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(54) Title: ANTI-CEACAM5 ANTIBODIES AND CONJUGATES AND USES THEREOF

(57) Abstract: The invention provides antibodies which bind human CEACAM5 protein, as well as isolated nucleic acids and host cells comprising a sequence encoding said antibodies. The invention also provides immunoconjugates comprising said antibodies linked to a growth-inhibitory agent, and pharmaceutical compositions comprising antibodies or immunoconjugates of the invention. The invention also provides use of the antibodies, immunoconjugates and pharmaceutical compositions of the invention for the treatment of cancer or for diagnostic purposes.

WO 2023/170240 A1

ANTI-CEACAM5 ANTIBODIES AND CONJUGATES AND USES THEREOF

TECHNICAL FIELD

The present invention relates to antibodies which bind human CEACAM5 protein, as well as to isolated nucleic acids and host cells comprising a sequence encoding said antibodies. The invention also relates to immunoconjugates comprising said antibodies linked to a growth-inhibitory agent, and to pharmaceutical compositions comprising antibodies or immunoconjugates of the invention. The invention also relates to the use of the antibodies, immunoconjugates and pharmaceutical compositions of the invention for the treatment of cancer or for diagnostic purposes.

BACKGROUND

Carcino-embryonic antigen (CEA) is a glycoprotein involved in cell adhesion. CEA was first identified in 1965 (Gold and Freedman, J Exp Med, 121, 439, 1965) as a protein normally expressed by fetal gut during the first six months of gestation and found in many cancers such as colorectal cancer or pancreatic cancer. The CEA family belongs to the immunoglobulin superfamily. The CEA family, which consists of 18 genes, is sub-divided in two sub-groups of proteins: the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) sub-group and the pregnancy-specific glycoprotein subgroup (Kammerer & Zimmermann, BMC Biology 2010, 8:12).

In humans, the CEACAM sub-group consists of 7 members: CEACAM1, CEACAM3, CEACAM4, CEACAM5, CEACAM6, CEACAM7 and CEACAM8. CEACAM5, identical to the originally identified CEA, has been reported to be highly expressed on the surface of cancer cells such as e.g. colorectal, gastric, lung, and pancreatic tumor cells, and expression in normal tissues is limited to a few normal epithelial cells such as colon and esophagus epithelial cells. Thus, CEACAM5 may constitute a therapeutic target suitable for tumor-specific targeting approaches, such as immunoconjugates.

The extracellular domains of CEACAM family members are composed of repeated immunoglobulin-like (Ig-like) domains which have been categorized in 3 types, A, B and N, according to sequence homologies. CEACAM5 contains seven such domains, namely N, A1, B1, A2, B2, A3 and B3. CEACAM5 A1, A2 and A3 domains, on the one hand, and B1, B2 and

B3 domains, on the other hand, show high sequence homologies, the A domains of human CEACAM5 presenting from 84 to 87% pairwise sequence similarity, and the B domains from 69 to 80%. Furthermore, other human CEACAM members presenting A and/or B domains in their structure, namely CEACAM1, CEACAM6, CEACAM7 and CEACAM8, show homology with human CEACAM5. In particular, the A and B domains of human CEACAM6 protein display sequence homologies with A1 and A3 domains and any of B1 to B3 domains of human CEACAM5, respectively, which are even higher than those observed among the A domains and the B domains of human CEACAM5.

Anti-CEA antibodies have been generated for CEA-targeted diagnostic or therapeutic purposes. Specificity towards related antigens has always been mentioned as a concern in this field, e.g. by Sharkey et al (1990, Cancer Research 50, 2823). Due to the above-mentioned homologies, some of the previously described antibodies may demonstrate binding e.g. to repetitive epitopes of CEACAM5 present in the different immunoglobulin domains and show cross-reactivity to other CEACAM family members such as CEACAM1, CEACAM6, CEACAM7, or CEACAM8, thus lacking specificity for CEACAM5. Specificity of an anti-CEACAM5 antibody, however, is desired for CEA-targeted therapies, such that it binds to human CEACAM5-expressing tumor cells but does not bind to certain normal tissues expressing the other CEACAM family members. It is noteworthy that CEACAM1, CEACAM6 and CEACAM8 have been described as being expressed by neutrophils of human and non-human primates (Ebrahimmnejad et al, 2000, Exp Cell Res, 260, 365; Zhao et al, 2004, J Immunol Methods 293, 207; Strickland et al, 2009 J Pathol, 218, 380) where they have been shown to regulate granulopoiesis and to play a role in the immune response. For therapeutic purposes, cross-reactivity of an anti-CEACAM5 antibody with CEACAM1, CEACAM6, CEACAM7, or CEACAM8 may thus decrease the therapeutic index of the compound due to increased toxicity in normal tissues. Accordingly, there is a need for antibodies specifically directed to CEACAM5 that do not cross-react with other molecules of the CEACAM family, e.g. for use as part of an antibody drug conjugate (ADC) or for use in any other way resulting in killing the target cell.

Moreover, as CEACAM5 is described to be expressed in some normal cell tissues, it is desirable to develop anti-CEACAM5 antibodies capable of binding to human CEACAM5 as well as to cynomolgus monkey (*Macaca fascicularis*) CEACAM5, as such antibodies may be readily tested in preclinical toxicological studies in cynomolgus monkeys to evaluate their safety profile.

Combining the need for a) species cross-reactivity with b) the specificity for human and *Macaca fascicularis* CEACAM5, i.e. no cross reactivity with other *Macaca fascicularis* and human CEACAM family members, adds a further degree of complexity to the development of novel

anti-CEACAM5 antibodies, not least in view of the overall sequence homologies between human and *Macaca fascicularis* CEACAM proteins.

Also, CEACAM5 is described in literature as a poorly internalizing surface protein (reviewed in Schmidt et al, 2008, Cancer Immunol. Immunother. 57, 1879), presenting a further challenge for antibody drug conjugates directed to this target protein.

Known anti-CEACAM5 antibodies include Immunomedics' labetuzumab (also known as hMN14; Sharkey et al, 1995, Cancer Research 55, 5935). This antibody has been shown not to bind to related antigens, but is also not cross-reacting with CEACAM5 from *Macaca fascicularis*. Labetuzumab has also been used as part of an antibody-drug conjugate (ADC), namely as labetuzumab govitecan. Labetuzumab govitecan is an ADC composed of the cytotoxic drug SN38 conjugated to the anti-CEACAM5 antibody labetuzumab via a linker (called CL2A) comprising a pH-sensitive carbonate and a short polyethylene glycol (PEG) chain. Labetuzumab govitecan is characterized by a significant instability of the linker structure used, resulting in an early systemic loss of the cytotoxic payload after parenteral application. This degradation process might limit antitumor activity and increases risks of side effects. Another known anti-CEACAM5 ADC is Sanofi's SAR408701 (tusamitamab ravtansine), comprising the anti-CEACAM5 antibody SAR408377 (tusamitamab; also referred to as huMab2-3) covalently linked to the cytotoxic agent DM4, a potent microtubule-destabilizing maytansinoid, via an *N*-succinimidyl 4-(2-pyridyldithio) butyrate (SPDB) linker. SAR408701 is associated with toxic side effects on several organs and tissues including the cornea of the eye (including keratitis and keratopathy). Also, effectiveness of microtubule inhibitor-based ADCs may be limited in certain cancer indications such as colorectal cancer. To date, no anti-CEACAM5 antibody or ADC has been approved for any therapeutic use in the clinic; in general, few ADCs have been approved for the treatment of solid tumors.

