 0.277 mg/ml

Drug:antibody ratio (DAR): 1.8

PM: 182 kDa

Purity: 87% (13% of free mAb)

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 (Figure 9), and cytotoxic effect on cell cultures (Figures 10A and 10B).

The RRL assay results show that the anti-FAP hu36-IgG1-recNgA conjugates (HPS131-001-1) presented similar IC50 values as native Nigrin-b or recNgA and were in the 3pM range, showing that antibody conjugation did not diminish the enzymatic activity of recNgA (see Figure 9).

The cell cytotoxicity results show that, on HT1080 wild-type cells, conjugated antibody HPS131-001-1 displays only slight toxicity (if any) and only at highest concentration, naked anti-FAP hu36-IgG1 does not have any effect, and recNgA shows cytotoxic effect only at 10^{-6}M and after 72h incubation (see Figure 10A).

However, on FAP-expressing cells, HT1080-FAP, only HPS131-001-1 conjugated anti-FAP antibodies strongly reduce HT-1080-FAP cell viability in the picomolar concentration range, with IC_{50} values of 5pM (see Figure 10B).

These results show that: 1) anti-FAP:recNgA immunotoxins are highly active *in vitro*, being cytotoxic at picomolar range; 2) Activity is highly specific to FAP-expression, since no significant effect was observed in HT1080-WT; 3) Anti-FAP hu36-IgG1 specificity for its target is not affected by the conjugation to recNgA, neither is the enzymatic RIP activity of recNgA; 4) Activity is specific of the conjugated anti-FAP hu36-IgG1, since no effect was observed with the naked IgG1; 5) Anti-FAP:recNgA immunotoxins are internalized, since non conjugated recNgA (lacking membrane binding domain) shows almost no cytotoxic effect (IC_{50}>1µM)(see Figure 8).

In summary, anti-FAP:recNgA immunotoxins have the ability *in vitro* to specifically recognize the target (FAP), to be internalized within the cytosol and release the recNgA effector moiety to actively inhibit ribosomes, resulting in cytotoxicity IC_{50} values within the picomolar range.

In vivo evaluation of anti-tumoral effect

Immunotoxin anti-FAP:recNgA has been tested *in vivo* in both cell-derived and patient-derived xenograft mouse models for pancreas cancer. A dose range study was first performed to define the maximum tolerated dose in normal mice and each of these models: doses from 5 to 0.1 mg/kg were administrated intraperitoneally once a week during 3 weeks, and animal weight was monitored every 2 days to detect possible weight loss due to toxic effect of the immunotoxin. Results are presented in Figure 13.

High doses (>0.5 mg/kg) induced hepatotoxicity in normal mice, while no FAP-dependent toxicity was observed after pathological analysis of uterus and skeletal muscle, where low FAP expression has been described (*Dolznig H., et al., Cancer Immun., 5:10,2005; Roberts*

E.W., et al., J. Exp.Med., 210:1137, 2013), nor in heart and kidney. Doses lower than 0.5 mg/kg did not induce any detectable non-specific toxicity in cell line-derived orthotopic (Figure 13) and patient-derived subcutaneous (Figure 14A) xenograft murine models of pancreas cancer.

In efficacy studies performed then at nontoxic doses from 0.5 to 0.1 mg/kg, anti-FAP:recNgA immunotoxin, applied as single agent or in combination with Gemcitabine (240mg/kg), has shown no *in vivo* antitumoral efficacy in FAP (-) cell line derived orthotopic xenograft murine models (not shown), while high *in vivo* antitumoral efficacy was evidenced at a dose of 0.5 mg/kg in FAP (+) patient-derived subcutaneous xenograft murine models of pancreas cancer (Figure 14B). When combined with Gemcitabine (150mg/kg), it even showed 100% tumor growth inhibition and tumor regression.

Example 5 – Cytolysins and their conjugation to anti-FAP antibodies

Tubulysins are recently discovered natural compounds isolated from Myxobacteria, able to destabilize the tubulin skeleton, inducing apoptosis with a very high activity.
Leading to a fast, irreversible and strong change in the cell morphology, tubulysins and their synthetic tetrapeptidic analogues, the cytolysins, are highly potent cell-killing agents (nM to pM activity). Tubulysin A inhibits tubulin polymerization *in vitro* with an IC_{50} of 0.75-1µM, thus blocking the formation of mitotic spindles and inducing cell cycle arrest in G2/M phase. Tubulysins compete strongly with vinblastine through binding on the vinblastine binding site of tubulin. Furthermore they are stable in lysosome enriched cell fractions (*45-48*).

Amenable to conjugation, many different tubulysin/cytolysin derivatives are accessible by total synthesis in sufficient quantities for preclinical and clinical development; functional groups in their structure can be adapted to several different linker technologies.

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).

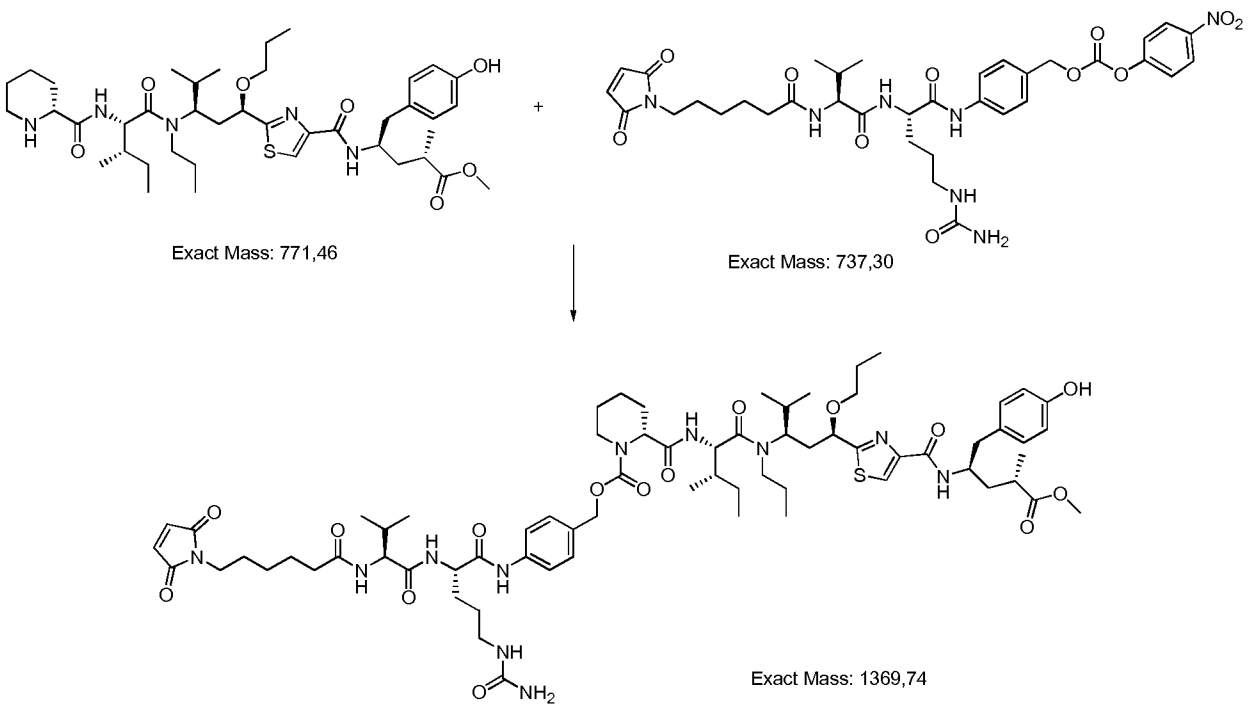
Various linker systems can be used and attached to either R^2 or R^{17} position of the molecule.

The general outline of the cytolysin conjugates, including the vcPABA linker and anti-FAP antibody, is shown in Figure 11 (in the structure depicted in Figure 11, 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 *49)*

The present inventors have used this linker (maleimide caproyl-vcPABA) to conjugate anti-FAP 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 (R1 or R4 positions of the cytolysin shown in Figure 11).

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

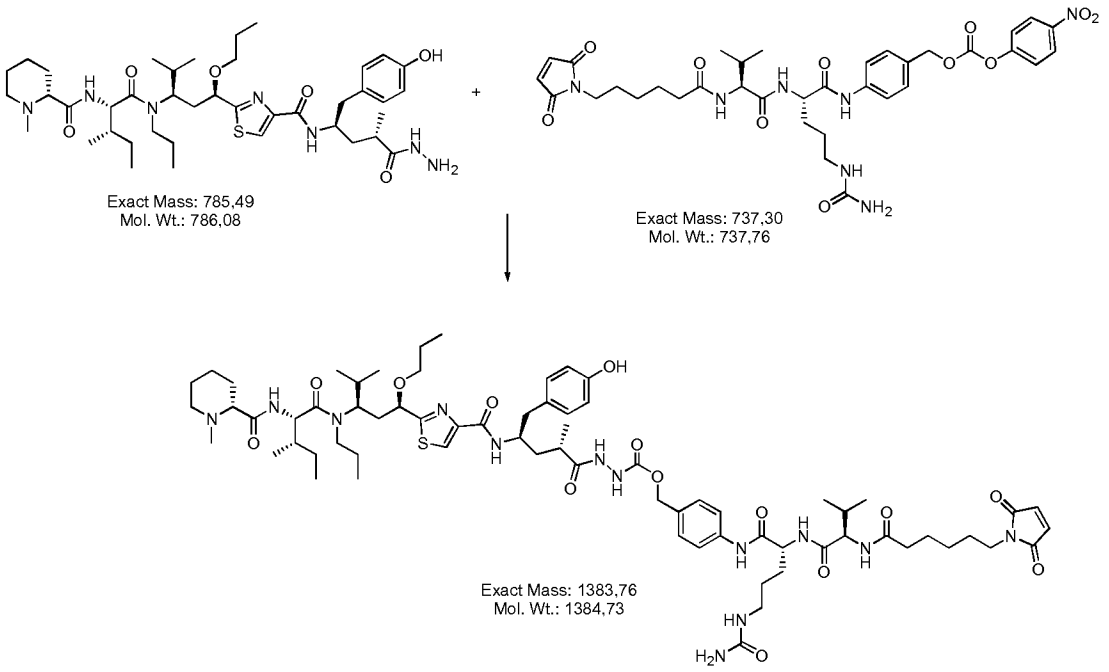


TAM467

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].

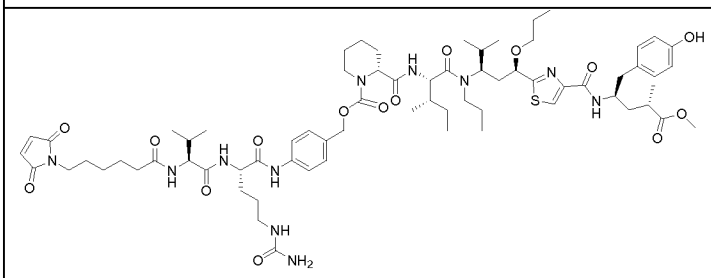
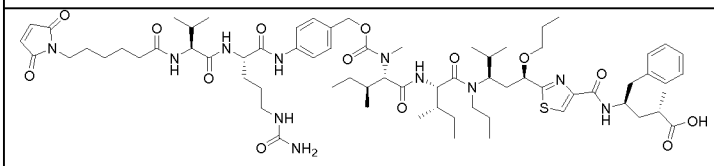
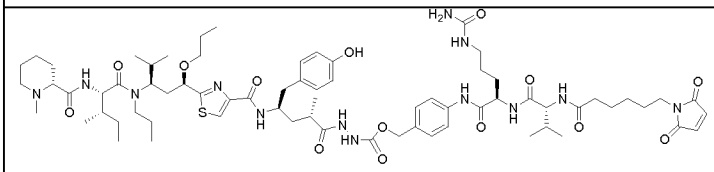
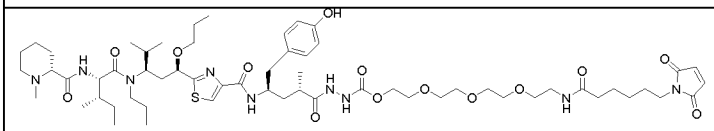
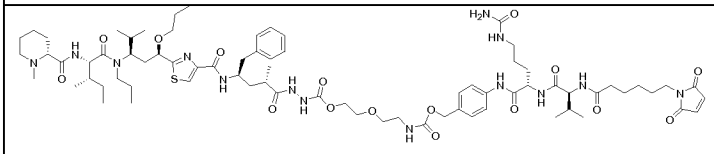
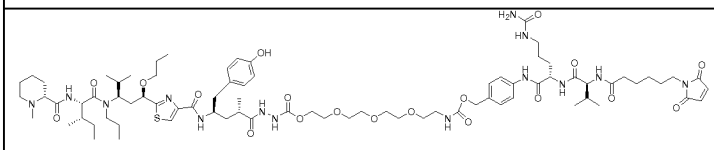
In vitro activity testing is performed. Functional activity will be evaluated through microtubule inhibition assay, while cytotoxic activity is determined through crystal violet viability assay.

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 structures 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 was evaluated through tubulin polymerization inhibition assay (TPI; Tubulin Polymerization assay kit; Cytoskeleton, Cat. #BK011P), and cell proliferation arrest on HT1080 cells (CPA; crystal violet). IC_{50} 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	**IC_{50} (TPI assay; µM)**	**IC_{50} (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.

Conjugation and Chemical characterization of ADCs

Each of the newly generated derivatives was conjugated to the anti-FAP hu36 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 follows: 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 (SEC, HIC and PRLP profiles) were determined for each ADC and for free antibody (data not shown). Biochemical characteristics of the ADCs is 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 apparently less than optimal for this type of cytolysin molecule under these conditions.

Target binding of conjugates

Anti-FAP hu36:TAM471 ADC binding to huFAP fusion protein was analysed by ELISA, and binding to HT1080-FAP cells by FACS (Figure

15). For FACS analysis, compounds were incubated either at serial dilutions (Fig. 15B) or at one dilution (Fig. 15C; 10nM) and detected with an anti-human IgG-PE (γ chain specific).

EC_{50} values obtained in both assays showed no significant difference with respect to naked anti-hu/moFAP hu36 antibody (Fig. 15A & 15B). No binding was observed in FAP(-) cells such as HT1080-wt and HEK293 cells (Fig. 15C).

Figure 16 shows that ADC-471 (Fig. 16B) specifically binds and gets fully internalized after 90min in HT1080-FAP cells, similarly to naked anti-FAP antibody (Fig. 16A). These results evidenced that conjugation did not affect target specificity and affinity, or internalization ability of the anti-FAP hu36 IgG1.

Example 6 – Evaluation of in vitro cytotoxic activity and in vivo anti-tumoral effect

Anti-FAP:cytolysin ADC candidates were evaluated *in vitro* through proliferation arrest assay (crystal violet staining). Results are presented in Figure 17 and IC_{50} values in Table 7. Anti-tumoral effect of each ADC candidate was evaluated in a patient-derived xenograft (PDX) mouse model for pancreas cancer (PAXF-736). This model was previously selected for FAP expression level and stroma expansion. ADC compounds were administrated once a week intraperitoneally at 2.5mg/kg. Tumor volume and body weight were measured twice a week. Vehicle-treated and Gemcitabine-treated (150mg/kg) PDX mice were used as negative and positive control groups, respectively. Results are shown in Figure 18.

Location of vcPABA linker alone in R1 position (ADC-551) generated conjugates with much less cytotoxic activity *in vitro* in comparison with conjugates utilizing the R4 position (ADC-471) (Figure 17; Table 7) and no anti-tumoral activity *in vivo* (Figure 18).

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 FAP-specific cytotoxic activity *in vitro* (Figure 17) and anti-tumoral effect *in vivo* (Figure 18). The TAM552 conjugate (ADC-552), having a 3 ethylene glycol spacer, but no vcPABA present in the linker was found to exhibit minimal or no *in vivo* anti-tumoral activity (data not shown). While ADC-471 and ADC-553 showed low and no FAP-specific cytotoxic activity (10nM and 100nM IC_{50} range, respectively) with no difference between HT1080-WT and FAP cells nor anti-tumoral effect *in vivo*, ADC-558 presented a 1 nM range FAP-specific cytotoxic activity with a specificity ratio of 500 between FAP(+) and FAP(-) HT1080 cells, and a 40% tumor growth inhibition effect at 2.5mg/kg dose in PDX mouse model for pancreas cancer. No weight loss, nor toxic effect was observed for none of the candidates at this dose (not shown).

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

Compound	**HT1080-WT**	**HT1080-FAP**
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

Further investigation was carried out using ADC-558. Maximum tolerated dose (MTD) was performed in normal mice and ADC-558 was found to be non-toxic within 2.5 to 25mg/kg dose range with a weekly treatment for 3 weeks. Doses from 20, 10, and 5 mg/kg were then administrated weekly for 4 weeks to a PDX mouse model (Panc185) with high FAP expression level and stroma expansion to confirm tumor growth inhibition and full regression efficacy of the ADC-558 conjugate.

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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.

References

1. Weinberg, R.A., et al., Garland science, Taylor & Francis Group LLC, New York, NY, USA, 2007
2. Nieman, K.M., et al., Nat. Med., 2011, 17: 1498-1503
3. Joyce, J.A., et al., Nat. Rev. Cancer, 2009, 9: 239-252
4. Hanahan, D., et al., Cancer Cell, 2012, 21: 309-322
5. Gupta, G.P., et al., Cell, 2006: 127: 679-695
6. Valastyan, S., et al., Cell, 2011, 147: 275-292
7. Meads, M.B, et al., Nat. Rev. Cancer, 2009, 9: 665-674
8. Olive, K.P., et al., Science, 2009, 324: 1457-1461
9. Acharyya, S., et al., Cell, 2012, 150: 165-178
10. Crawford, Y., et al. Cancer Cell, 2009, 15: 21-34
11. Straussman, R., Nature, 2012, 487: 500-504
12. Joyce, J.A., et al., Cancer Cell, 2005, 7: 513-520
13. Hanahan, D., et al., Cell, 2011, 144: 646-674
14. Kalluri, R., Nat. Rev. Cancer, 2006, 6: 392-401
15. Pietras, K., et al., Exp. Cell Res., 2010, 316: 1324-1331
16. Orimo, A., et al., Cell, 2005, 121: 335-348
17. Erez, N., et al., Cancer Cell, 2010, 17: 135-147
18. Olumi, A.F., et al., Cancer Res., 1999, 59: 5002-5011
19. Yang, G., et al., Proc. Natl. Acad. Sci. USA, 2006, 103: 16472-16477
20. Hwang, R.F., et al., Cancer Res., 2008, 68: 918-926
21. Hu, M., et al., Proc. Natl. Acad. Sci. USA, 2009, 106: 3372-3377
22. Medema, J.P., et al., Nature, 2011, 474: 318-326
23. Malanchi, I., et al., Nature, 2012, 481: 85-89
24. Strell, C., et al., Ups. J. Med. Sci., 2012, 117: 187-195
25. Horimoto, Y., et al., Cell Adhes. Migr., 2012, 6: 193202
26. Wu, et al., J. Cancer Mol., 2008, 4: 37-45
27. Mersmann M., et al., Int. J. Cancer, 2001, 92: 240-248
28. Brocks B., et al., Molecular Medicine, 2001, 7: 461-469
29. Schmidt A., et al., Eur. J. Biochem., 2001, 268: 1730-1738
30. Messerschmidt, S.K., et al., J Control Release, 2009, 137: 69-77
31. Ostermann E., et al., Clin. Cancer Res., 2008, 14: 4584-4592
32. Shi, M., et al., World J. Gastroenterology, 2012, 28: 840-846.