Some ADCs might be dose-limited in patients because of side effects of released payload by cellular catabolism, resulting in toxicities in the bone marrow and circulating blood cells (e.g. neutropenia, reticulocytopenia, lymphopenia).

Because of the drug coupling to the antibody, ADCs frequently have a suboptimal half-life in humans of only a few days in circulation, which is significantly lower compared to the half-life of the corresponding unconjugated antibodies. The relatively high clearance of these ADCs is related to cellular degradation after target-independent uptake, and leads to substantial release of toxic drug payload which can trigger side effects. Thus, it is an objective of the ADCs disclosed herein to improve their half-life in order to reduce unwanted side-effects.

In view of the above, there remains a need for new and improved therapeutic agents for the treatment of cancer, e.g. for different solid tumor indications including e.g. CRC, pancreatic cancer, gastric cancer, NSCLC, esophageal cancer and prostate cancer.

SUMMARY OF THE INVENTION

The present invention addresses this need and other needs in the art *inter alia* by providing monoclonal antibodies directed against CEACAM5 (reactive with both the human and *Macaca fascicularis* proteins) and by providing immunoconjugates (also referred to as antibody-drug conjugates (ADC) herein) comprising said antibodies; these immunoconjugates have a cytotoxic effect, killing tumor cells *in vitro* and inhibiting tumor growth *in vivo*. The present invention relates to embodiments described in the claims as well as in the further description herein below.

In an attempt to produce new antibodies against CEACAM5 with optimal characteristics for therapeutic purposes, particularly in the format of an immunoconjugate, the inventors have performed extensive research and development, in order to select antibodies with an advantageous profile and to develop immunoconjugates on that basis.

The inventors were able to select and produce optimized IgGs that unexpectedly comprise several desired features. These antibodies bind to the A2-B2 domain of human CEACAM5 with a high affinity and do not recognize human CEACAM1, CEACAM6, CEACAM7 and CEACAM8 proteins. In a cellular context, these antibodies display high affinity for CEACAM5-expressing tumor cells and are internalized. Moreover, these antibodies also bind to *Macaca fascicularis* CEACAM5 protein, with affinities to the monkey and human proteins, within 10-fold of each other. Antibodies of the invention bind to the A2-B2 domain of *Macaca fascicularis* CEACAM5 but they do not recognize another *Macaca fascicularis* CEACAM protein, CEACAM6.

The inventors have also shown that the antibodies they have produced are able to induce cytotoxic effects on tumor cells *in vitro* when combined with a cytotoxic drug in an immunoconjugate. The antibodies conjugated to a cytotoxic drug (i.e. immunoconjugates of the invention) are also able to markedly inhibit tumor growth in mice bearing CEACAM5-expressing tumors. The linkers connecting drug and antibody were designed to maximize systemic stability after parenteral application. The release of exatecan from the immunoconjugates of the invention within target cells leads to very high potency and outstanding bystander effects. A potent bystander effect may be beneficial for the treatment of patients with heterogeneous target expression.

Generally, a high systemic exposure is desired. High systemic exposure of an ADC drug leads to a more effective tumor targeting and an improved cytotoxic payload disposition in tumor tissues and cell, and finally in an enhanced tumor cell killing compared to compounds with lower systemic exposure.

Furthermore, the inventive antibody-drug-conjugates are improved by including molecular modifications to reduce target-independent, cellular degradation leading to molecules with lower clearance, higher systemic exposure and reduced payload release.

The present invention relates to antibody modifications and payload conjugation strategies which significantly reduce the off-target cellular catabolism of such ADCs, thereby reducing the levels of released payload while improving the efficacy driven by higher ADC exposure. Therefore, these modifications will provide drugs with an improved therapeutic window by reduction of side effects and increase of antitumor activity.

As described herein, the exposure and half-life of the ADCs according to the invention will be improved for example

(1) by modification of the antibody heavy-chain by including YTE mutations and linking it to the drug using interchain cysteine conjugation or

(2) by modification of the antibody heavy-chain with YTE mutations and using an innovative enzymatic conjugation method that uses a microbial transglutaminase (herein also referred to as “TGase” and “mTG”) enzyme to attach the drug to the antibody

Surprisingly, high exposure leading to low clearance values in human predictive animal models have been obtained by approach (2) above. With such modifications the exposure of ADCs has been improved by ca. factor 10x compared to variants not comprising these modifications, which consequently results in a significant reduction of released payload levels and a significant increase of antitumor active ADC concentrations in circulation. The improved ADCs resembled the PK profile of the original YTE antibodies not comprising a drug linker and demonstrate an improved half-life over prior art ADCs.

These results will reduce toxicities and improve potencies in clinical cancer therapy compared to prior art molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Binding of mAb1 to recombinant human (rh) CEACAM5 ECD or its domains N-A1-B1, A2-B2, A3-B3 or to recombinant Macaca fascicularis (mf) CEACAM5 ECD in an ELISA assay.

Fig. 2: EC50 of anti CEACAM5 antibodies binding to MKN-45 cells: Cellular binding of mAb1 compared to antibodies huMab2-3 and hmn-14 on MKN45 cell line which expresses CEACAM5.

Fig. 3: Internalization of pHrodo labeled antibodies into the late endosomes and lysosomes of cells (sum fluorescence intensity per cell, average of triplicates).

Fig. 4: Fluorescence intensity per cell from time of 700 minutes to 1200 minutes, which is the linear part of the curve. Linear slope was measured and compared between samples (see Example 1.6.5).

Fig. 5: IHC staining with antibody rb8G4 on FFPE cancer cell lines.

Fig. 6: Correlation of CEACAM5 mRNA expression and IHC staining for 104 cancer cell lines.

Fig. 7: IHC staining with the antibody rb8G4 on normal human tissue.

Fig. 8: CEACAM5 mRNA expression in normal human tissues.

Fig. 9: IHC staining with the antibody rb8G4 on human colorectal cancer tissue.

Fig. 10: IHC staining with the antibody rb8G4 on human gastric cancer tissue.

Fig. 11: IHC staining with the antibody rb8G4 on human esophageal cancer tissue.

Fig. 12: IHC staining with the antibody rb8G4 on human non-small cell lung cancer tissue.

Fig. 13: Binding of mAb1 (Fig. 13A) and rb8G4 (Fig. 13B) to CEACAM5 in cancer cell line lysates investigated by Western Blots.

Fig. 14: Typical SEC chromatogram showing the purity of the stock mAb, the conjugate post UF and the final bulk drug substance (BDS).

Fig. 15: Typical RP-HPLC chromatogram showing the separation of light and heavy chains. The chromatogram shows an overlay of the stock mAb, the crude ADC and the final BDS.

Fig. 16: Typical chromatogram showing the NAC standard and the free-drug levels of the final BDS.

Fig. 17: Typical SEC chromatogram showing the purity of the stock mAb and the final BDS.

Fig. 18: Typical RP-HPLC chromatogram showing the separation of light and heavy chains. The chromatogram shows an overlay of the stock mAb and the final BDS.

Fig. 19: Typical chromatogram showing the NAC standard and the free-drug levels of the final BDS.

Fig. 20: ADC stability for human, mouse and cynomolgus sera. Conjugated Exatecan concentrations were calculated (initial dose ~10 µM) using free Exatecan (normalized data).

Fig. 21: ADC3 control stability for mouse serum and buffer. Conjugated SN38 concentrations were calculated (initial dose 50 µg/mL ADC protein concentration) using free SN38 (not normalized).

Fig. 22: Payload liberation profiles for ADC1 and ADC2 in human liver lysosomes (pH 5.0). Conjugated drug concentrations were calculated using e.g. free Exatecan (initial conc. ~10 µM Exatecan), normalized data.

Fig. 23: ADC catabolite profiling confirms free exatecan as lysosomal release product.

Fig. 24: *In vitro* potency of ADC1, ADC2 and free payload against antigen-positive SK-CO-1 (Fig. 24A) and SNU-16 (Fig. 24B) cell lines in comparison to antigen-negative MDA-MB-231 (Fig. 24C) cell line. One representative experiment is shown, mean of triplicates ±SD. The legend assigning the three different series of data points to ADC1, ADC2 and payload, respectively, as shown in Fig. 24C, also applies to Fig. 24A and Fig. 24B.

Fig. 25: Comparison of ADC1 and ADC2 with respective isotype controls on SK-CO-1 cell line. One representative experiment is shown, mean of triplicates ±SD.

Fig. 26: *In vitro* potency of ADC1, ADC2, ADC SAR DM4, ADC mAb1 DM4 and free payloads against antigen-positive SK-CO-1 (Fig. 26A) in comparison to antigen-negative MDA-MB-231 (Fig. 26B) cell line. One representative experiment is shown, mean of triplicates ±SD; legend shown in Fig. 26B also applies to Fig. 26A.

Fig. 27: Potent bystander effect of ADC1 and ADC2 on antigen-negative MDA-MB-231 cells in co-culture with antigen-positive SK-CO-1 cells (Fig. 27A). No unspecific effects of ADC1 or ADC2 on MDA-MB-231 cells alone (Fig. 27B). One representative experiment is shown, mean of duplicates ±SD.

Fig. 28: Bystander effect of ADC1 and ADC2 on antigen-negative MDA-MB-231 cells in co-culture with antigen-positive SK-CO-1 cells is more potent than for ADC SAR DM4 (Fig. 28A and Fig. 28B). No non-specific effects of tested ADCs on MDA-MB-231 cells alone (Fig. 28C). One representative experiment is shown, mean of duplicates ±SD.

Fig. 29: Efficacy of ADC1 and ADC2 in a CRC PDX model (COPF217) after single treatment.

Fig. 30: Efficacy of ADC1 in a NSCLC PDX model (LUPF160151) after single treatment.

Fig. 31: Efficacy of ADC1 in a gastric cancer PDX model (GAX066) after single treatment

Fig. 32: Efficacy of ADC1 compared to ADC3 in a pancreatic xenograft model (HPAF-II).

Fig. 33: Efficacy of ADC1 compared to ADC SAR DM4 in a CRC PDX model (COPF230)

Fig. 34: Efficacy of ADC1 compared to ADC SAR DM4 in a CRC PDX model (REPF210)

Fig. 35: Efficacy of ADC1 compared to ADC SAR DM4 in a gastric PDX model, GAPF313 (interim analysis of an ongoing experiment)

Fig. 36: Typical SEC chromatograms showing the purity of the input mAb and the final BDS for mAb1-M / ADC1-M

Fig. 37: Typical RP-HPLC chromatograms illustrating DAR determination of the final BDS for mAb1-M / ADC1-M

Fig. 38: Typical SEC chromatograms showing the purity of the input mAb and the final BDS for ADC7-M, ADC2-M and ADC5-M

Fig. 39: Typical RP-HPLC chromatograms illustrating DAR determination of the final BDS for ADC7-M, ADC2-M and ADC5-M

Fig. 40: *In vitro* potency of ADC1-M, ADC4-M and free payload against antigen-positive SK-CO-1 **(Fig. 40a)**, SNU-16 **(Fig. 40b)**, MKN-45 **(Fig. 40c)** and LS174T **(Fig. 40d)** cell lines in comparison to antigen-negative MDA-MB-231 **(Fig. 40e)** cell line. One representative experiment is shown, mean of duplicates ±SD.

Fig. 41: *In vitro* potency of ADC2-M, ADC5-M and free payload against antigen-positive SK-CO-1 **(Fig. 41a)**, SNU-16 **(Fig. 41b)**, MKN-45 **(Fig. 41c)** and LS174T **(Fig. 41d)** cell lines in comparison to antigen-negative MDA-MB-231 **(Fig. 41e)** cell line. One representative experiment is shown, mean of duplicates ±SD.

Fig. 42 Pharmacokinetic profile (total antibody) in huFcRn Tg276 mice for ADC1, ADC1-M, ADC2-M, ADC6-M and ADC7-M.

Fig. 43 Tumor volume changes after treatment with ADC1-M and ADC2-M versus vehicle control.

Fig. 44 More potent bystander effect of ADC1-M, ADC2-M, ADC6-M and ADC7-M compared with ADC SAR DM4 on antigen-negative MDA-MB-231 cells in co-culture with antigen-positive SK-CO-1 cells. One representative experiment is shown, mean of duplicates ±SD.

Fig. 45 Efficacy of ADC1-M and ADC3-M compared to ADC8 in an HPAF-II xenograft model.

Fig. 46 Efficacy of ADC1-M, ADC3-M in comparison to ADC SAR DM4 in a CRC PDX model (COPF230) after single treatment.