33. Muñoz et al., Cancer Res., 2001

34. *Trush et al., Annu. Rev. Immunol., 1996, 14:49-71*
35. Lambert *et al.*, 1988,
36. *Barbieri, et al., Methods in Mol. Biol., 2001, 166: 71-85*
37. *Ghetie and Vitetta, Mol. Biotechnol., 2001, 18: 251-286*
38. Muñoz R., et al., Cancer Lett., 2007, 256: 73-80.
39. Muñoz R., et al., Cancer Immunol. Immunother., 2012
40. *Thorpe et al., Cancer Res., 1987, 47:5924-5931*
41. *Fracasso et al., Mini Rev. Med. Chem., 2004, 4: 545-562*
42. *Marsh et al., "Immunotoxins", Frankel A.E. ed., Kluwer Academic Publishers, Boston, MA, 1988, 213-237*
43. *Riddles et al., Anal. Biochem., 1979, 94:75-81*
44. *Riener et al., Anal. Bioanal. Chem, 2002, 373:266-276*
45. Sasse, F., et al., Journal of Antibiotics, 2000, 53:879-885.
46. Kaur, G., et al., Biochem. J., 2006, 396:235-242.
47. Schluep, T., et al., Clin. Cancer Res., 2009, 15:181-189
48. Reddy, J.A., et al., Mol. Pharmaceutics, 2009.
49. *Gualberto A. Expert Opin Investig Drugs. 2012; 21(2): 205-16*
50. *Perez-Soler et al., Clin. Cancer Res., 2000, 6: 4932-4938;*
51. *Yabuchi et al., Cancer Letters, 2013*

Claims:

1. 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 FAP;
L is a linker;
D is a drug comprising a cytolysin or a Nigrin-b A-chain; and
p is 1 to 10.

2. The conjugate of claim 1, wherein A is a monoclonal antibody or binding fragment thereof that selectively binds to an extracellular region of human FAP and/or murine FAP.

3. The conjugate of claim 2, 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 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.

4. The conjugate of claim 3, wherein CDRH1-3 comprise the amino acid sequences of SEQ ID NOS: 7-9, respectively and wherein CDRL1-3 comprise the amino acid sequences of SEQ ID NOS: 10-12, respectively.

5. The conjugate of any one of the preceding claims, wherein 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.

6. The conjugate of claim 5, wherein A comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO: 5.

7. The conjugate of any one of the preceding claims, wherein 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.

8. The conjugate of claim 7, wherein A comprises a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 6.

9. The conjugate of any one of the preceding claims, wherein 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.

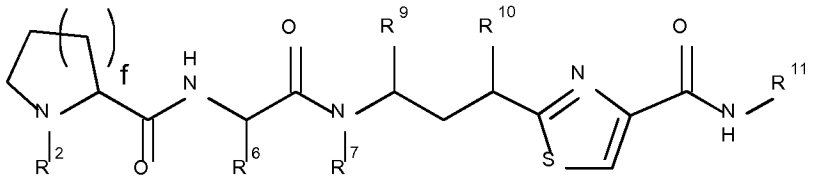
10. The conjugate of claim 9, wherein A comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 3.

11. The conjugate of any one of the preceding claims, wherein 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.

12. The conjugate of claim 11, wherein A comprises a light chain comprising the amino acid sequence of SEQ ID NO: 4.

13. The conjugate of claim 1 or claim 2, wherein A competes with the anti-FAP IgG1 antibody having the heavy chain amino acid sequence of SEQ ID NO: 3 and the light chain amino acid sequence of SEQ ID NO: 4 for binding to immobilized recombinant human and/or murine FAP.

14. The conjugate of any one of the preceding claims, wherein D is a cytolysin of formula IV:



(IV)

wherein:

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

R^6 is C_1-C_6 alkyl;

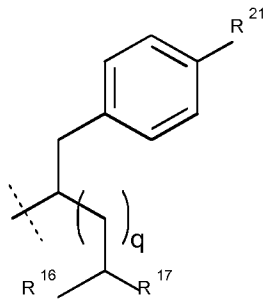
R^7 is C_1-C_6 alkyl, CH_2OR^{19} or CH_2OCOR^{20}, wherein R^{19} is alkyl, R^{20} is C_2-C_6-alkenyl, phenyl, or CH_2-phenyl;

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} (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.

15. The conjugate of claim 14, wherein R^2 is a bond to linker L.

16. The conjugate of claim 14, wherein R^{17} is C(O)X, CONHNHX, OX, NHX or SX, wherein X is a bond to linker L.

17. The conjugate of any one of the preceding claims, wherein linker L further comprises a spacer.

18. The conjugate of claim 17, wherein the spacer has a chain length of 2 to 30 atoms.

19. The conjugate of claim 17 or claim 18, wherein the spacer comprises or consists of an alkylene (i.e. divalent alkyl) or heteroalkylene (i.e. divalent heteroalkyl) group.

20. The conjugate of any one of claims 17 to 19, wherein the spacer comprises or consists of an alkylene or oxyalkylene group.

21. The conjugate of claim 20, wherein the spacer comprises or consists of a group $-(CH_2)_n-$ or $-(OCH_2CH_2)_n-$, wherein $n \geq 1$.

22. The conjugate of claim 21, wherein the spacer comprises or consists of a group $-(OCH_2CH_2)_n-$, wherein $n \geq 1$.

23. The conjugate of claim 21 or claim 22, wherein n = 1 to 15, 1 to 10, 1 to 6, or 2 to 5.

24. The conjugate of claim 23, wherein n = 3 or 4.

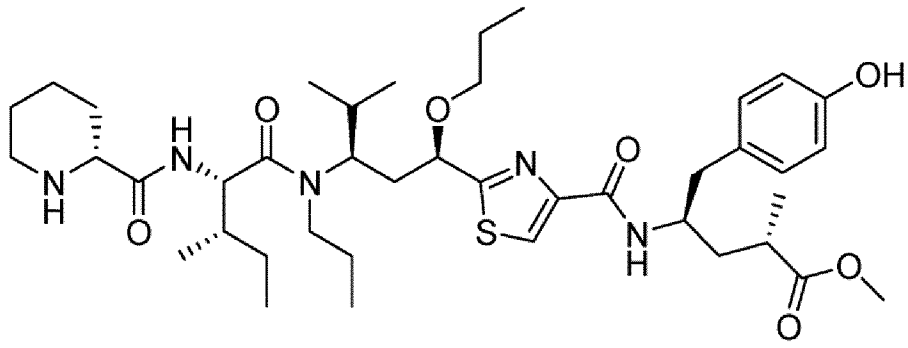
25. The conjugate of any one of claims 17 to 24, wherein the spacer is directly attached to group R^{17}, or is attached to group R^{17} via a bridging group.

26. The conjugate of claim 25, wherein the spacer is attached to group R^{17} via a -C(O)X bridging group, wherein X is a bond to R^{17}.

27. The conjugate of claim 26, wherein 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}.

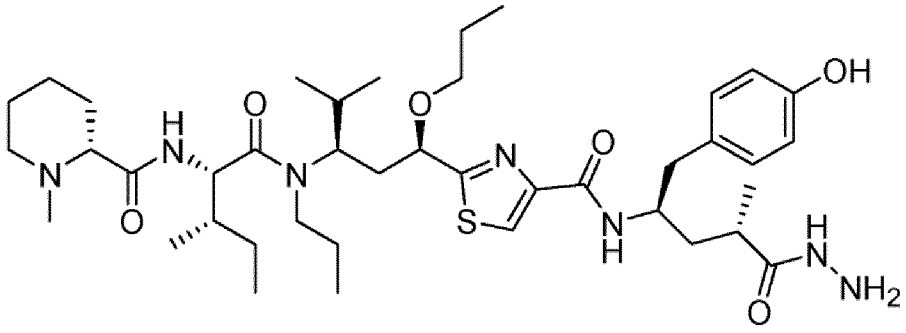
28. The conjugate of claim 27, wherein R^{17} is CONHNHX and the spacer is a $-(OCH_2CH_2)_n-$ attached to R^{17} via a -C(O)X bridging group, wherein n = 2, 3 or 4.

29. The conjugate of claim 14, wherein D comprises a cytolysin having the following structure:



.

30. The conjugate of claim 14, wherein D comprises a cytolysin having the following structure:



.

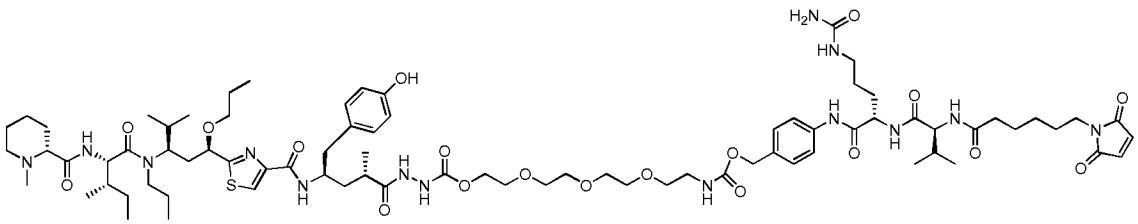
31. The conjugate of any one of claims 14 to 30, wherein L comprises an attachment group for attachment to A and a protease cleavable portion.

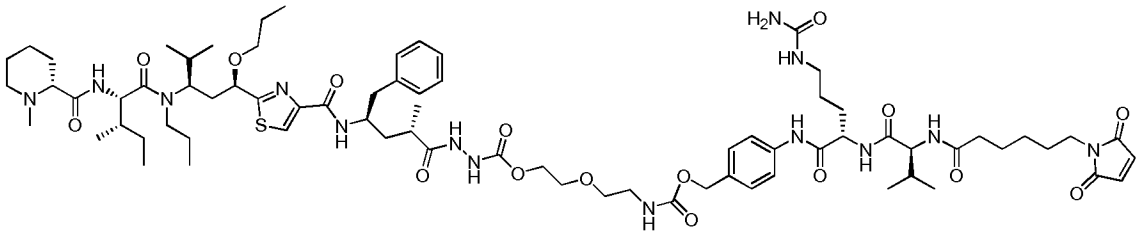
32. The conjugate of claim 31, wherein L comprises a valine-citrulline unit.

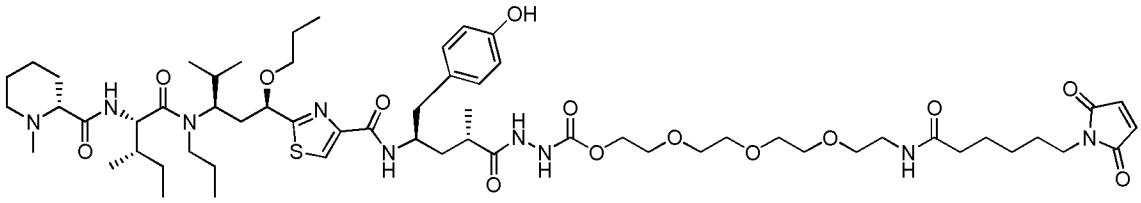
33. The conjugate of claim 32, wherein L comprises maleimidocaproyl-valine-citrulline-p-aminobenzylcarbamate.

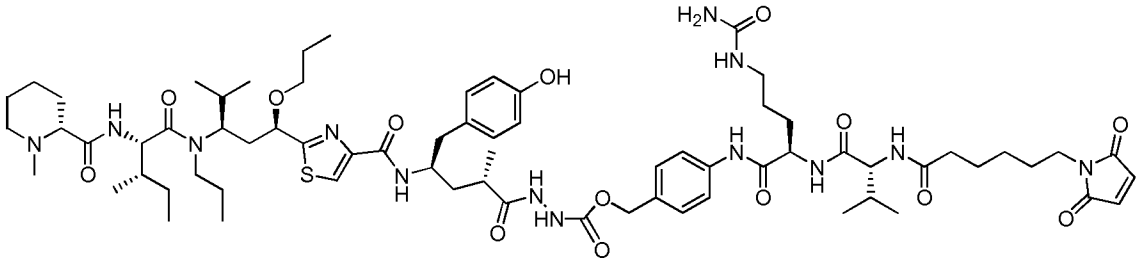
34. The conjugate of claim 33, wherein the double bond of the maleimide is reacted with a thiol group of a cysteine residue of the antibody A to form a sulphur-carbon bond in order to effect linkage of the linker L to the antibody A.

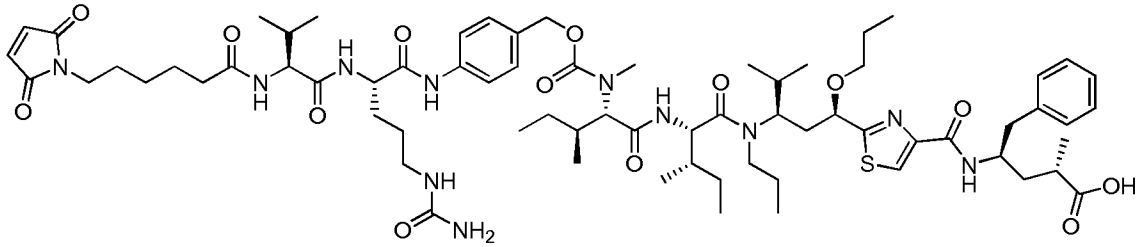
35. The conjugate of any one of the preceding claims, wherein -L-D has a structure selected from the group consisting of:



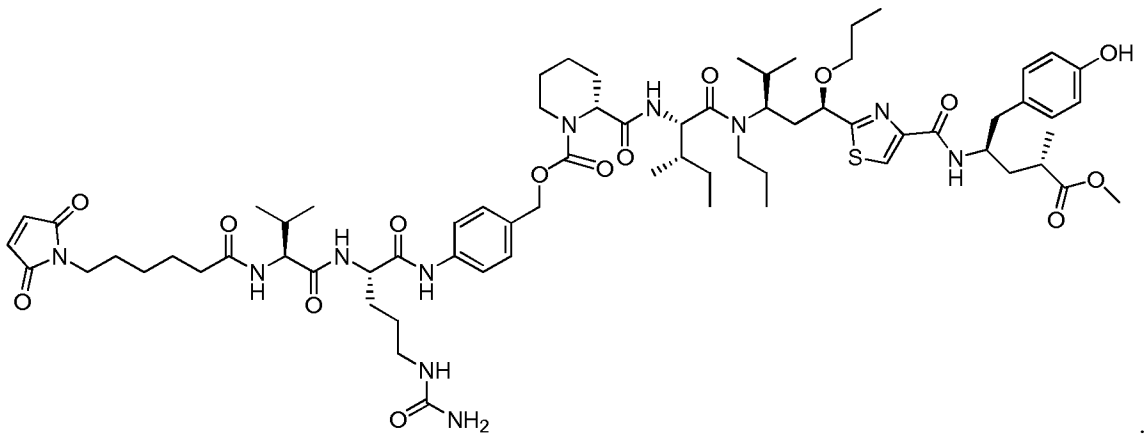






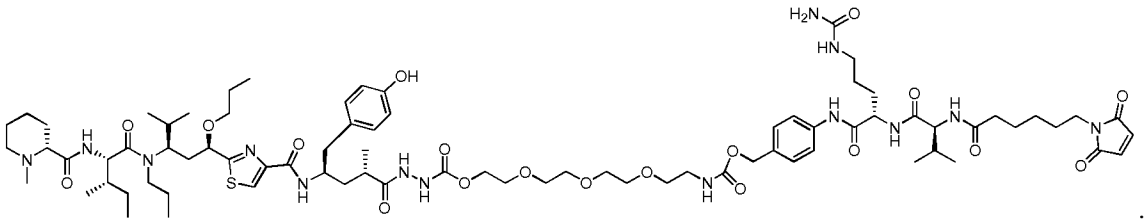


and



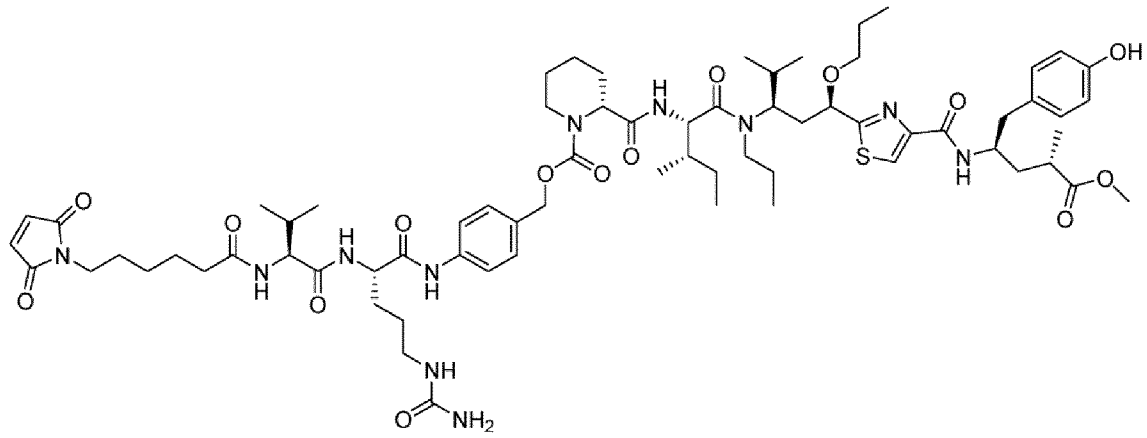
.

36. The conjugate of claim 35, wherein -L-D has the following structure:



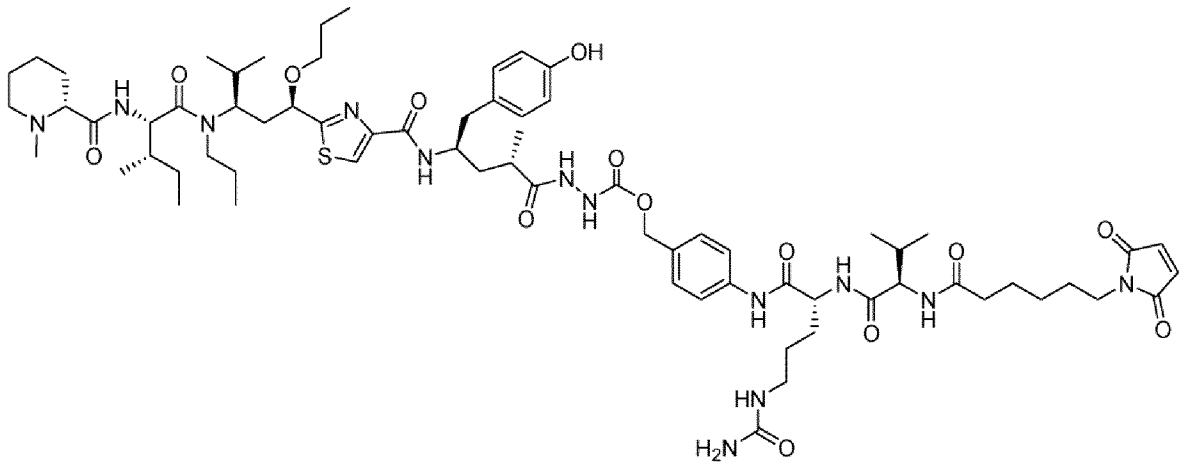
.