Fig. 47 Efficacy of ADC1-M, ADC3-M in comparison to ADC SAR DM4 in a GC PDX model (GAPF313) after multiple treatment.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein "CEACAM5" designates the "carcino-embryonic antigen-related cell adhesion molecule 5", also known as "CD66e" (Cluster of Differentiation 66e) . CEACAM5 is a glycoprotein involved in cell adhesion. CEACAM5 is highly expressed especially on the surface of e.g. colorectal cancer, gastric cancer, non-small cell lung cancer, pancreatic cancer, esophageal cancer, prostate cancer and other solid tumors. A reference sequence of full length human CEACAM5, including signal peptide (positions 1-34) and propeptide (positions 686-702), is available from the GenBank database under accession number AAA51967.1; this amino acid sequence reads as follows: MESPSAPPHRWCIPWQRLLLTASLLTFWNPPTTAKLTIESTPFNVAEGKEVLLLVHNLPQHL FGYSWYKGERVDGNRQIIGYVIGTQQATPGPAYSGREIIYPNASLLIQNIIQNDTGFYTLHVIK SDLVNEEATGQFRVYPELPKPSISSNNSKPVEDKDAVAFTCEPETQDATYLWWVNNQSLPV SPRLQLSNGNRTLTLFNVTRNDTASYKCETQNPVSARRSDSVILNVLYGPDAPTISPLNTSY RSGENLNLSCHAASNPPAQYSWFVNGTFQQSTQELFIPNITVNNSGSYTCQAHNSDTGLNR TTVTTITVYAEPPKPFITSNNSNPVEDEDAVALTCEPEIQNTTYLWWVNNQSLPVSPRLQLSN DNRTLTLLSVTRNDVGPYECGIQNELSVDHSDPVILNVLYGPDDPTISPSYTYYRPGVNLSLS CHAASNPPAQYSWLIDGNIQQHTQELFISNITEKNSGLYTCQANNSASGHSRTTVKTITVSAE LPKPSISSNNSKPVEDKDAVAFTCEPEAQNTTYLWWVNGQSLPVSPRLQLSNGNRTLTLFN VTRNDARAYVCGIQNSVSANRSDPVTLDVLYGPDTPIISPPDSSYLSGANLNLSCHSASNPS PQYSWRINGIPQQHTQVLFIAKITPNNNGTYACFVSNLATGRNNSIVKSITVSASGTSPGLSA GATVGIMIGVLVGVALI (SEQ ID NO: 1). Five non-synonymous SNPs have been identified with a frequency higher than 2% in Caucasian population, four of them being localized in the N domain (at positions 80, 83, 112, 113), the last one in the A2 domain (at position 398) of human CEACAM5. GenBank AAA51967.1 contains the major haplotype (I80, V83, I112, I113 and E398).

A "domain" or “region” may be any region of a protein, generally defined on the basis of sequence homologies and often related to a specific structural or functional entity. CEACAM family members are known to be composed of Ig-like domains. The term domain is used in

this document to designate either individual Ig-like domains, such as "N-domain" or for groups of consecutive domains, such as "A2-B2 domain".

Domain organization of human CEACAM5 is as follows (based on GenBank AAA51967.1 sequence; SEQ ID NO: 1):

Human CEACAM5 domains	Positions in SEQ ID NO: 1
N	35-142
A1	143-237
B1	238-320
A2	321-415
B2	416-498
A3	499-593
B3	594-685

Accordingly, the A2-B2 domain of human CEACAM5 consists of amino acids 321-498 of SEQ ID NO: 1.

A reference sequence of *Macaca fascicularis* CEACAM5 protein is available (NCBI Reference Sequence XP_005589491.1), and this amino acid sequence reads as follows: *mgspsaplhrwcipwqtlllltaslltfwnpptta*qltiesrpfnvaegkevlllahnvsqnlfgyiwykgervdasrrigscvirtqqitpg pahsgretidfnasllihnvtqsdtgsytiqvikedlvneeatgqfrvypelpkpyissnnsnpvedkdavaltcepetqdttylwwv nnqslpvsprlelssdnrtltvfniprndttsykcetqnpvsvrrsdpvtlnvlygpdaptisplntpyragenlnlschaasnptaqyf wfvngtfqqstqelfipnitvnnsgsymcqahnsatglnrttvtaitvya**elpkpyitsnnsnpiedkdavtltcepetqdttylw wvnnqslsvssrlelsndnrtltvfniprndttfyecetqnpvsvrrsdpvtlnvlygpdaptisplntpyragenlnlsch aasnpaaqyswfvngtfqqstqelfipnitvnnsgsymcqahnsatglnrttvtaitvyv**elpkpyissnnsnpiedkdav tltcepvaenttylwwvnnqslsvsprlqlsngnriltllsvtrndtgpyecgiqnsesakrsdpvtlnvtygpdtpiisppdlsyrsgan lnlschsdsnpspqyswlingtlrqhtqvlfiskitsnnngayacfvsnlatgrnnsivknisvssgdsapgssglsaratvgiiigmlv gvalm (SEQ ID NO: 2) (signal peptide in *italics*; A2-B2 domain in **bold** letters; GPI anchor underlined; N-A1-B1 domain in regular font between signal peptide and A2-B2 domain; A3-B3 domain in regular font between A2-B2 domain and GPI anchor).

A "coding sequence" or a sequence "encoding" an expression product, such as a polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that polypeptide, protein, or enzyme, i.e., the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

As used herein, references to specific proteins (e.g. antibodies) can include a polypeptide having a native amino acid sequence, as well as variants and modified forms regardless of their origin or mode of preparation. A protein which has a native amino acid sequence is a protein having the same amino acid sequence as obtained from nature. Such native sequence proteins can be isolated from nature or can be prepared using standard recombinant and/or synthetic methods. Native sequence proteins specifically encompass naturally occurring truncated or soluble forms, naturally occurring variant forms (e.g. alternatively spliced forms), naturally occurring allelic variants and forms including post-translational modifications. Native sequence proteins include proteins carrying post-translational modifications such as glycosylation, or phosphorylation, or other modifications of some amino acid residues.

The term "gene" means a DNA sequence that codes for, or corresponds to, a particular sequence of amino acids which comprises all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription. In particular, the term gene may be intended for the genomic sequence encoding a protein, i.e. a sequence comprising regulator, promoter, intron and exon sequences.

Herein, a sequence "at least 85% identical" to a reference sequence is a sequence having, over its entire length, 85% or more, for instance 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with the entire length of the reference sequence. The percentage of "sequence identity" may thus be determined by comparing two such sequences over their entire length by global pairwise alignment using the algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443 (1970)), e.g. using the program Needle (EMBOSS) with the BLOSUM62 matrix and the following parameters: gap open=10, gap extend=0.5, end gap penalty=false, end gap open=10, end gap extend=0.5 (which are standard settings).

A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain with similar chemical properties (e.g., charge, size or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartic acid and glutamic acid; and 7) sulfur-containing side chains:

cysteine and methionine. Conservative amino acid substitution groups can also be defined on the basis of amino acid size.

An "antibody" (also referred to as an “immunoglobulin”) may e.g. be a natural or conventional type of antibody in which two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (l) and kappa (k). There are five main heavy chain classes (or isotypes) which determine aspects of the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each antibody chain contains distinct sequence domains (or regions). The light chain of a typical IgG antibody includes two regions, a variable region (VL) and a constant region (CL). The heavy chain of a typical IgG antibody includes four regions, namely a variable region (VH) and a constant region (CH), the latter being made up of three constant domains (CH1, CH2 and CH3). The variable regions of both light and heavy chains determine binding and specificity to the antigen. The constant regions of the light and heavy chains can confer important biological properties, such as antibody chain association, secretion, trans-placental mobility, complement binding, and binding to Fc receptors (FcR). The Fv fragment is the N-terminal part of the Fab fragment of an antibody and consists of the variable portions of one light chain and one heavy chain.