37. The conjugate of claim 14, wherein -L-D has the following structure:



.

38. The conjugate of claim 14, wherein -L-D has the following structure:



.

39. The conjugate of claim 1, wherein the conjugate is the conjugate described herein as ADC-558.

40. The conjugate of any one of the preceding claims, wherein p is 1, 2, 3, 4 or 5.

41. The conjugate of any one of claims 1 to 13, wherein D is a Nigrin-b A-chain in the absence of a Nigrin-b B-chain.

42. The conjugate of claim 41, wherein the amino acid sequence of the Nigrin-b A-chain comprises or consists of the sequence of SEQ ID NO: 13.

43. The conjugate of claim 41 or claim 42, wherein the Nigrin-b A-chain is recombinantly-produced in a bacterial host cell.

44. The conjugate of any one of claims 41 to 43, wherein L comprises or is a disulphide bond.

45. The conjugate of any one of claims 41 to 44, wherein p is 1 to 5.

46. A conjugate as defined in any one of the preceding claims for use in medicine.

47. A conjugate as defined in any one of the preceding claims for use in a method of treatment of a tumor in a mammalian subject.

48. The conjugate for use according to claim 47, wherein said conjugate is for simultaneous, sequential or separate administration with one or more other antitumor drugs.

49. The conjugate for use according to claim 48, wherein said one or more other antitumor drugs comprise a cytotoxic chemotherapeutic agent or an anti-angiogenic agent or an immunotherapeutic agent.

50. The conjugate for use according to claim 49, 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.

51. The conjugate for use according to claim 50, wherein said anti-PD-1 molecule or anti-PD-L1 molecule comprises nivolumab or pembrolizumab.

52. The conjugate for use according to any one of claims 47 to 51, wherein the tumor is a solid tumor.

53. The conjugate for use according to claim 52, wherein the treatment is of pancreatic cancer, breast cancer, melanoma, lung cancer, head and neck cancer, ovarian cancer, bladder cancer or colon cancer.

54. A method of treating a tumor in a mammalian subject, comprising administering a therapeutically effective amount of a conjugate as defined in any one of claims 1 to 45 to the subject in need thereof.

55. The method of claim 54, wherein said conjugate is administered simultaneously, sequentially or separately with one or more other antitumor drugs.

56. The method of claim 55, wherein said one or more other antitumor drugs comprise a cytotoxic chemotherapeutic agent or an anti-angiogenic agent or an immunotherapeutic agent.

57. The method of claim 56, wherein said one or more other antitumor drugs comprise Gemcitabine, Abraxane, bevacizumab, itraconazole, or carboxyamidotriazole, an anti-PD-1 molecule or an anti-PD-L1 molecule.

58. The method of claim 57, wherein said anti-PD-1 molecule or anti-PD-L1 molecule comprises nivolumab or pembrolizumab.

59. The method of any one of claims 54 to 58, wherein the tumor is a solid tumor.

60. The method of claim 59, wherein the tumor is of pancreatic cancer, breast cancer, melanoma, lung cancer, head and neck cancer, ovarian cancer, bladder cancer or colon cancer.

61. Use of a cytolysin as defined in any one of claims 14 to 18 in the preparation of an antibody-drug conjugate that comprises an FAP-specific antibody.

62. Use according to claim 61, wherein the FAP-specific antibody is as defined in any one of claims 2 to 13.

63. A conjugate as defined in any one of claims 1 to 45 for use in the treatment of an inflammatory condition.

64. A method of treating an inflammatory condition in a mammalian subject, comprising administering a therapeutically effective amount of a conjugate as defined in any one of claims 1 to 45 to the subject in need thereof.

65. The conjugate for use according to claim 63 or the method according to claim 64, wherein said inflammatory condition is rheumatoid arthritis.

66. An isolated Nigrin-b A-chain in the absence of the Nigrin-b B-chain.

67. The isolated Nigrin-b A-chain of claim 66, wherein the amino acid sequence of the Nigrin-b A-chain comprises or consists of the sequence of SEQ ID NO: 13.

68. Use of an isolated Nigrin-b A-chain according to claim 66 or claim 67 in the preparation of an immunotoxin.

69. Use according to claim 68, wherein the immunotoxin comprises an FAP-specific antibody.

70. Use according to claim 69, wherein the FAP-specific antibody is as defined in any one of claims 2 to 13.

71. A human monoclonal antibody that selectively binds FAP 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.

72. The antibody of claim 71 for use in medicine.

73. The antibody of claim 71 for use in a method of treatment of an inflammatory condition.

74. The antibody for use according to claim 73, wherein said inflammatory condition comprises rheumatoid arthritis.

75. Use of a monoclonal antibody as defined in claim 71 in the preparation of an antibody-drug conjugate or an immunotoxin.

76. 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 and 13.

77. The host cell of claim 76, wherein the polynucleotide comprises the nucleic acid sequence of SEQ ID NO: 14.

78. A process for the production of a conjugate as defined in any one of claims 41-45, comprising:

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

(b) reacting the derivatised antibody and Nigrin-b A-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; and

(c) optionally, purifying and/or isolating the conjugate.

79. The process of claim 78, wherein step (a) comprises 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.

80. A process for the production of a conjugate as defined in any one of claims 14-40, comprising:

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

(b) linking the cytolysin to the linker; and

(c) optionally, purifying and/or isolating the conjugate,

wherein steps (a) and (b) can be performed in any order.

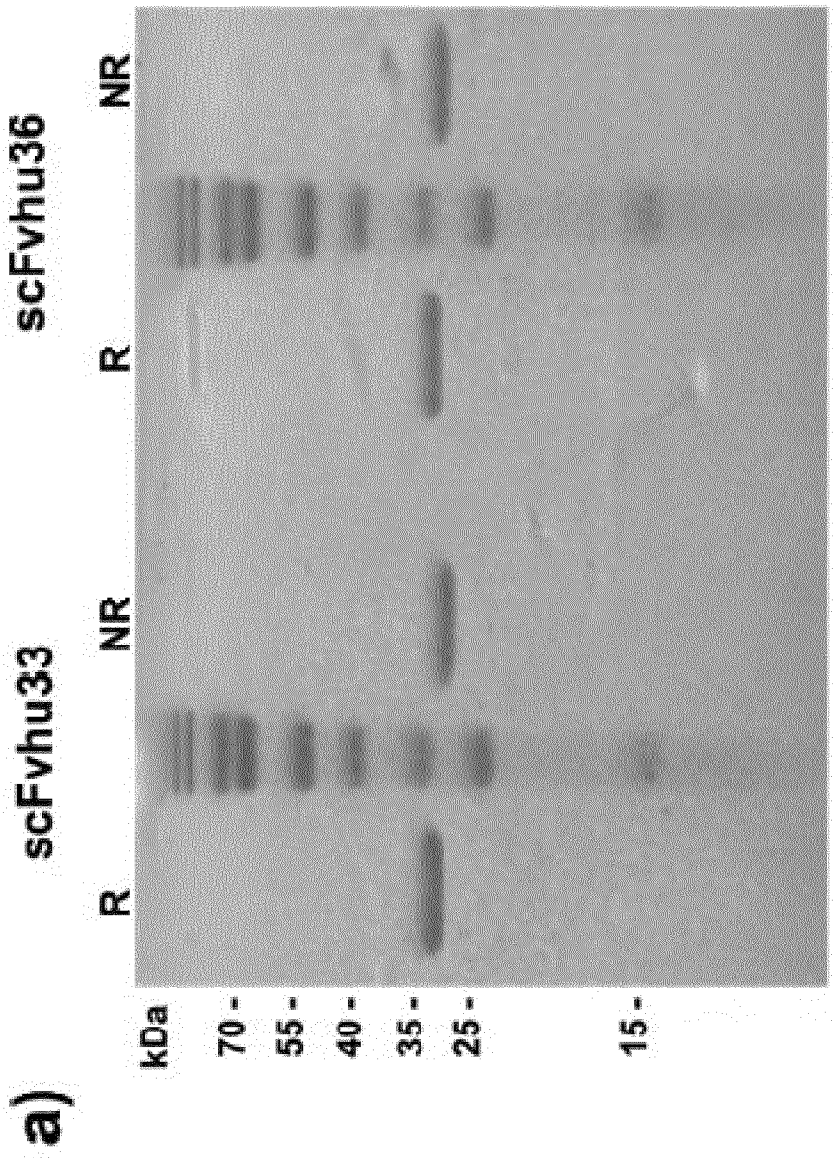


Figure 1A

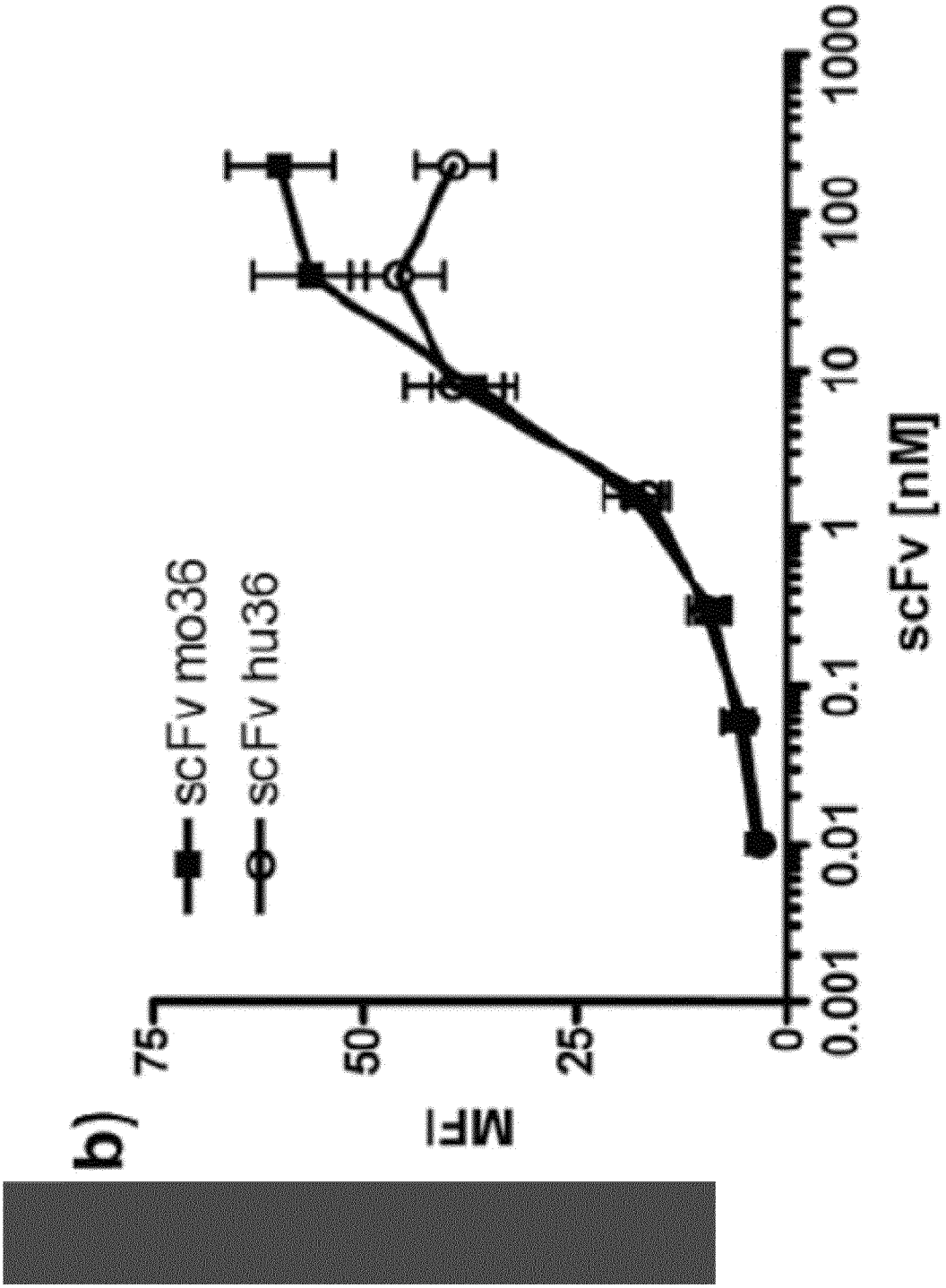

b)
MFI
75
50
25
0
0.001
0.01
0.1
1
10
100
1000
scFv [nM]
scFv mo36
scFv hu36

Figure 1B

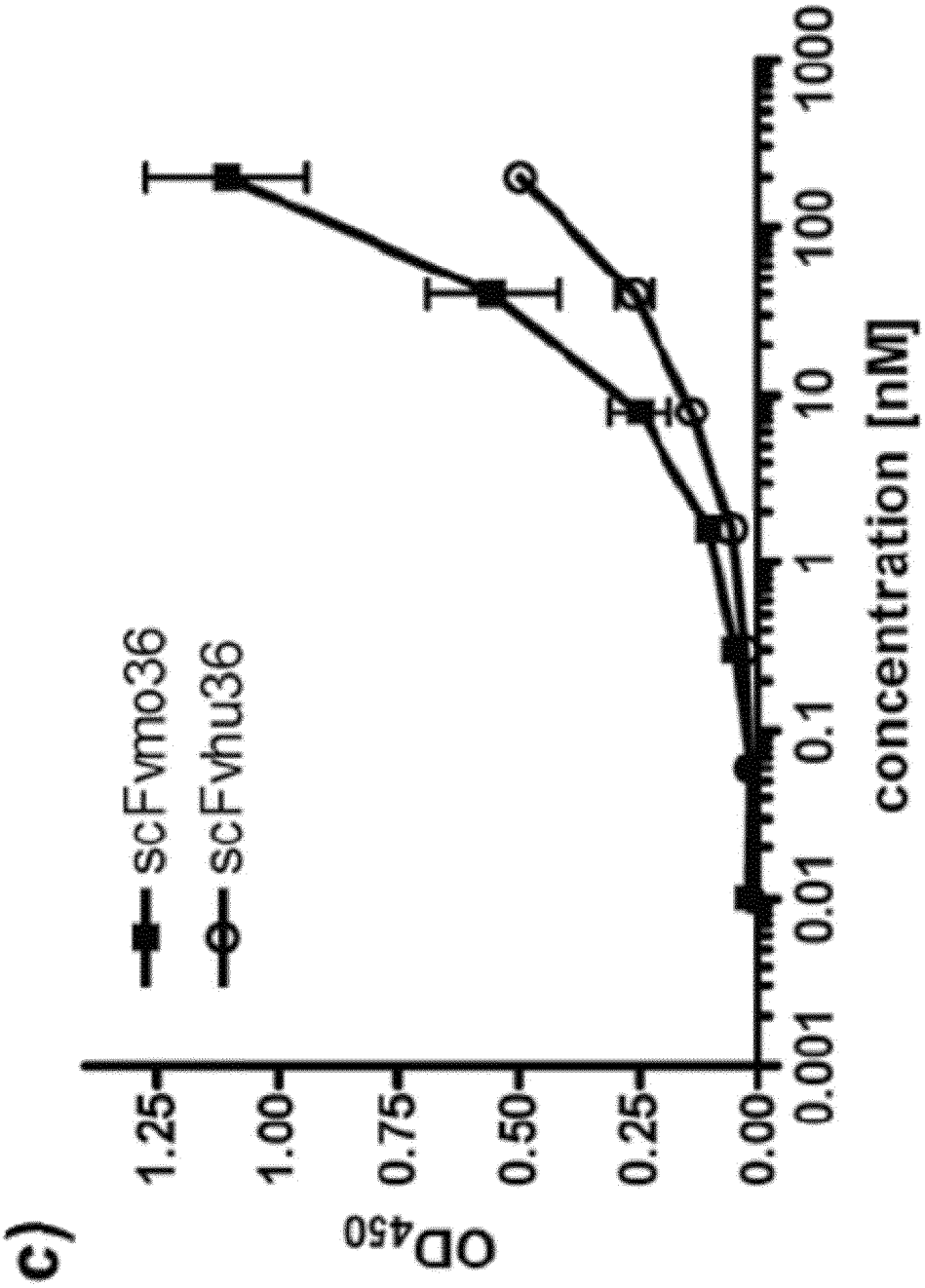

c)
OD450
1.25
1.00
0.75
0.50
0.25
0.00
0.001
0.01
0.1
1
10
100
1000
concentration [nM]
scFvmo36
scFvhu36

Figure 1C

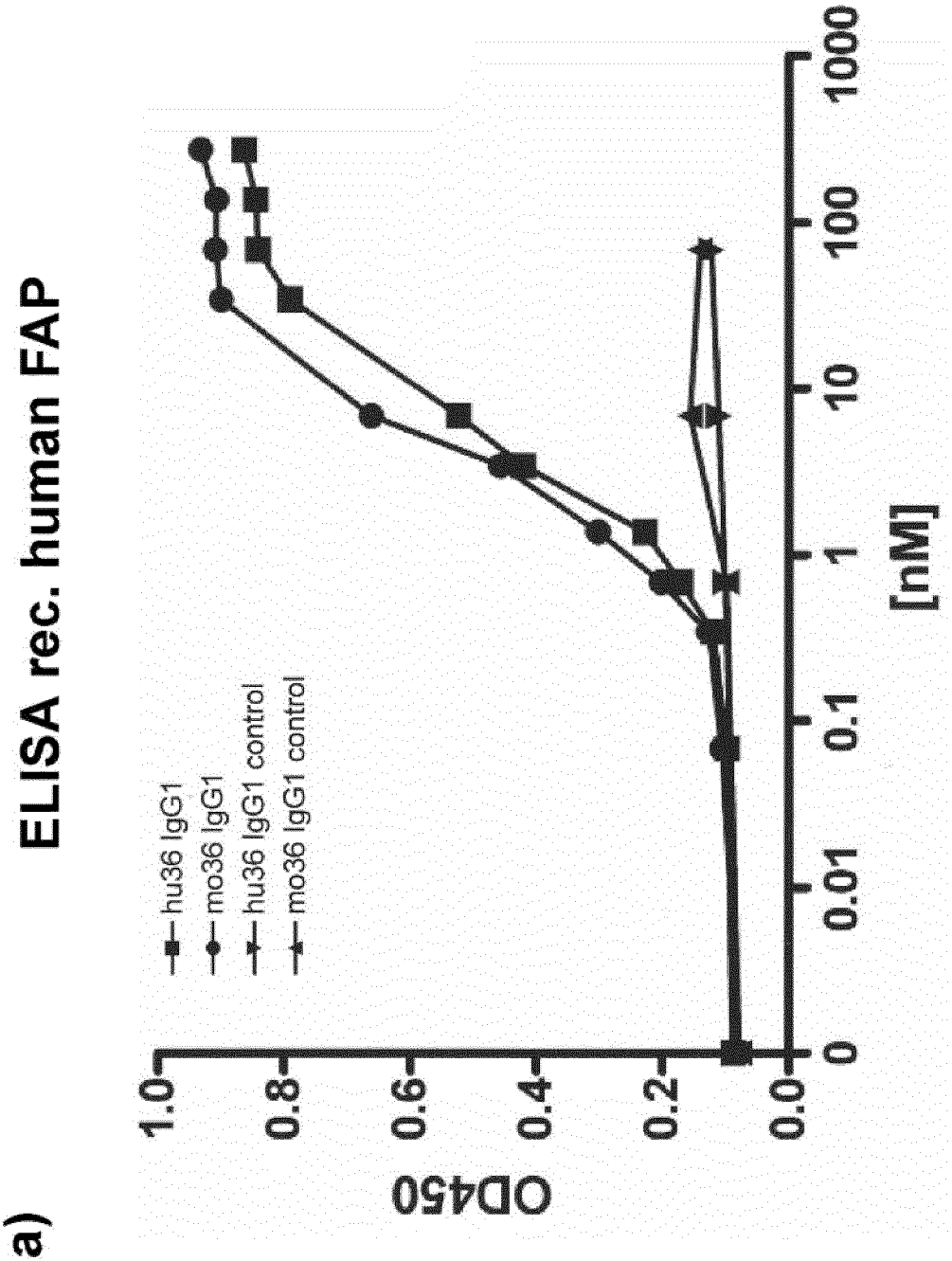

a)
ELISA rec. human FAP
hu36 IgG1
mo36 IgG1
hu36 IgG1 control
mo36 IgG1 control
OD450
1.0
0.8
0.6
0.4
0.2
0.0
0
0.01
0.1
1
10
100
1000
[nM]