The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the so-called hypervariable or complementarity determining regions (CDRs). Complementarity determining regions (CDRs) therefore refer to amino acid sequences which together define the binding affinity and specificity of the Fv region of an antibody. The light (L) and heavy (H) chains of an antibody each have three CDRs, designated CDR1-L, CDR2-L, CDR3-L and CDR1-H, CDR2-H, CDR3-H, respectively. A conventional antibody’s antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain variable region.

"Framework regions" (FRs) refer to amino acid sequences interposed between CDRs, i.e. to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved among different immunoglobulins in a single species. The light and heavy chains of an immunoglobulin each have four FRs, designated FR1-L, FR2-L, FR3-L, FR4-L, and FR1-H, FR2-H, FR3-H, FR4-H, respectively. As used herein, a "human framework region" is a framework region that is substantially identical (about 85%, or more, for instance 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) to the framework region of a naturally occurring human antibody.

In the context of the invention, CDR/FR definition in an immunoglobulin light or heavy chain is determined based on the IMGT definition (Lefranc et al. Dev. Comp. Immunol., 2003, 27(1):55-77; www.imgt.org).

As used herein, the term "antibody" includes conventional antibodies and fragments thereof, as well as single domain antibodies and fragments thereof, such as variable heavy chain of single domain antibodies; the term “antibody” as used herein also includes chimeric, humanized, bispecific or multispecific antibodies, as well as other types of engineered antibodies. The term “antibody” includes monoclonal antibodies.

The term "monoclonal antibody" or "mAb" as used herein refers to an antibody molecule of a single amino acid sequence, which is directed against a specific antigen, and is not to be construed as requiring production of the antibody by any particular method. A monoclonal antibody may be produced e.g. by a single clone of B cells or hybridoma, but may also be recombinant, e.g. produced by methods involving genetic or protein engineering.

The term "chimeric antibody" refers to an engineered antibody which, in its broadest sense, contains one or more regions from one antibody and one or more regions from one or more other antibodies. In an embodiment, a chimeric antibody comprises a VH and a VL of an antibody derived from a non-human animal, in association with a CH and a CL of another antibody which is, in some embodiments, a human antibody. As the non-human animal, any animal such as mouse, rat, hamster, rabbit or the like can be used. A chimeric antibody may also denote a multispecific antibody having specificity for at least two different antigens.

The term "humanized antibody" refers to an antibody which is wholly or partially of non-human origin and which has been modified to replace certain amino acids, for instance in the framework regions of the VH and VL, in order to avoid or minimize an immune response in humans. The constant regions of a humanized antibody are typically human CH and CL regions.

"Fragments" of antibodies (e.g. of conventional antibodies) comprise a portion of an intact antibody such as an IgG, in particular an antigen binding region or variable region of the intact antibody. Examples of antibody fragments include Fv, Fab, F(ab')2, Fab', dsFv, (dsFv)2, scFv, sc(Fv)2, diabodies, as well as bispecific and multispecific antibodies formed from antibody fragments. A fragment of a conventional antibody may also be a single domain antibody, such as a heavy chain antibody or VHH.

The term "Fab" denotes an antibody fragment having a molecular weight of about 50,000 Da and antigen binding activity, in which about a half of the N-terminal side of the heavy chain and the entire light chain are bound together through a disulfide bond. It is usually obtained among fragments by treating IgG with a protease, papaine.

The term "F(ab')2" refers to an antibody fragment having a molecular weight of about 100,000 Da and antigen binding activity, which is slightly larger than 2 identical Fab fragments bound via a disulfide bond of the hinge region. It is usually obtained among fragments by treating IgG with a protease, pepsin.

The term "Fab' " refers to an antibody fragment having a molecular weight of about 50,000 Da and antigen binding activity, which is obtained by cutting a disulfide bond of the hinge region of the F(ab')2.

A single chain Fv ("scFv") is a covalently linked VH::VL heterodimer which is usually expressed from a gene fusion including VH and VL encoding genes linked by a peptide-encoding linker. The human scFv fragments of the invention include CDRs that are held in appropriate conformation, for instance by using gene recombination techniques. Divalent and multivalent antibody fragments can form either spontaneously by association of monovalent scFvs, or can be generated by coupling monovalent scFvs by a peptide linker, such as divalent sc(Fv)2. "dsFv" is a VH::VL heterodimer stabilised by a disulphide bond. "(dsFv)2" denotes two dsFv coupled by a peptide linker.

The term "bispecific antibody" or "BsAb" denotes an antibody which comprises two different antigen binding sites. Thus, BsAbs are able to e.g. bind two different antigens simultaneously. Genetic engineering has been used with increasing frequency to design, modify, and produce antibodies or antibody derivatives with a desired set of binding properties and effector functions as described for instance in EP 2 050 764 A1.

The term "multispecific antibody" denotes an antibody which comprises two or more different antigen binding sites.

The term "diabodies" refers to small antibody fragments with two antigen binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains of the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites.

The term "hybridoma" denotes a cell, which is obtained by subjecting a B cell prepared by immunizing a non-human mammal with an antigen to cell fusion with a myeloma cell derived from a mouse or the like which produces a desired monoclonal antibody having an antigen specificity.

By "purified" or "isolated" it is meant, when referring to a polypeptide (e.g. an antibody) or a nucleotide sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. The term "purified" as used herein means at least 75%, 85%, 95%, 96%, 97%, or 98% by weight, of biological macromolecules of the same type

are present. An "isolated" nucleic acid molecule which encodes a particular polypeptide refers to a nucleic acid molecule which is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include some additional bases or moieties which do not deleteriously affect the basic characteristics of the composition.

As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, a primate or a human. In embodiments of the invention, the subject (or patient) is a human.

Antibodies of the invention

The inventors have succeeded in generating, screening and selecting specific anti-CEACAM5 antibodies surprisingly displaying a combination of several characteristics that make them ideally suited for use in cancer therapy, in particular as part of an immunoconjugate (antibody-drug conjugate). For instance, the antibodies of the invention display high affinity for both human and *Macaca fascicularis* CEACAM5 protein, and they do not significantly cross-react with human CEACAM1, CEACAM6, CEACAM7 and CEACAM8 proteins, or with *Macaca fascicularis* CEACAM6 protein. The inventors have determined the amino acid sequence of such monoclonal antibodies according to the present invention.