Figure 2A

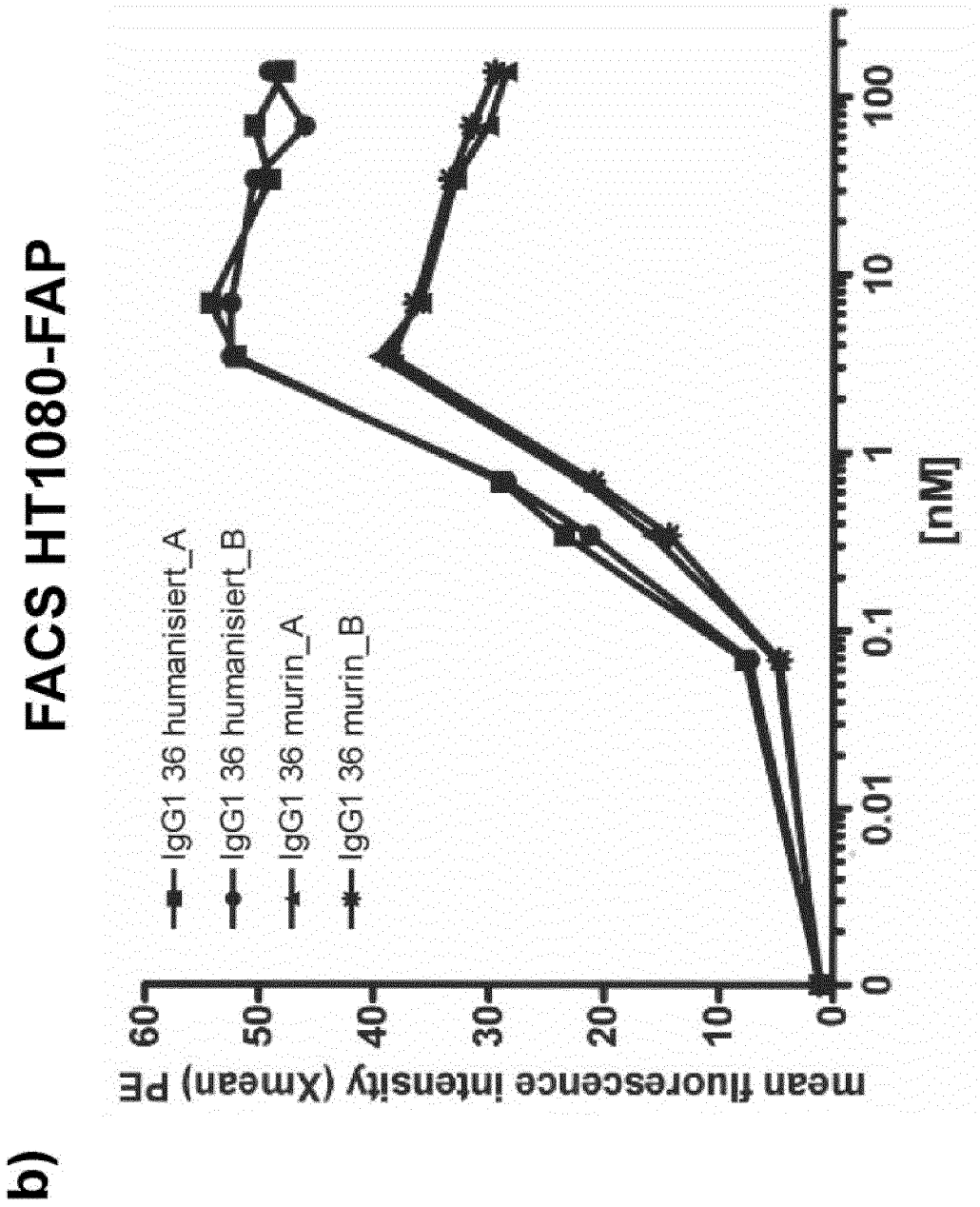

b)
FACS HT1080-FAP
mean fluorescence intensity (Xmean) PE
60
50
40
30
20
10
0
0
0.01
0.1
1
10
100
[nM]
IgG1 36 humanisiert_A
IgG1 36 humanisiert_B
IgG1 36 murin_A
IgG1 36 murin_B

Figure 2B

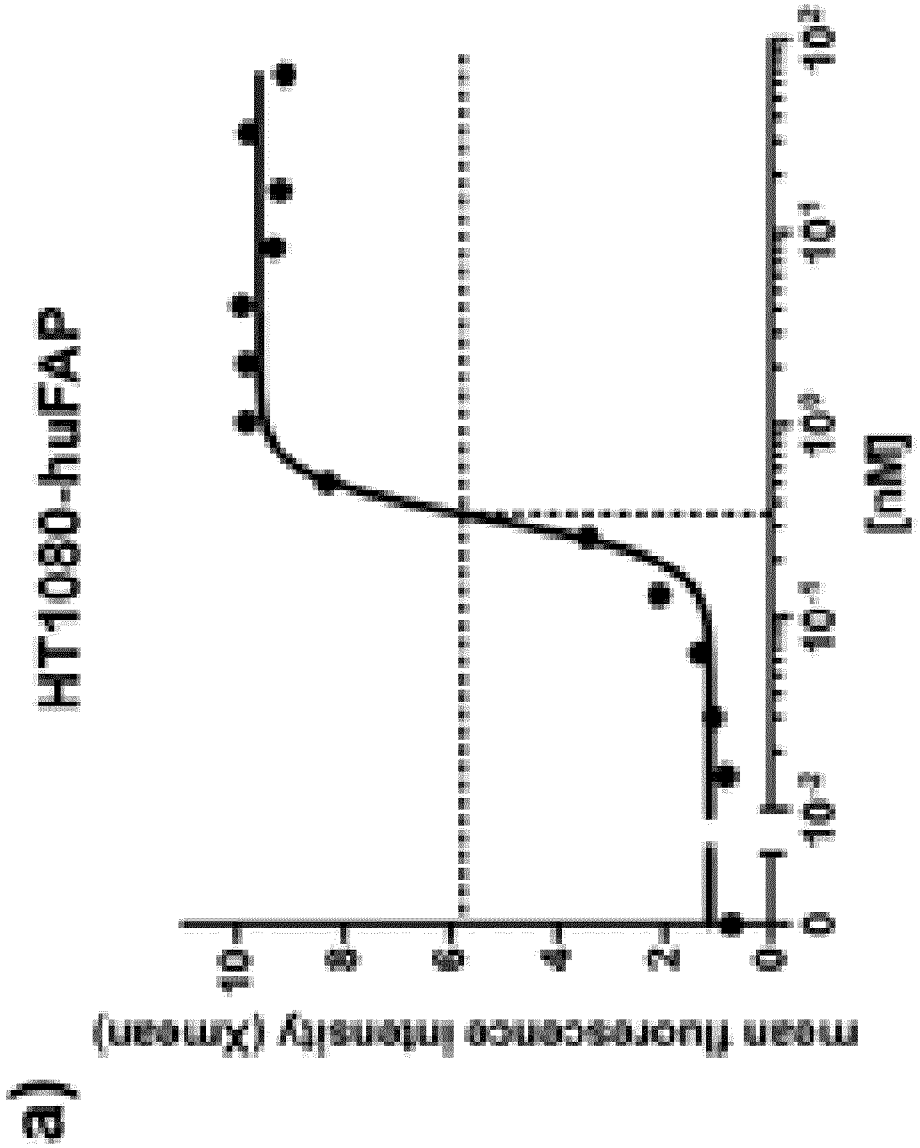

a)
HT1080-huFAP
mean fluorescence intensity (xmean)
0
2
4
6
8
10
0
10⁻²
10⁻¹
10⁰
10¹
10²
[nM]

Figure 3A

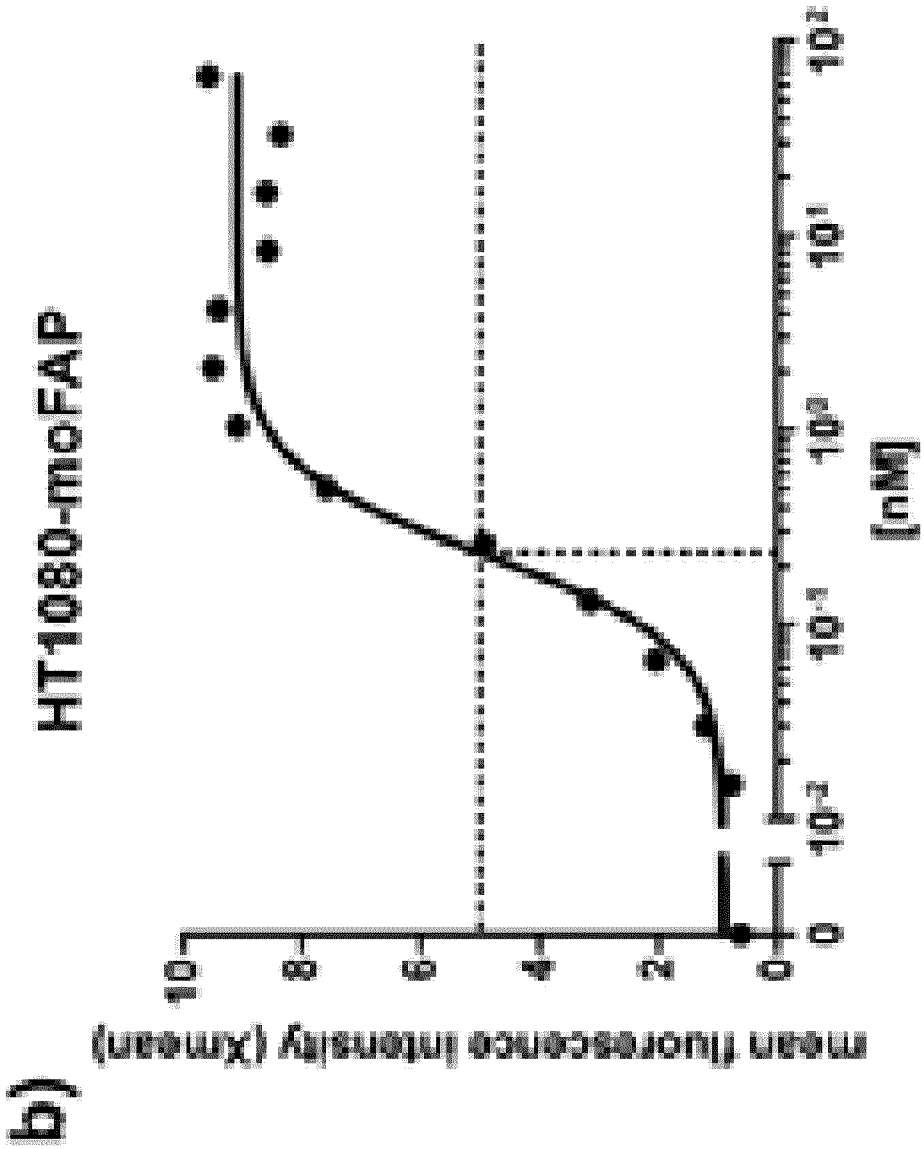

b)
HT1080-moFAP
mean fluorescence intensity (xmean)
0
2
4
6
8
10
0
10-2
10-1
101
102
103
[nM]

Figure 3B

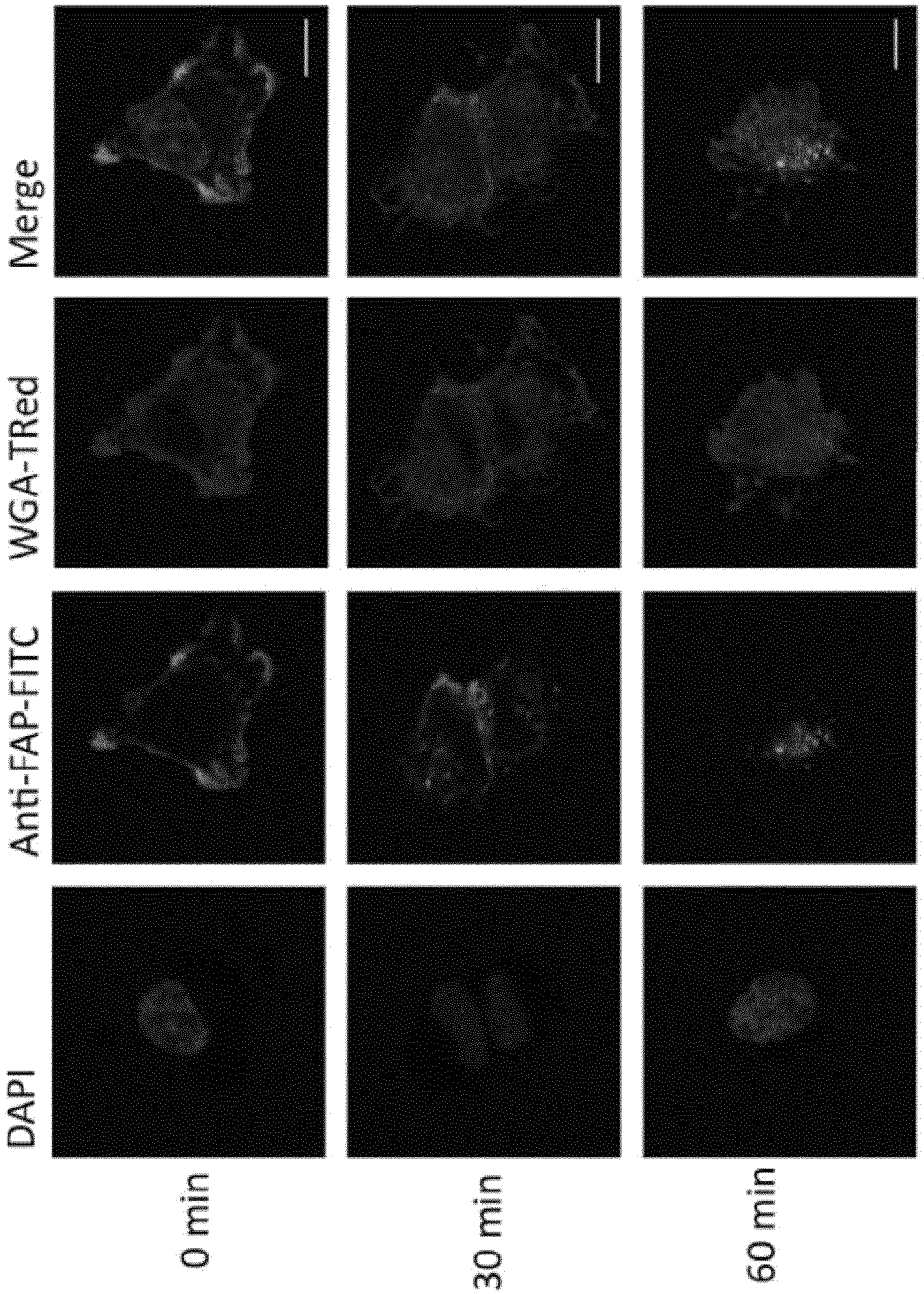


Figure 4

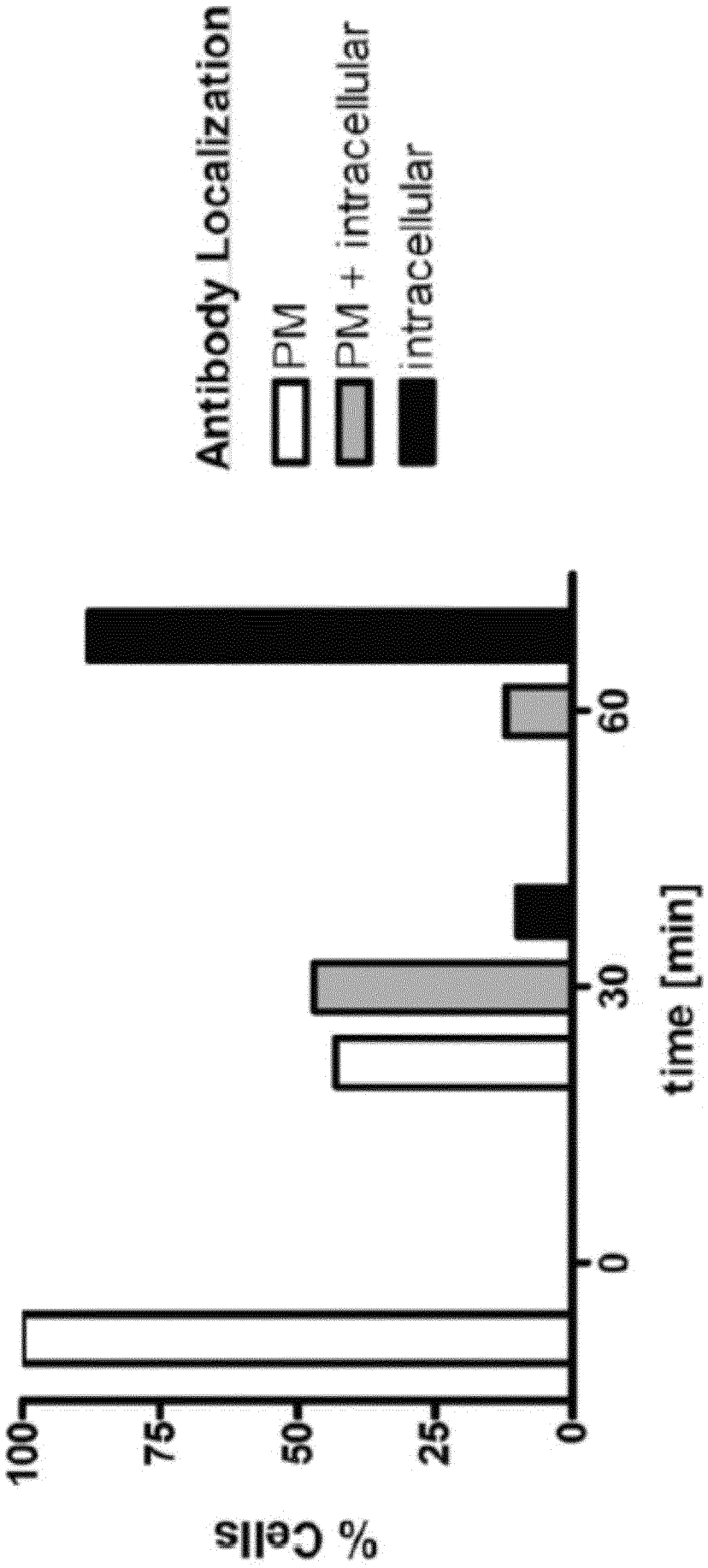

Antibody Localization
PM
PM + intracellular
intracellular
% Cells
100
75
50
25
0
0
30
60
time [min]