The present invention provides an isolated antibody which binds to human CEACAM5 protein;

and wherein the isolated antibody comprises

(i) at least one light chain constant region (CL) that comprises a sequence selected from the group consisting of GGTLQSPP, LLQGA, GGLLQGPP, TLQSG, TLQSPP and TLQSA (preferably GGTLQSPP) and preferably comprising this sequence at the C-terminus of said light chain constant region;

and/or

(ii) at least one heavy chain constant region (CH) comprising one or more of the following amino acid substitutions:

(a) L234A and L235A (LALA mutation);

(b) L234A and L235A and P329G (LALA-PG mutation);

(c) L235A and G237A (LAGA mutation);

(d) M252Y and S254T and T256E (YTE mutation);

(e) K222R;

and wherein Eu numbering is used for said amino acid substitutions;

and wherein said isolated antibody comprises a CDR1-H consisting of the amino acid sequence of SEQ ID NO: 3, a CDR2-H consisting of the amino acid sequence of SEQ ID NO: 4, a CDR3-H consisting of the amino acid sequence of SEQ ID NO: 5, a CDR1-L consisting of the amino acid sequence of SEQ ID NO: 6, a CDR2-L consisting of the amino acid sequence of SEQ ID NO: 7, and a CDR3-L consisting of the amino acid sequence of SEQ ID NO: 8.

The present invention further provides an isolated antibody which binds to human CEACAM5 protein; and wherein the isolated antibody comprises

(i) at least one light chain constant region (CL) that comprises a sequence selected from the group consisting of GGTLQSPP, LLQGA, GGLLQGPP, TLQSG, TLQSPP and TLQSA (preferably GGTLQSPP) and preferably comprising this sequence at the C-terminus of said light chain constant region;

and

(ii) at least one heavy chain constant region (CH) comprising one or more of the following amino acid substitutions:

(a) L234A and L235A (LALA mutation);

(b) L234A and L235A and P329G (LALA-PG mutation);

(c) L235A and G237A (LAGA mutation);

(d) M252Y and S254T and T256E (YTE mutation);

(e) K222R;

and wherein Eu numbering is used for said amino acid substitutions;

and wherein said isolated antibody comprises a CDR1-H consisting of the amino acid sequence of SEQ ID NO: 3, a CDR2-H consisting of the amino acid sequence of SEQ ID NO: 4, a CDR3-H consisting of the amino acid sequence of SEQ ID NO: 5, a CDR1-L consisting of the amino acid sequence of SEQ ID NO: 6, a CDR2-L consisting of the amino acid sequence of SEQ ID NO: 7, and a CDR3-L consisting of the amino acid sequence of SEQ ID NO: 8.

Preferably, the isolated antibody of the invention comprises framework regions FR1, FR2, FR3, FR4, FR5, FR6, FR7 and FR8 having the structure FR1 - CDR1-H – FR2 – CDR2-H – FR3 – CDR3-H – FR4 and FR5 - CDR1-L – FR6 – CDR2-L – FR7 – CDR3-L – FR8; wherein FR1 consists of SEQ ID NO: 54, FR2 consists of SEQ ID NO: 55, FR3 consists of SEQ ID NO: 56, FR4 consists of SEQ ID NO: 57, FR5 consists of SEQ ID NO: 58, FR6 consists of SEQ ID NO: 59, FR7 consists of SEQ ID NO: 60 and FR8 consists of SEQ ID NO: 61.

The present invention further provides an isolated antibody which binds to human CEACAM5 protein,

wherein said isolated antibody preferably comprises a CDR1-H consisting of the amino acid sequence of SEQ ID NO: 3, a CDR2-H consisting of the amino acid sequence of SEQ ID NO: 4, a CDR3-H consisting of the amino acid sequence of SEQ ID NO: 5, a CDR1-L consisting of the amino acid sequence of SEQ ID NO: 6, a CDR2-L consisting of the amino acid sequence of SEQ ID NO: 7, and a CDR3-L consisting of the amino acid sequence of SEQ ID NO: 8;

and wherein said isolated antibody comprises framework regions FR1, FR2, FR3, FR4, FR5, FR6, FR7 and FR8 having the structure FR1 - CDR1-H – FR2 – CDR2-H – FR3 – CDR3-H – FR4 and FR5 - CDR1-L – FR6 – CDR2-L – FR7 – CDR3-L – FR8; wherein FR1 consists of SEQ ID NO: 54 (EVQLQESGPGLVKPSQTLSLTCTVS), FR2 consists of SEQ ID NO: 55 (LTWIRQHPGKGLEWIGY), FR3 consists of SEQ ID NO: 56 (YFNPSLRSRVTMSVDTSKNQFSLKLSSVTAADTAVYYC), FR4 consists of SEQ ID NO: 57 (WGQGTLVTVSS), FR5 consists of SEQ ID NO: 58 (EIVLTQSPATLSVSPGERATLSCRTS), FR6 consists of SEQ ID NO: 59 (LAWYQQKPGQAPRLLIY), FR7 consists of SEQ ID NO: 60 (TRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYC) and FR8 consists of SEQ ID NO: 61 (FGPGTKVDIK).

The invention also provides an isolated antibody which binds to human CEACAM5 protein, wherein the isolated antibody comprises

(i) at least one light chain constant region (CL) that comprises a sequence selected from the group consisting of GGTLQSPP, LLQGA, GGLLQGPP, TLQSG, TLQSPP and TLQSA and most preferably the sequence GGTLQSPP, and preferably comprising this sequence at the C-terminus of said light chain constant region;

and/or

(ii) at least one heavy chain constant region (CH) comprising one or more of the following amino acid substitutions:

(a) L234A and L235A (LALA mutation);

(b) L234A and L235A and P329G (LALA-PG mutation);

(c) L235A and G237A (LAGA mutation);

(d) M252Y and S254T and T256E (YTE mutation);

(e) K222R;

and wherein Eu numbering is used for said amino acid substitutions;

and wherein preferably said isolated antibody comprises a CDR1-H consisting of the amino acid sequence of SEQ ID NO: 3, a CDR2-H consisting of the amino acid sequence of SEQ ID NO: 4, a CDR3-H consisting of the amino acid sequence of SEQ ID NO: 5, a CDR1-L consisting of the amino acid sequence of SEQ ID NO: 6, a CDR2-L consisting of the amino acid sequence of SEQ ID NO: 7, and a CDR3-L consisting of the amino acid sequence of SEQ ID NO: 8;

and wherein preferably said isolated antibody comprises framework regions FR1, FR2, FR3, FR4, FR5, FR6, FR7 and FR8 having the structure FR1 - CDR1-H – FR2 – CDR2-H – FR3 – CDR3-H – FR4 and FR5 - CDR1-L – FR6 – CDR2-L – FR7 – CDR3-L – FR8; wherein FR1 consists of SEQ ID NO: 54, FR2 consists of SEQ ID NO: 55, FR3 consists of SEQ ID NO: 56, FR4 consists of SEQ ID NO: 57, FR5 consists of SEQ ID NO: 58, FR6 consists of SEQ ID NO: 59, FR7 consists of SEQ ID NO: 60 and FR8 consists of SEQ ID NO: 61.