Figure 5

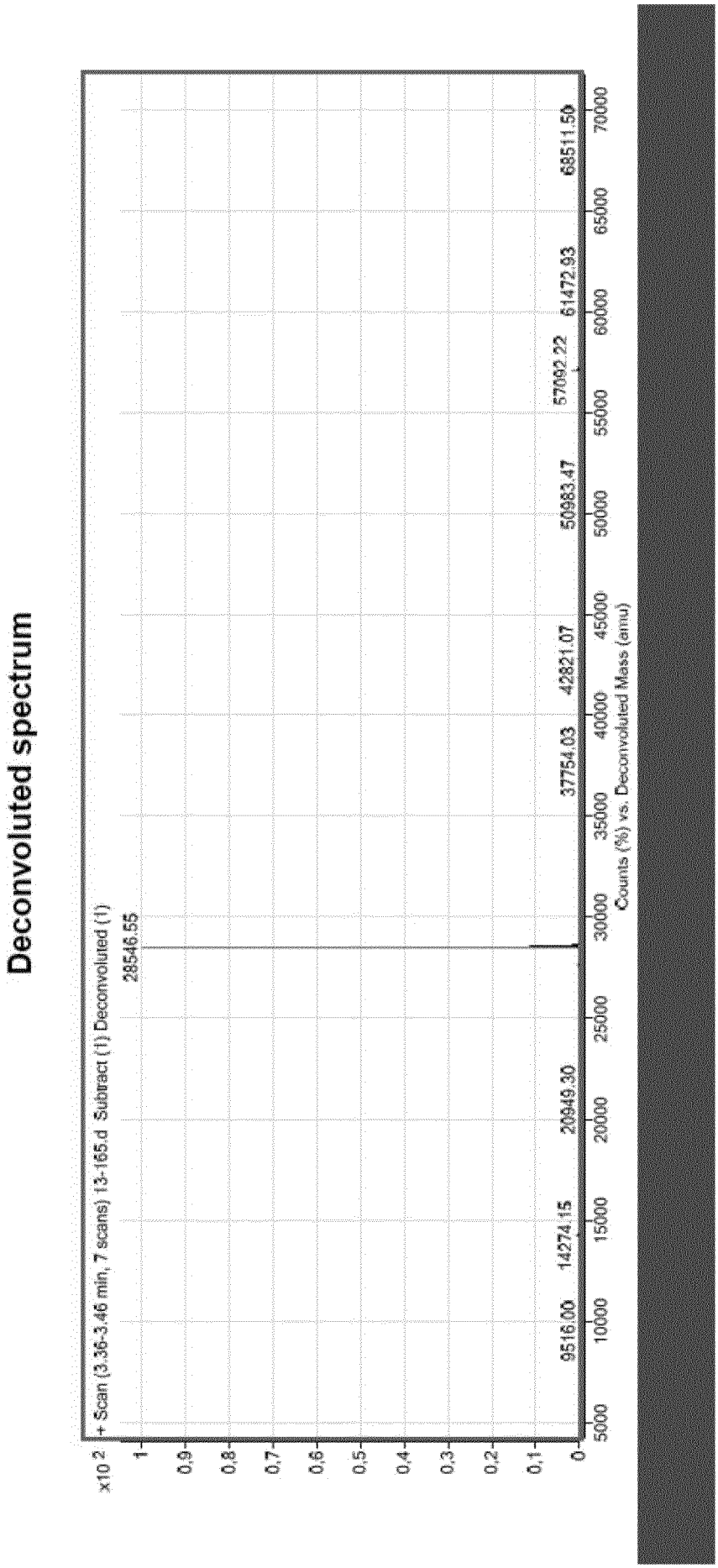

Deconvoluted spectrum
+ Scan (3.36-3.46 min, 7 scans) 13-165.d Subtract (1) Deconvoluted (1)
x10 2
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
28546.55
9516.00
14274.15
20949.30
37754.03
42821.07
50983.47
57092.22
61472.93
68511.50
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000
55000
60000
65000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

Figure 6

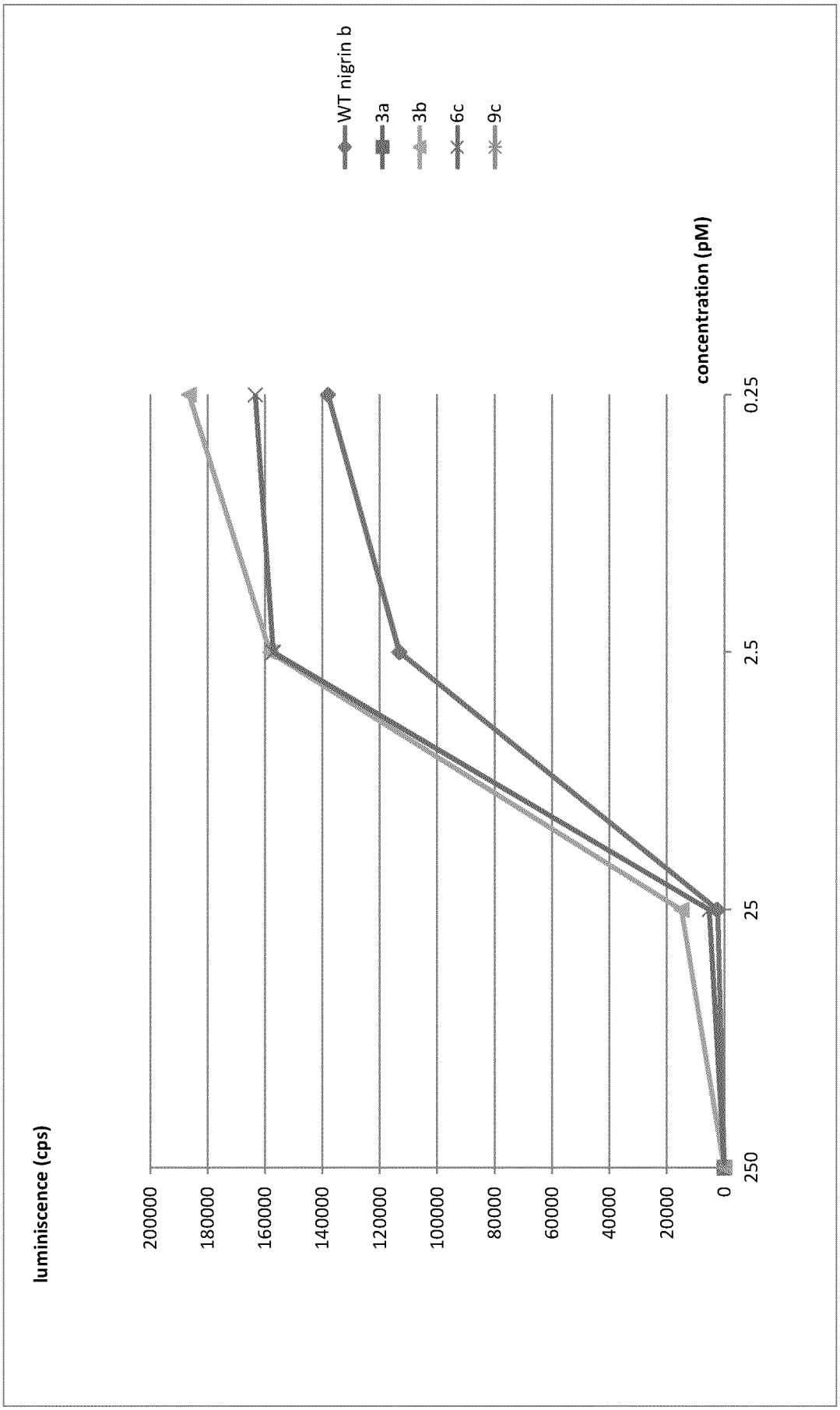

luminiscence (cps)
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
250
25
2.5
0.25
concentration (pM)
WT nigrin b
3a
3b
6c
9c

Figure 7

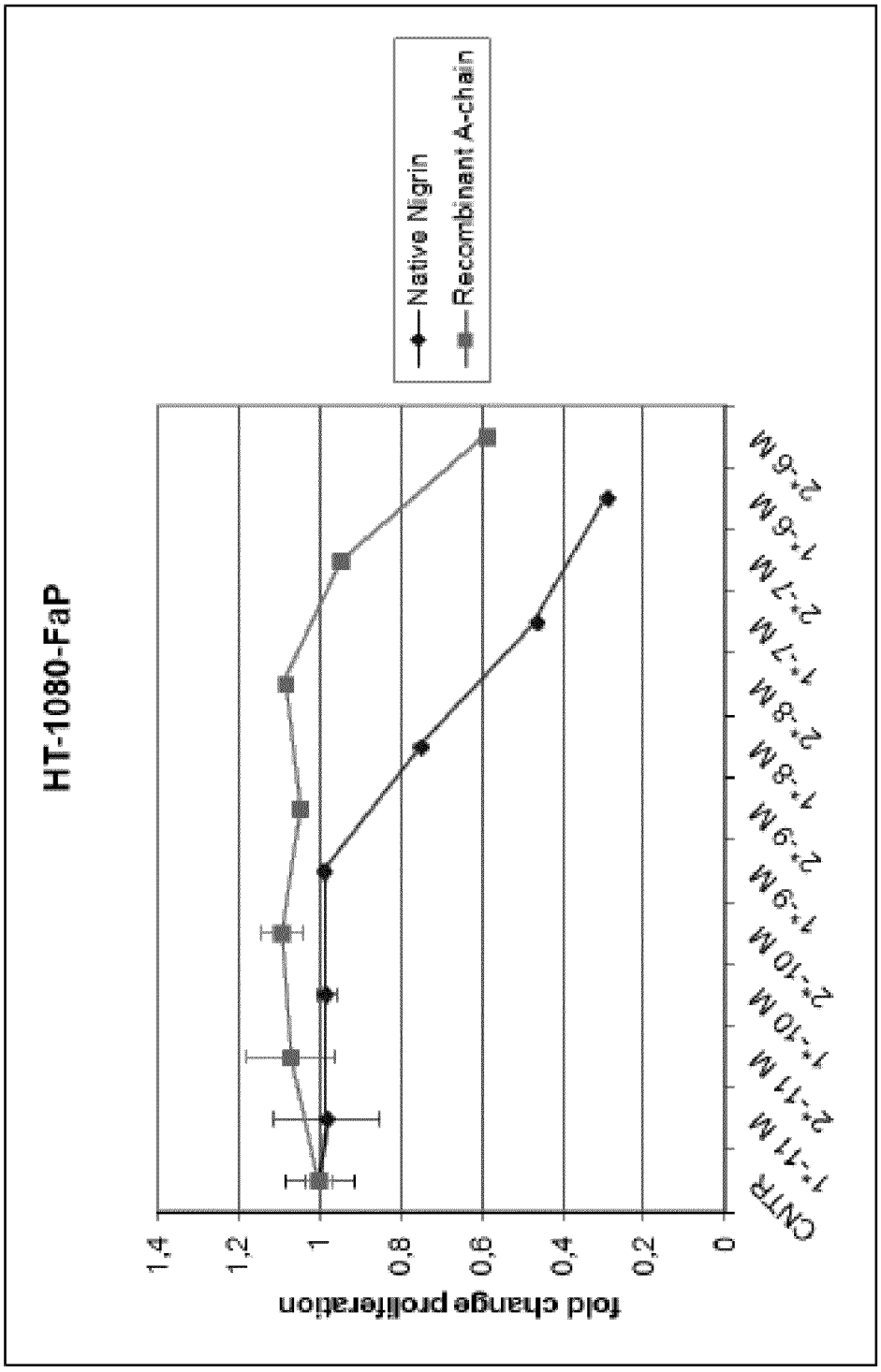

HT-1080-FaP
Native Nigrin
Recombinant A-chain
fold change proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CNTR
1-11M
2-11M
1-10M
2-10M
1-9M
2-9M
1-8M
2-8M
1-7M
2-7M
1-6M
2-6M

Figure 8

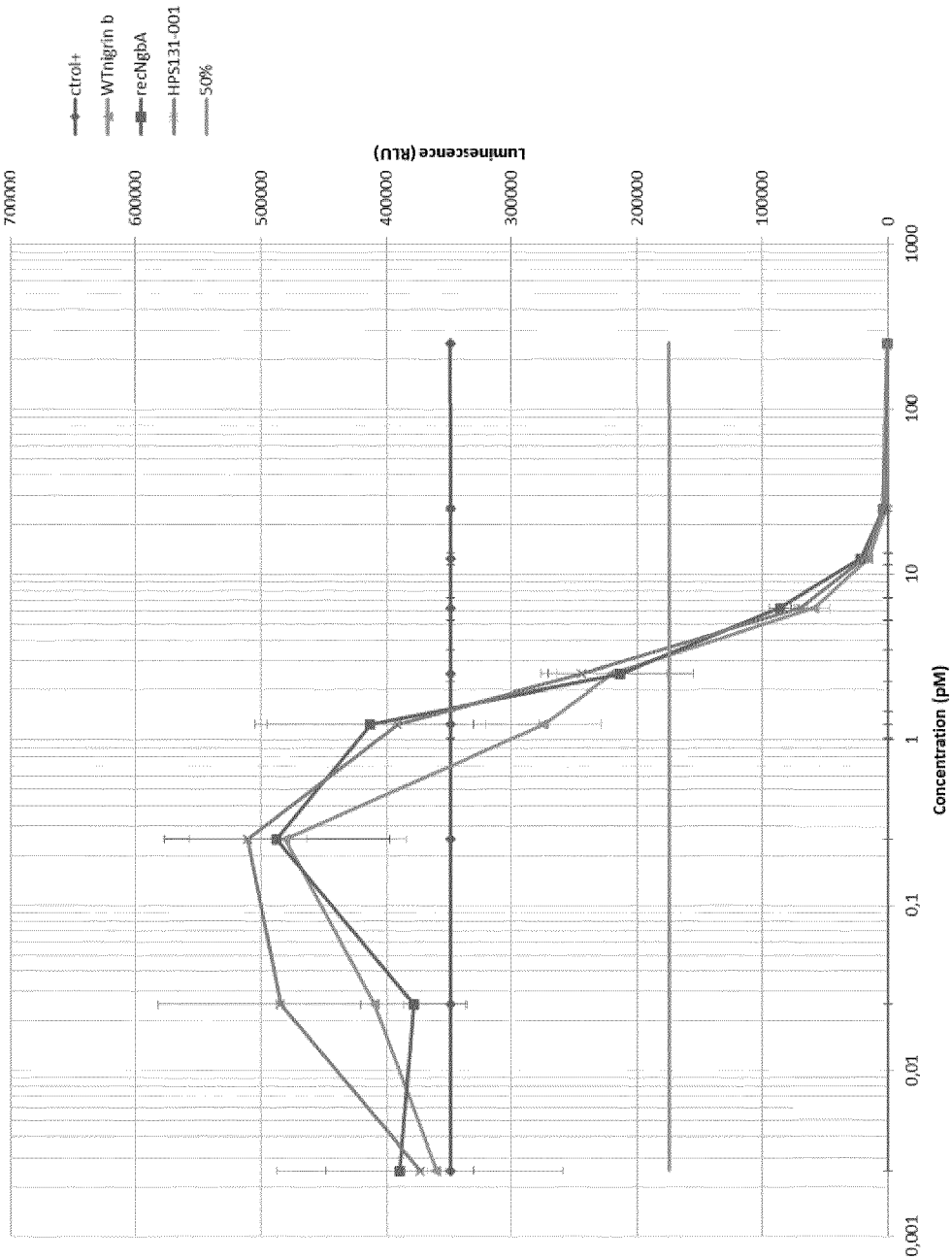

ctrol+
WTnitgrin b
recNgbA
HPS131-001
50%
Luminescence (RLU)
700000
600000
500000
400000
300000
200000
100000
0
1000
100
10
1
0,1
0,01
0,001
Concentration (pM)

Figure 9

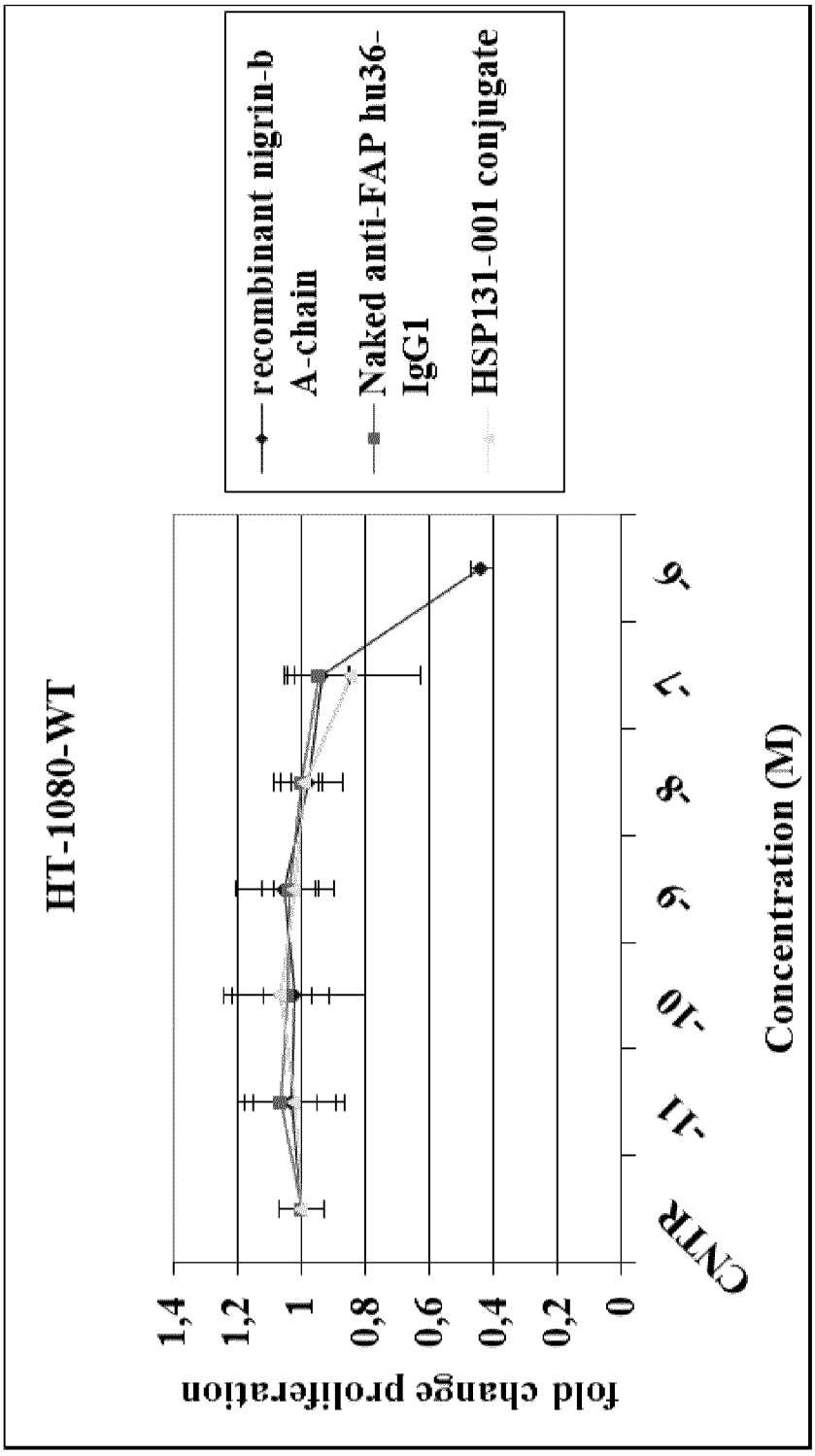

HT-1080-WT
recombinant nigrin-b A-chain
Naked anti-FAP hu36-IgG1
HSP131-001 conjugate
fold change proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CNTR
-11
-10
-9
-8
-7
-6
Concentration (M)