The present invention also provides an isolated antibody which binds to human CEACAM5 protein and which comprises a CDR1-H consisting of the amino acid sequence DGSVSRGGYY (SEQ ID NO: 3), a CDR2-H consisting of the amino acid sequence IYYSGST (SEQ ID NO: 4), a CDR3-H consisting of the amino acid sequence ARGIAVAPFDY (SEQ ID NO: 5), a CDR1-L consisting of the amino acid sequence QSVRSN (SEQ ID NO: 6), a CDR2-L consisting of the amino acid sequence AAS (SEQ ID NO: 7), and a CDR3-L consisting of the amino acid sequence QQYTNWPFT (SEQ ID NO: 8); and wherein the isolated antibody comprises

(i) at least one heavy chain constant region (CH) comprising one or more of the following amino acid substitutions:

(a) L234A and L235A (LALA mutation);

(b) L234A and L235A and P329G (LALA-PG mutation);

(c) L235A and G237A (LAGA mutation);

(d) M252Y and S254T and T256E (YTE mutation);

(e) K222R;

and/or

(ii) at least one light chain constant region (CL) that comprises the sequence GGTLQSPP, and preferably comprising this sequence at the C-terminus of said light chain constant region;

and wherein Eu numbering is used for said amino acid substitutions. The EU numbering system is well known (cf. Edelman et al., Proc. Natl. Acad. Sci. USA 1969, 63, 78–85 and Kabat, E.A. et al., National Institutes of Health (U.S.) Office of the Director. Sequences of Proteins of Immunological Interest, 5th ed.; DIANE Publishing: Collingdale, PA, USA, 1991)

and the positions of the amino acid substitutions that are indicated follow this numbering system. The amino acid substitutions are specified using the single letter amino acid code. The GGTLQSPP can also be comprised in the light chain constant region (CL) several times and can alternatively or additionally also be comprised in the heavy chain constant region (CH). Preferably, the GGTLQSPP is comprised once per light chain constant region (CL) in both light chain constant regions (CL) of the antibody of the invention.

The antibodies of the invention can preferably also bind to *Macaca fascicularis* CEACAM5 protein.

In an embodiment of the isolated antibody of the invention, both heavy chain constant regions (CH) comprise one or more of said amino acid substitutions (a) through (e) and/or wherein both light chain constant regions comprise said sequence GGTLQSPP. Preferred combinations of modifications of the CL and CH chains are outlined in Table 4 below that indicates the modification combinations for antibodies mAb1-M, mAb2-M, mAb3-M, mAb6-M and mAb7-M. Preferably, the antibody of the invention comprises any of the following heavy chain constant region (CH) and light chain constant regions (CL) modifications:

(a) the CH comprises the amino acid substitutions L234A, L235A (LALA mutation) and M252Y, S254T and T256E (YTE mutation); or

(b) the CH comprises the amino acid substitutions L234A, L235A (LALA mutation) and M252Y, S254T and T256E (YTE mutation); and the light chain constant region (CL) that comprises the sequence GGTLQSPP (preferably at the C-terminus); or

(c) the CH comprises the amino acid substitutions L234A, L235A (LALA mutation) and M252Y, S254T and T256E (YTE mutation) and K222R; and the light chain constant region (CL) that comprises the sequence GGTLQSPP (preferably at the C-terminus); or

(d) the CH comprises the amino acid substitutions L234A, L235A (LALA mutation); or

(e) the CH comprises the amino acid substitutions L234A, L235A (LALA mutation); and the light chain constant region (CL) that comprises the sequence GGTLQSPP (preferably at the C-terminus).

Preferably, both CL and both CH regions of the antibody of the invention comprise a modification as outlined in (a) through (e) above.

In a further embodiment of the isolated antibody of the invention, at least one heavy chain constant regions (CH) comprises the amino acid sequence ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**AA**GGPS VFLFPPKPKDTL**Y**I**T**R**E**PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY

RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 31) or ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCD**R**THTCPPCPAPE**AA**GGPS VFLFPPKPKDTL**Y**I**T**R**E**PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 32) and/or at least one of said light chain constant regions (CL) comprises the amino acid sequence RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**GGTLQSPP** (SEQ ID NO: 33).

In yet a further embodiment of the isolated antibody of the invention, said heavy chain constant regions (CH) and light chain constant regions (CL) have any of the following sequence combinations:

(1) both CH comprise a sequence of SEQ ID NO: 31 and both CL comprise a sequence of SEQ ID NO: 12; or

(2) both CH comprise a sequence of SEQ ID NO: 31 and both CL comprise a sequence of SEQ ID NO: 33; or

(3) both CH comprise a sequence of SEQ ID NO: 32 and both CL comprise a sequence of SEQ ID NO: 33; or

(4) both CH comprise a sequence of ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**AA**GGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 50) and both CL comprise a sequence of SEQ ID NO: 12; or

(5) both CH comprise a sequence of SEQ ID NO: 50 and both CL comprise a sequence of SEQ ID NO: 33; or

(6) at least one CH comprise a sequence of SEQ ID NO: 31 and one CL comprise a sequence of SEQ ID NO: 12; or

(7) at least one CH comprise a sequence of SEQ ID NO: 31 and one CL comprise a sequence of SEQ ID NO: 33; or

(8) at least one CH comprise a sequence of SEQ ID NO: 32 and one CL comprise a sequence of SEQ ID NO: 33; or

(9) at least one CH comprise a sequence of SEQ ID NO: 50 and one CL comprise a sequence of SEQ ID NO: 12; or

10) at least one CH comprise a sequence of SEQ ID NO: 50 and one CL comprise a sequence of SEQ ID NO: 33.

In embodiments of the invention, the antibody having the above-mentioned six CDR sequences comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence EVQLQESGPGLVKPSQTLSLTCTVS**DGSVSRGGYY**LTWIRQHPGKGLEWIGY**IYYSGST**YF NPSLRSRVTMSVDTSKNQFSLKLSSVTAADTAVYYC**ARGIAVAPFDY**WGQGTLVTVSS (SEQ ID NO: 9) (CDRs shown in bold characters) and a light chain variable region (VL) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence EIVLTQSPATLSVSPGERATLSCRTS**QSVRSN**LAWYQQKPGQAPRLLIY**AAS**TRATGIPARF SGSGSGTEFTLTISSLQSEDFAVYYC**QQYTNWPFT**FGPGTKVDIK (SEQ ID NO: 10) (CDRs shown in bold characters).

In embodiments of the invention, the antibody having the above-mentioned six CDR sequences comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 10.

In embodiments of the invention, the antibody further comprises a heavy chain constant region (CH) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPPVAGPSVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV VSVLTVLHQDWLNGKEYKCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 11) and a light chain constant region (CL) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 12).

In embodiments of the invention, the antibody comprises a heavy chain constant region (CH) comprising the amino acid sequence of SEQ ID NO: 11 and a light chain constant region (CL) comprising the amino acid sequence of SEQ ID NO: 12.