Figure 10A

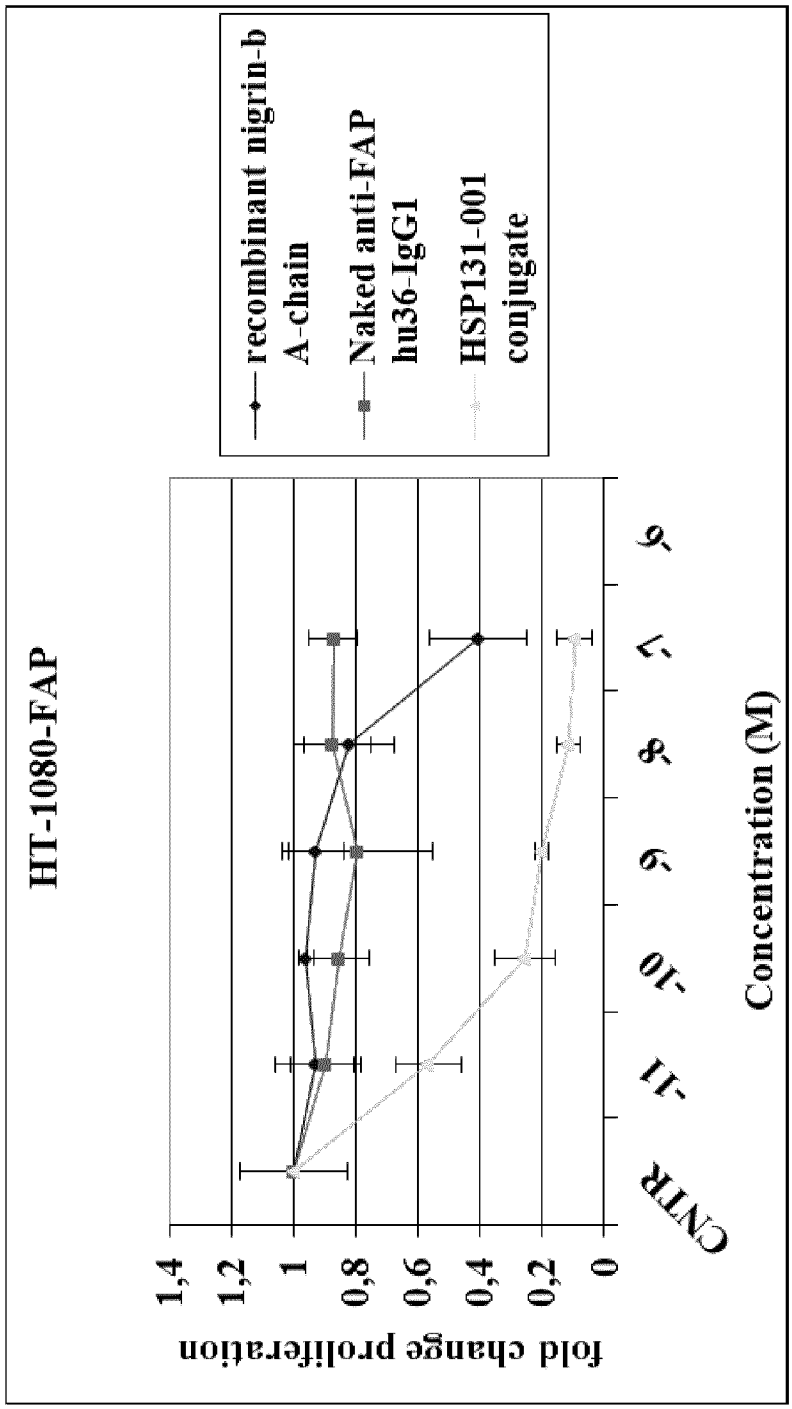

HT-1080-FAP
recombinant nigrin-b A-chain
Naked anti-FAP hu36-IgG1
HSP131-001 conjugate
fold change proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CNTR
-11
-10
-9
-8
-7
-6
Concentration (M)

Figure 10B

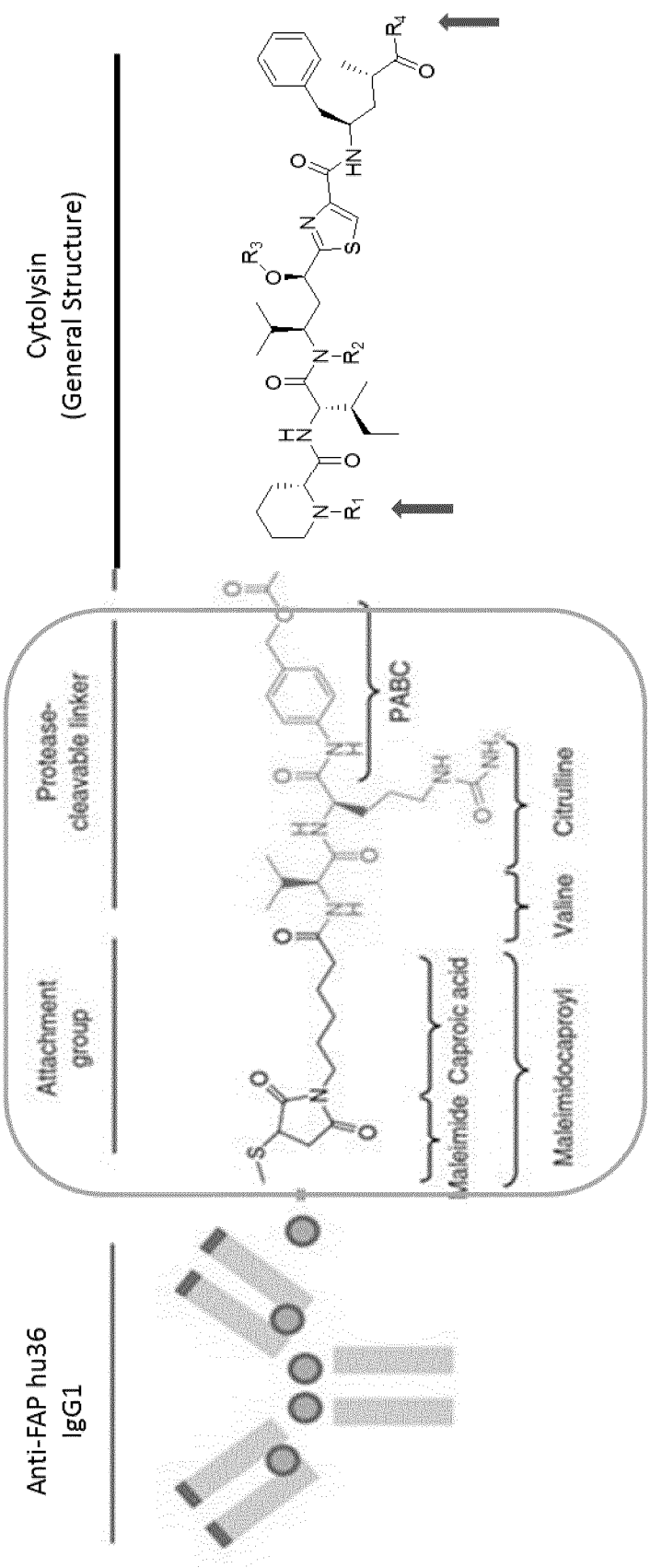

Anti-FAP hu36
IgG1
Attachment group
Protease-cleavable linker
Maleimide
Caproic acid
Maleimidocaproyl
Valine
Citrulline
PABC
Cytolysin
(General Structure)
vcPABA linker

Figure 11

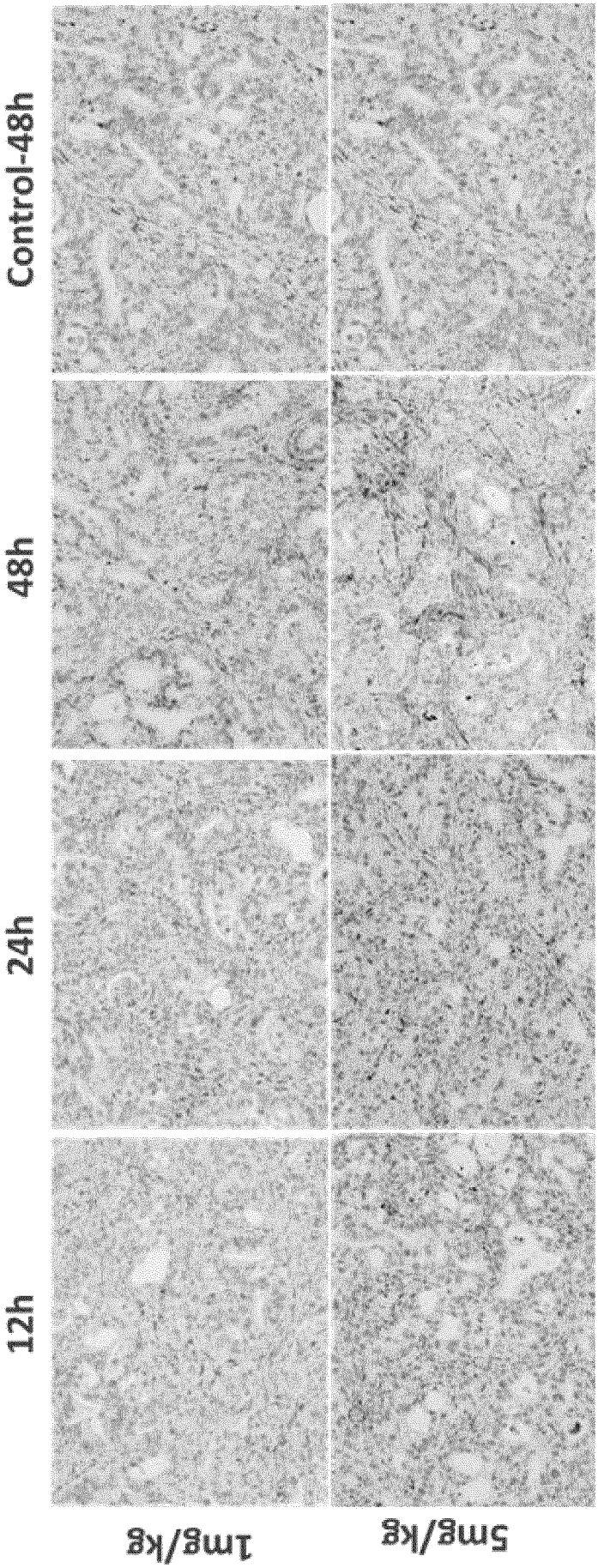


Figure 12

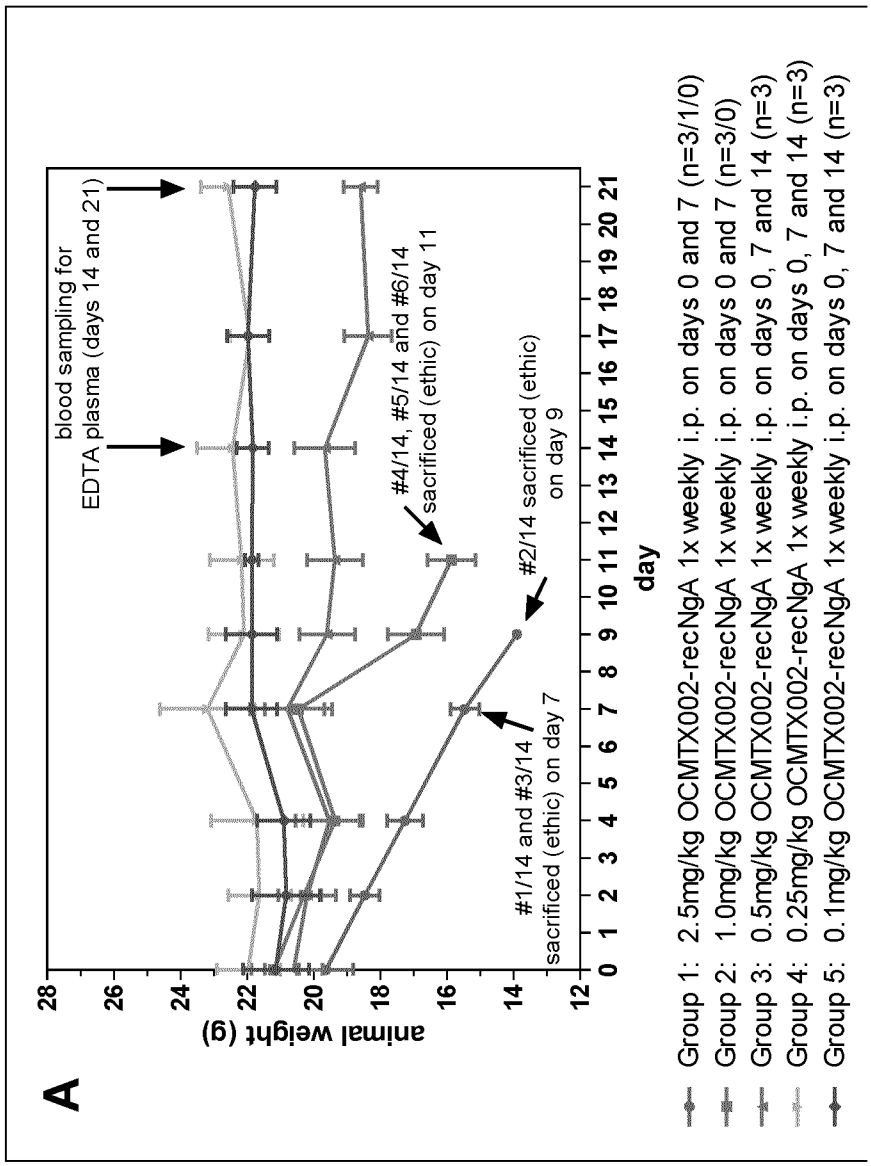

A
animal weight (g)
28
26
24
22
20
18
16
14
12
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
day
blood sampling for
EDTA plasma (days 14 and 21)
#4/14, #5/14 and #6/14
sacrificed (ethic) on day 11
#2/14 sacrificed (ethic)
on day 9
#1/14 and #3/14
sacrificed (ethic) on day 7
Group 1: 2.5mg/kg OCMTX002-recNgA 1x weekly i.p. on days 0 and 7 (n=3/1/0)
Group 2: 1.0mg/kg OCMTX002-recNgA 1x weekly i.p. on days 0 and 7 (n=3/0)
Group 3: 0.5mg/kg OCMTX002-recNgA 1x weekly i.p. on days 0, 7 and 14 (n=3)
Group 4: 0.25mg/kg OCMTX002-recNgA 1x weekly i.p. on days 0, 7 and 14 (n=3)
Group 5: 0.1mg/kg OCMTX002-recNgA 1x weekly i.p. on days 0, 7 and 14 (n=3)

Figure 13

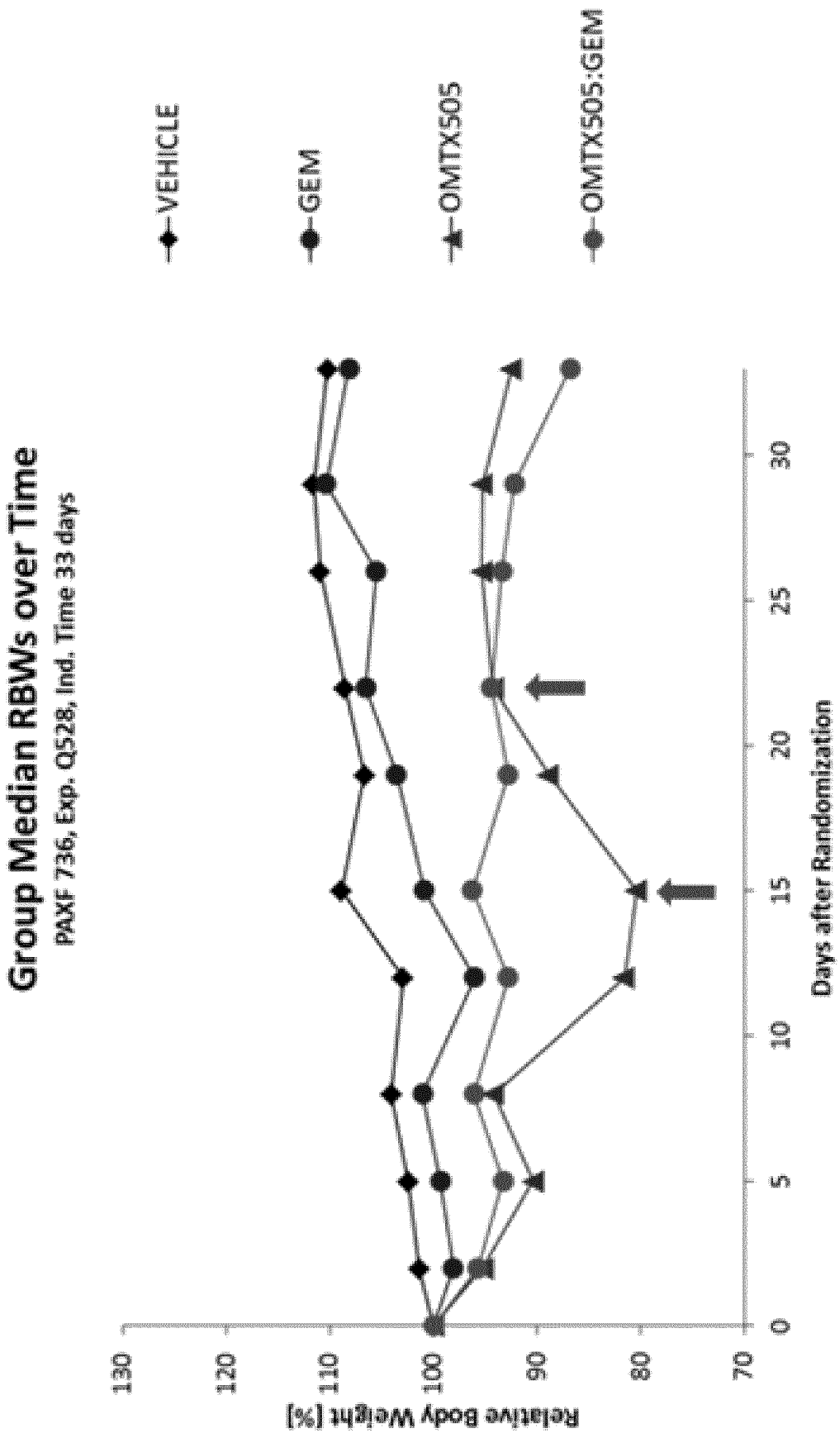

Group Median RBWs over Time
PAXF 736, Exp. Q528, Ind. Time 33 days
VEHICLE
GEM
OMTX505
OMTX505:GEM
Relative Body Weight [%]
130
120
110
100
90
80
70
0
5
10
15
20
25
30
Days after Randomization

Figure 14A

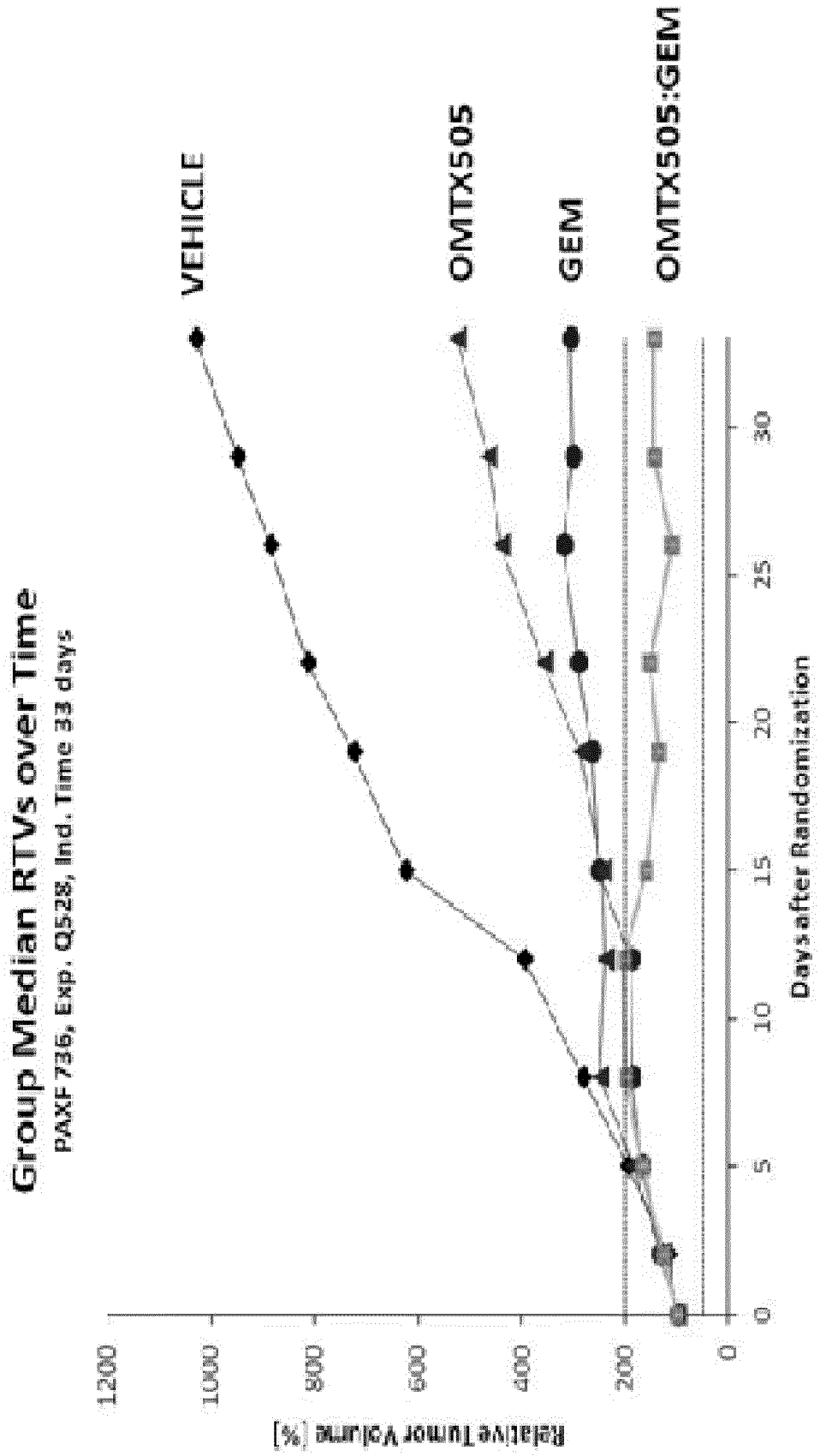
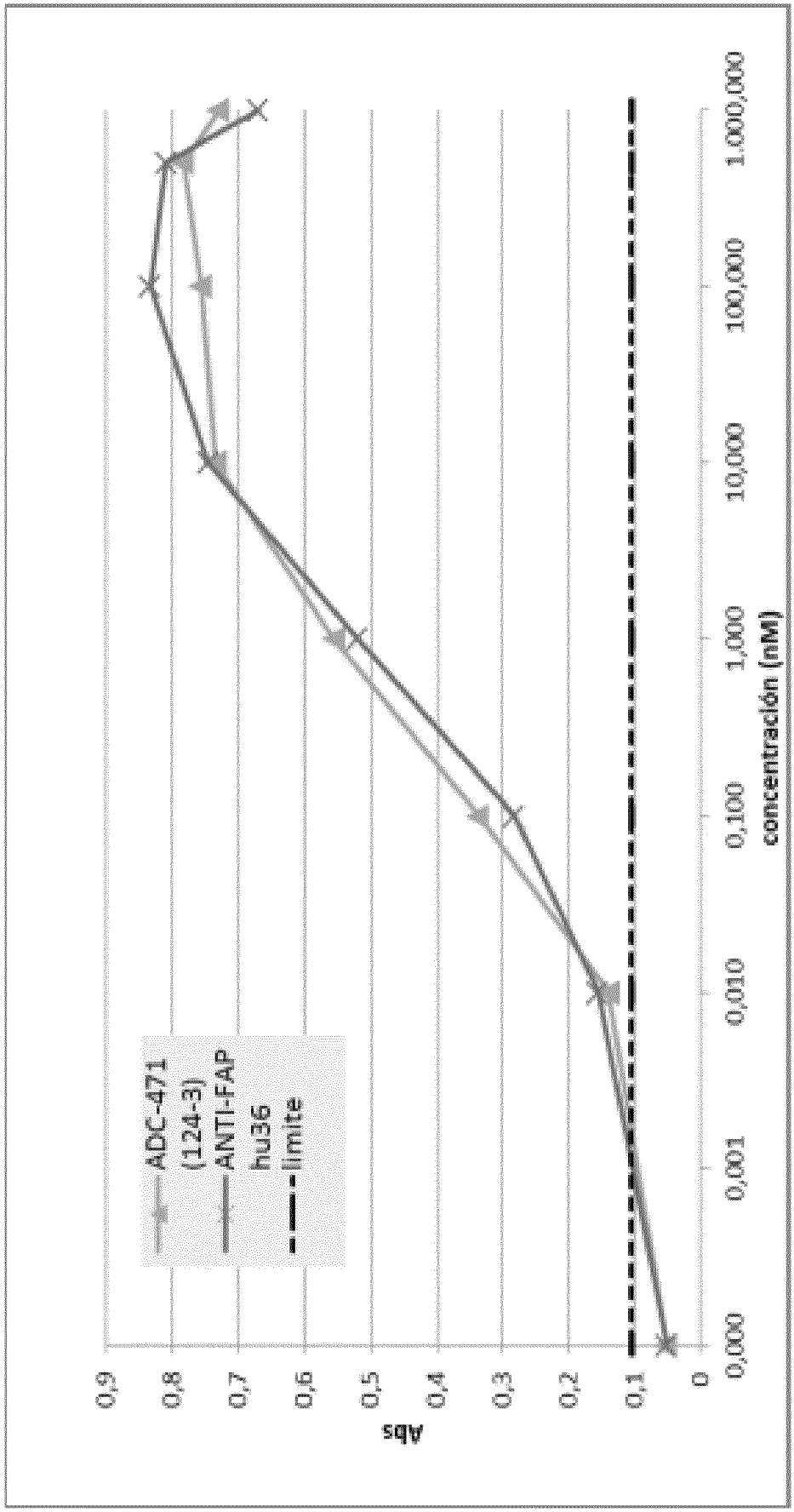
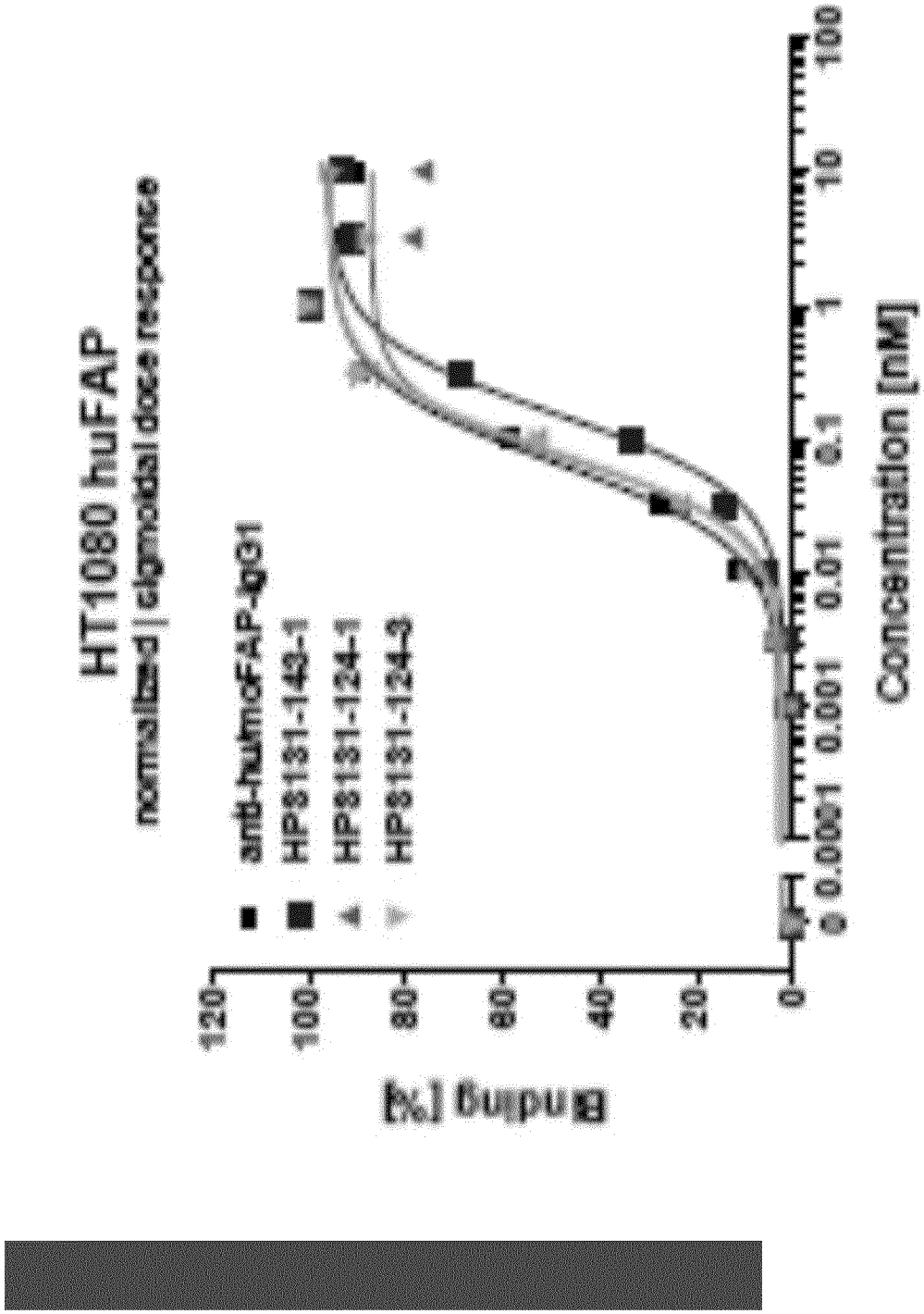

Group Median RTVs over Time
PAXF 736, Exp. Q528, Ind. Time 33 days
VEHICLE
OMTX505
GEM
OMTX505:GEM
Relative Tumor Volume [%]
Days after Randomization
0
200
400
600
800
1000
1200
0
5
10
15
20
25
30

Figure 14B



EC_{50} (nM): 0.210 (anti-FAP hu36) - 0.153 (ADC-471; HPS124-3; DAR 3.48)

Figure 15A



EC_{50} (nM): 0.067 (anti-FAP hu36) - 0.081 (ADC-471; HPS124-3; DAR 3.48)

Figure 15B

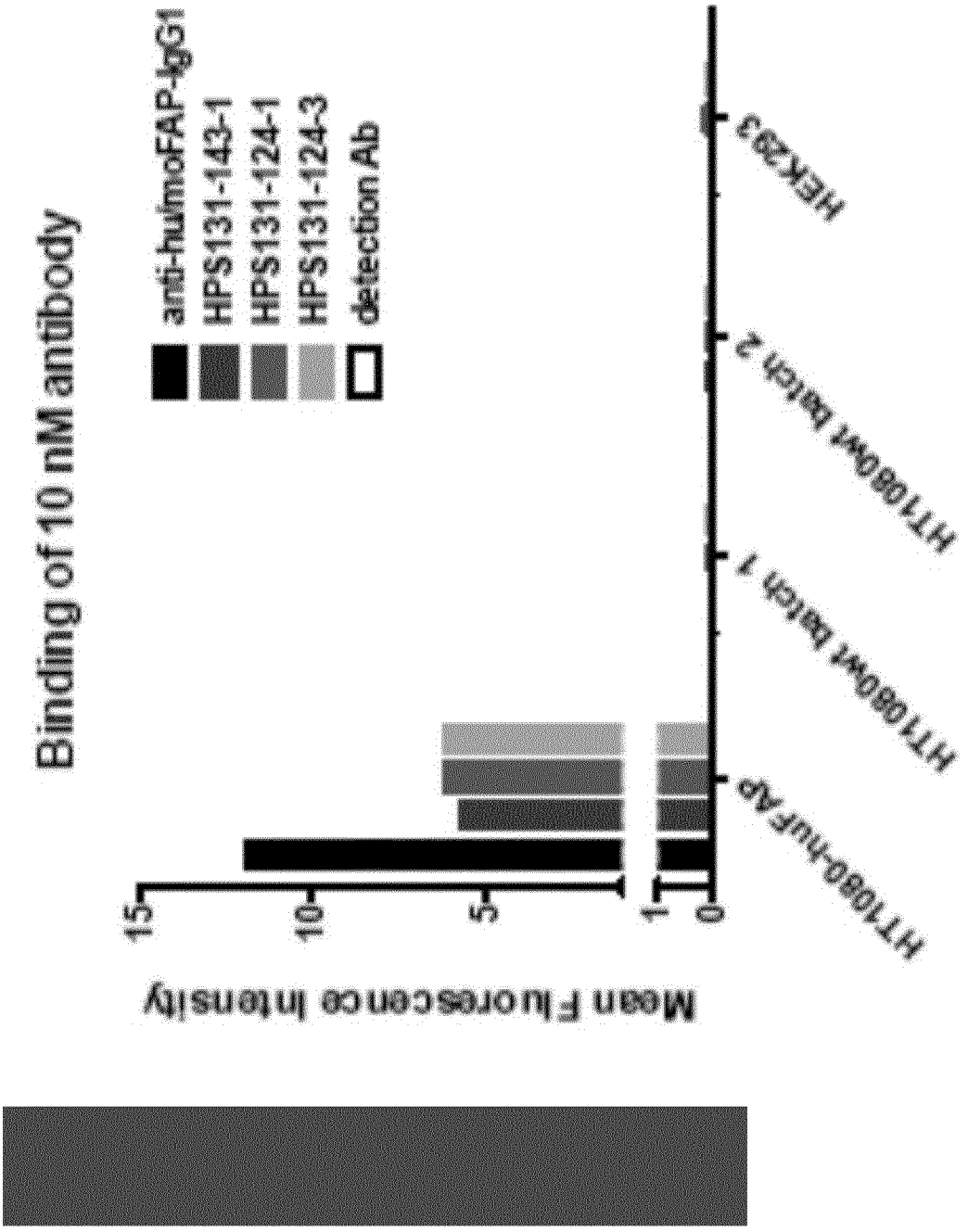

Binding of 10 nM antibody
anti-humanFAP-IgG1
HPS131-143-1
HPS131-124-1
HPS131-124-3
detection Ab
Mean Fluorescence Intensity
15
10
5
1
0
HT1080-huFAP
HT1080wt batch 1
HT1080wt batch 2
HEK293

Figure 15C

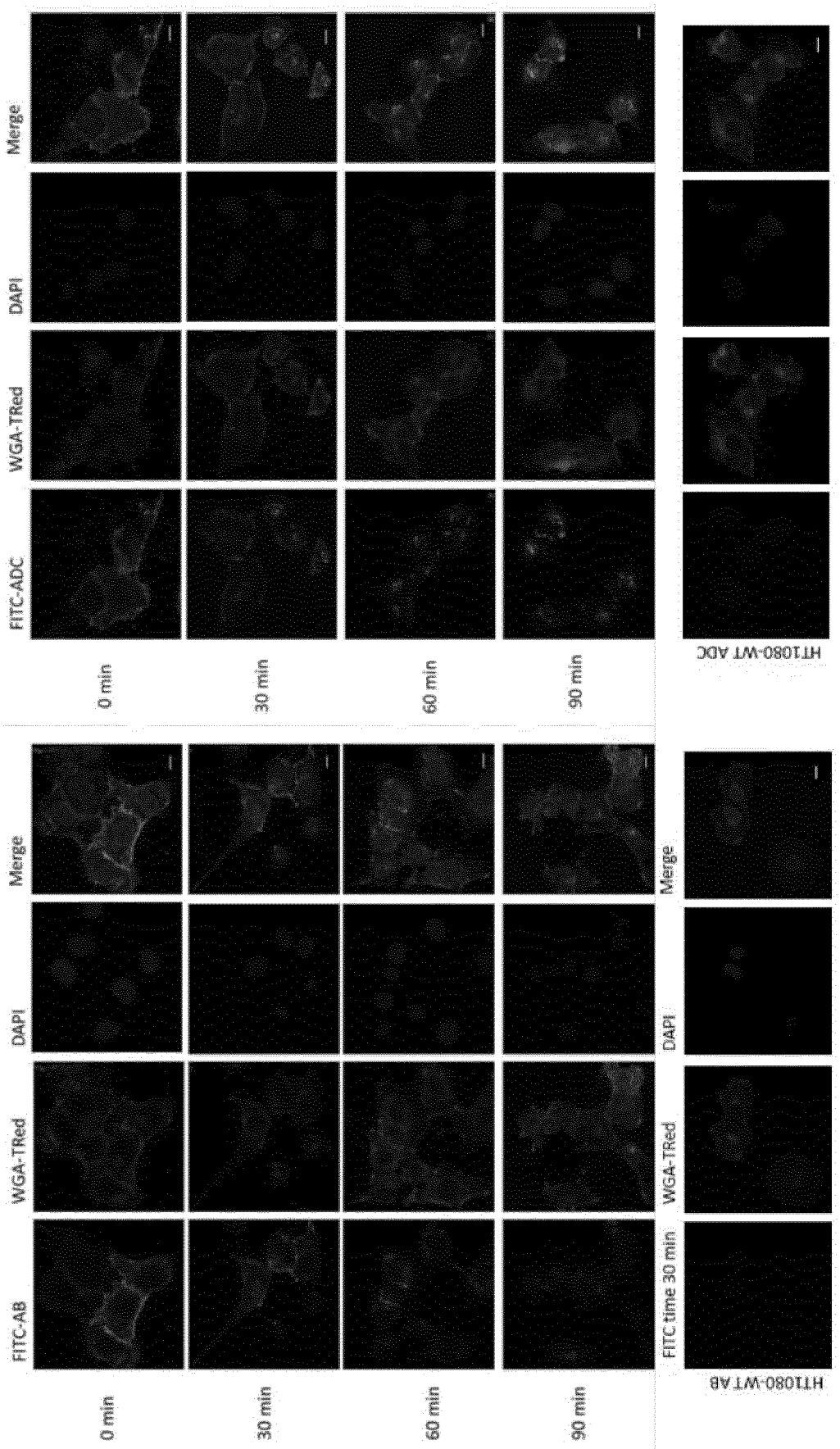

FITC-AB
WGA-TRed
DAPI
Merge
0 min
30 min
60 min
90 min
FITC time 30 min
HT1080-WT AB
FITC-ADC
HT1080-WT ADC

Figure 16

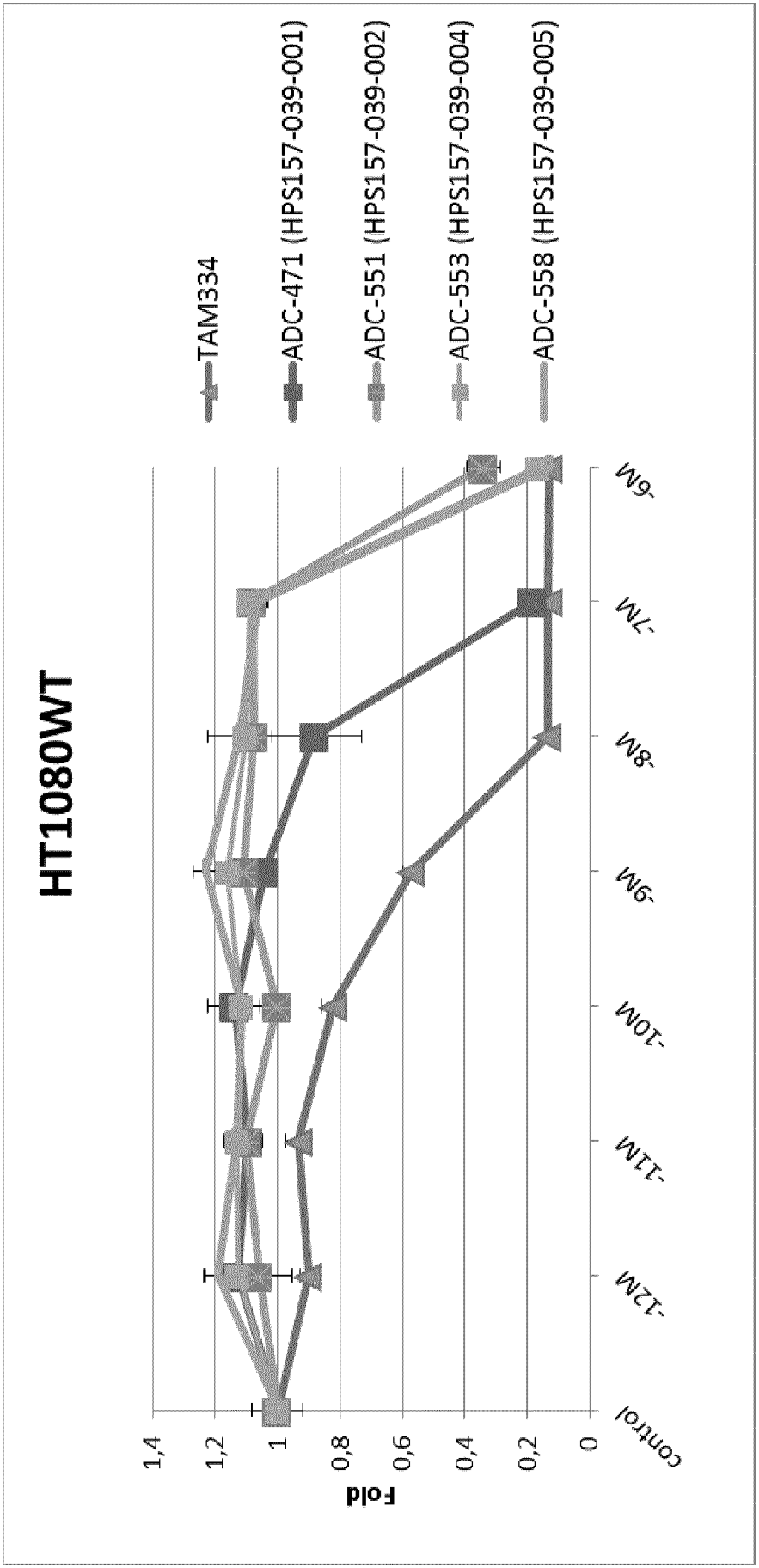

HT1080WT
TAM334
ADC-471 (HPS157-039-001)
ADC-551 (HPS157-039-002)
ADC-553 (HPS157-039-004)
ADC-558 (HPS157-039-005)
Fold
1,4
1,2
1
0,8
0,6
0,4
0,2
0
control
12M
11M
10M
9M
8M
7M
6M

Figure 17A

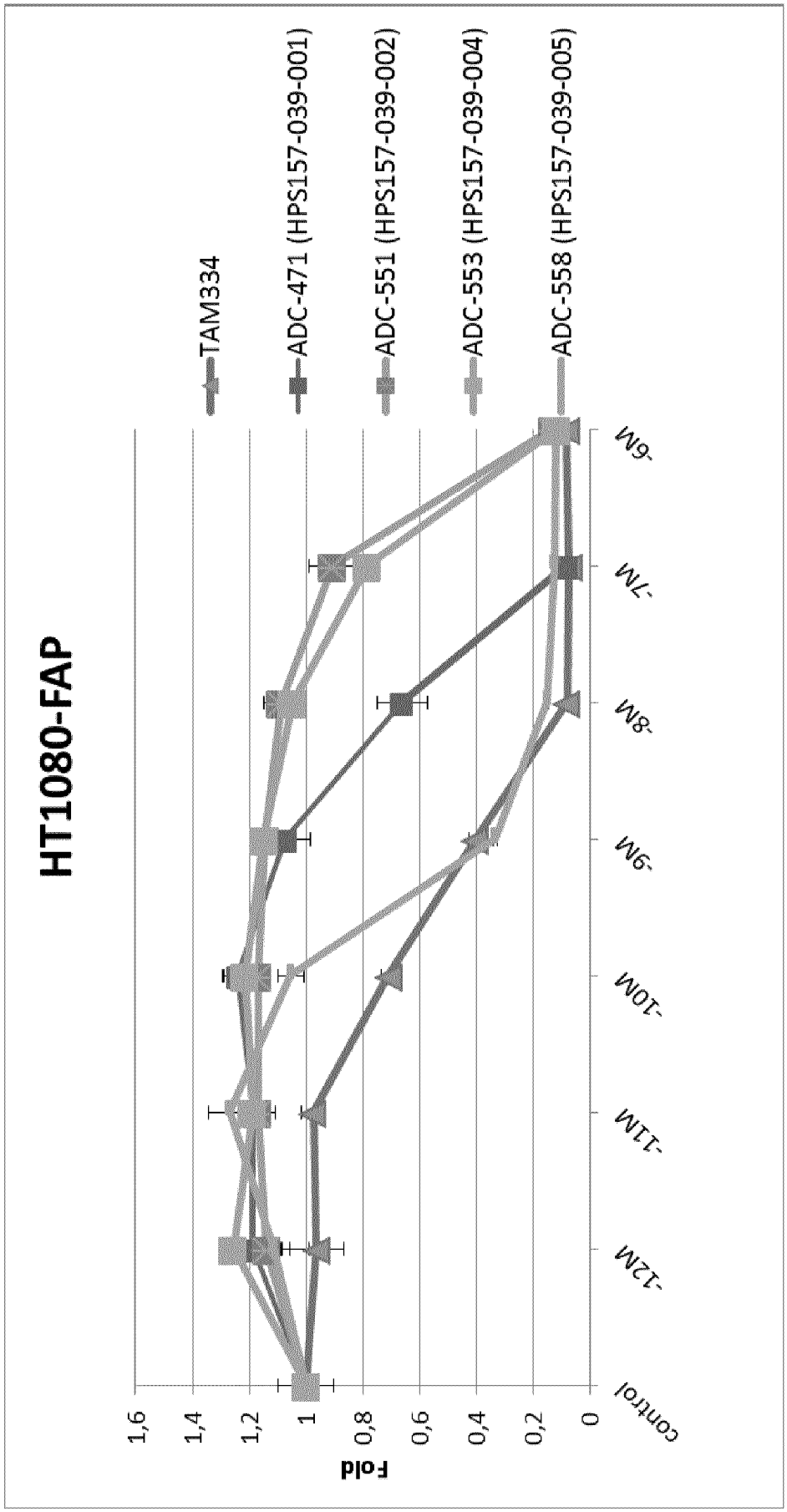

HT1080-FAP
TAM334
ADC-471 (HPS157-039-001)
ADC-551 (HPS157-039-002)
ADC-553 (HPS157-039-004)
ADC-558 (HPS157-039-005)
Fold
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
control
12M
11M
10M
9M
8M
7M
6M

Figure 17B

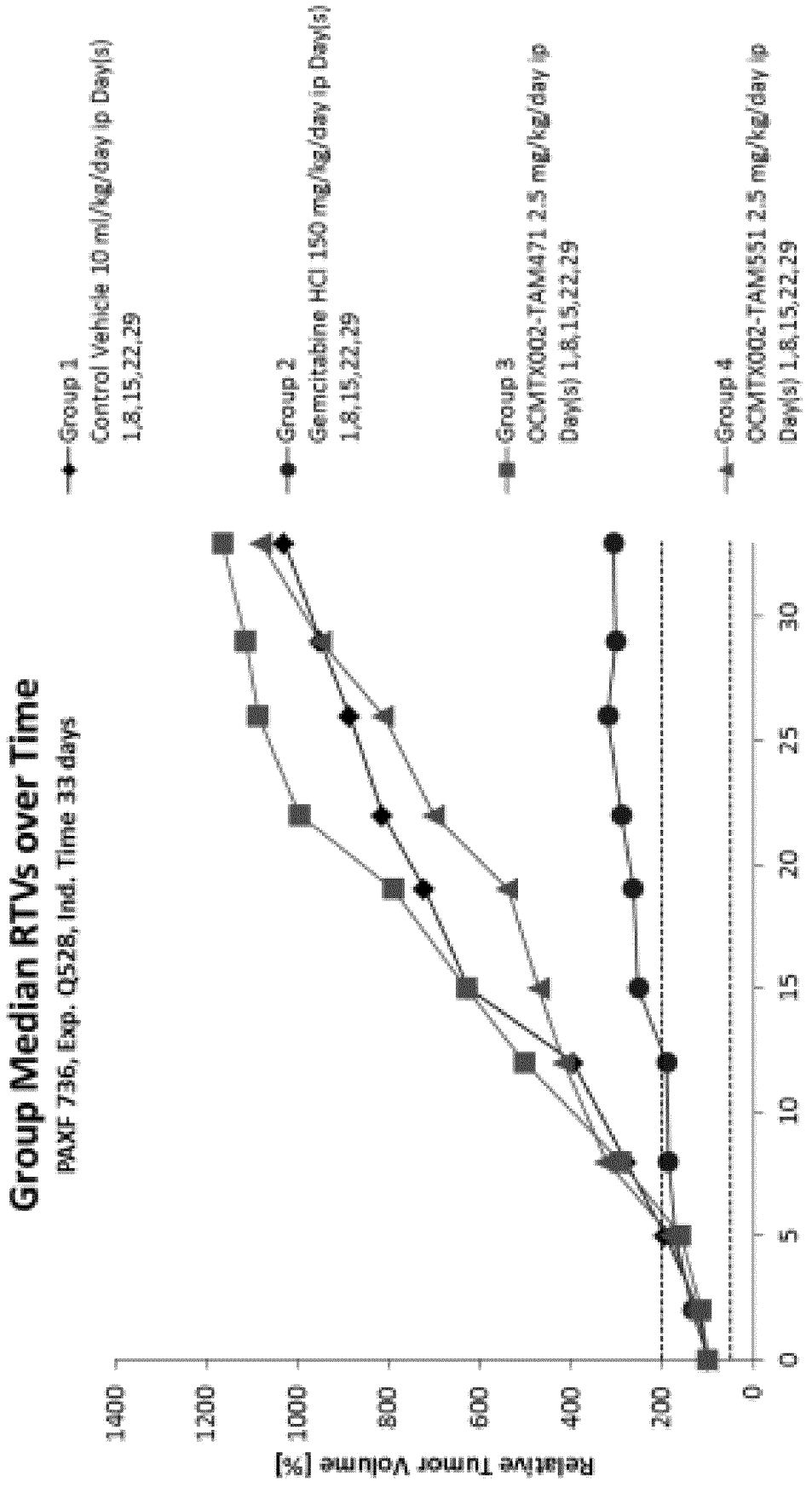

Group Median RTVs over Time
PAXF 736, Exp. Q528, Ind. Time 33 days
Group 1
Control Vehicle 10 ml/kg/day ip Day(s) 1,8,15,22,29
Group 2
Gemcitabine HCl 150 mg/kg/day ip Day(s) 1,8,15,22,29
Group 3
OOMTX002-TAM471 2.5 mg/kg/day ip Day(s) 1,8,15,22,29
Group 4
OOMTX002-TAM551 2.5 mg/kg/day ip Day(s) 1,8,15,22,29
Relative Tumor Volume [%]
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
Days after Randomization

Figure 18A

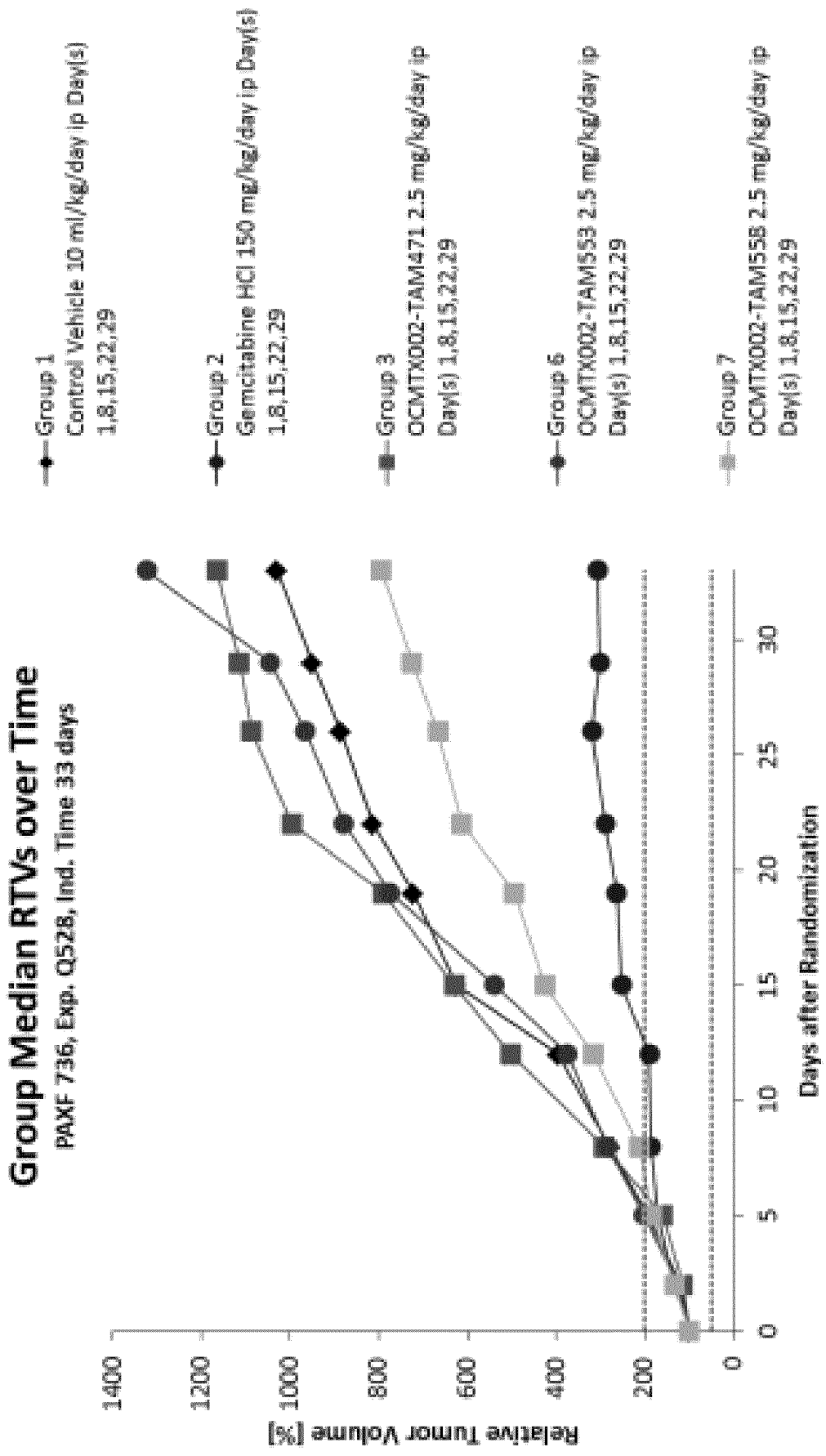

Group Median RTVs over Time
PAXF 736, Exp. Q528, Ind. Time 33 days
Relative Tumor Volume [%]
Days after Randomization
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
30
Group 1
Control Vehicle 10 ml/kg/day ip Day(s) 1,8,15,22,29
Group 2
Gemcitabine HCl 150 mg/kg/day ip Day(s) 1,8,15,22,29
Group 3
OCMTX002-TAM471 2.5 mg/kg/day ip Day(s) 1,8,15,22,29
Group 6
OCMTX002-TAM553 2.5 mg/kg/day ip Day(s) 1,8,15,22,29
Group 7
OCMTX002-TAM558 2.5 mg/kg/day ip Day(s) 1,8,15,22,29

Figure 18B