It is known that heavy chain constant regions (CH) of antibodies can comprise a C-terminal lysine (K) without losing any binding functionality. Accordingly, in the sequences described herein for the inventive antibodies, the heavy chain constant regions (CH) can optionally comprise an additional lysine (K) at the C-terminus. Heavy chain constant regions (CH) without lysine are preferred for the antibody-drug conjugates disclosed herein.

In more specific embodiments, the antibody of the invention is an isolated antibody which binds to human CEACAM5 protein and which comprises a heavy chain (HC) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence EVQLQESGPGLVKPSQTLSLTCTVSDGSVSRGGYYLTWIRQHPGKGLEWIGYIYYSGSTYF NPSLRSRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARGIAVAPFDYWGQGTLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPPVAGPSVFLFP PKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPG (SEQ ID NO: 13) and a light chain (LC) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence EIVLTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF SGSGSGTEFTLTISSLQSEDFAVYYCQQYTNWPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 14). In even more specific embodiments of the invention, the antibody comprises (i) a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 34 and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 14; or (ii) a heavy chain (HC) comprising the amino acid sequence EVQLQESGPGLVKPSQTLSLTCTVSDGSVSRGGYYLTWIRQHPGKGLEWIGYIYYSGSTYF NPSLRSRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARGIAVAPFDYWGQGTLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**AA**GGPSVFL FPPKPKDTL**Y**I**T**R**E**PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 34) and a light chain (LC) comprising the amino acid sequence (SEQ ID NO: 36);

or (iii) a heavy chain (HC) comprising the amino acid sequence EVQLQESGPGLVKPSQTLSLTCTVSDGSVSRGGYYLTWIRQHPGKGLEWIGYIYYSGSTYF NPSLRSRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARGIAVAPFDYWGQGTLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCD**R**THTCPPCPAPE**AA**GGPSVFL FPPKPKDTL**Y**I**T**R**E**PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 35) and a light chain (LC) comprising the amino acid sequence EIVLTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF SGSGSGTEFTLTISSLQSEDFAVYYCQQYTNWPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC**GGTLQSPP** (SEQ ID NO: 36);

or .

(iv) a heavy chain (HC) comprising the amino acid sequence of EVQLQESGPGLVKPSQTLSLTCTVSDGSVSRGGYYLTWIRQHPGKGLEWIGYIYYSGSTYF NPSLRSRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARGIAVAPFDYWGQGTLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**AA**GGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 51) and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 14; or

(v) a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 51 and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 36.

In yet more specific embodiments of the invention, the antibody consists of (i) two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 34 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 14; or (ii) two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 34 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 36; or (iii) two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 35 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 36; or (iv) of two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 51 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 14; or (v) of two identical

heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 51 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 36.In some embodiments, one or more individual amino acids of an antibody of the invention may be altered by substitution, in particular by conservative substitution, in one or more of the above-mentioned sequences, including the CDR sequences. Such an alteration may be intended for example to remove a glycosylation site or a deamidation site, e.g. in connection with humanization of the antibody.

In some embodiments, the antibody of the invention is an isolated antibody which binds to human CEACAM5 protein and which consists of two identical heavy chains (HC) consisting of the amino acid sequence of SEQ ID NO: 13 and two identical light chains (LC) consisting of the amino acid sequence of SEQ ID NO: 14; this particular antibody is also referred to as “mAb1” herein.

In some embodiments, the antibody of the invention is an isolated antibody which binds to human CEACAM5 protein and which consists of two identical heavy chains (HC) consisting of the amino acid sequence of SEQ ID NO: 34 and two identical light chains (LC) consisting of the amino acid sequence of SEQ ID NO: 14; this particular antibody is also referred to as “mAb1-M” herein.

In some embodiments, the antibody of the invention is an isolated antibody which binds to human CEACAM5 protein and which consists of two identical heavy chains (HC) consisting of the amino acid sequence of SEQ ID NO: 34 and two identical light chains (LC) consisting of the amino acid sequence of SEQ ID NO: 36; this particular antibody is also referred to as “mAb2-M” herein.

In some embodiments, the antibody of the invention is an isolated antibody which binds to human CEACAM5 protein and which consists of two identical heavy chains (HC) consisting of the amino acid sequence of SEQ ID NO: 35 and two identical light chains (LC) consisting of the amino acid sequence of SEQ ID NO: 36; this particular antibody is also referred to as “mAb3-M” herein.

In some embodiments, the antibody of the invention is an isolated antibody which binds to human CEACAM5 protein and which consists of two identical heavy chains (HC) consisting of the amino acid sequence of SEQ ID NO: 51 and two identical light chains (LC) consisting of the amino acid sequence of SEQ ID NO: 14; this particular antibody is also referred to as “mAb6-M” herein.

In some embodiments, the antibody of the invention is an isolated antibody which binds to human CEACAM5 protein and which consists of two identical heavy chains (HC) consisting of the amino acid sequence of SEQ ID NO: 51 and two identical light chains (LC) consisting of

the amino acid sequence of SEQ ID NO: 36; this particular antibody is also referred to as “mAb7-M” herein.

In some embodiments, the antibody of the invention binds to the A2-B2 domains of human and *Macaca fascicularis* CEACAM5. The invention also provides an antibody which competes for binding to A2-B2 domain of human and/or *Macaca fascicularis* CEACAM5 proteins with an antibody comprising the heavy and light chain variable regions of mAb1 (i.e. the VH and VL corresponding to SEQ ID NO: 9 and 10, respectively) and a heavy chain constant regions (CH) and light chain constant regions (CL) from any of mAb1-M, of mAb2-M, of mAb3-M, of mAb6-M or mAb7-M.

The ability of a candidate antibody to compete for binding to A2-B2 domain of human and/or *Macaca fascicularis* CEACAM5 proteins with an antibody comprising the VH and VL of mAb1 (hereafter, in the context of competition with a candidate antibody, referred to as a "reference" antibody) may be readily assayed, for instance, by competitive ELISA wherein the antigen (i.e. the A2-B2 domain of human or *Macaca fascicularis* CEACAM5, or a polypeptide comprising or consisting of a fragment of human or *Macaca fascicularis* CEACAM5 including the A2-B2 domain, in particular the extracellular domain of human or *Macaca fascicularis* CEACAM5) is bound to a solid support and two solutions containing the candidate antibody and the reference antibody, respectively, are added and the antibodies are allowed to compete for binding to the antigen. The amount of reference antibody bound to the antigen may then be measured, and compared to the amount of reference antibody bound to the antigen when measured against a negative control (e.g. solution containing no antibody). An amount of bound reference antibody in the presence of the candidate antibody decreased as compared to the amount of bound reference antibody in presence of the negative control indicates that the candidate antibody has competed with the reference antibody. Conveniently, the reference antibody may be labeled (e.g. fluorescently) to facilitate detection of bound reference antibody. Repeated measurements may be performed with serial dilutions of the candidate and/or reference antibody.

In some embodiments, the antibody of the invention does not bind to, or does not significantly cross-react with human CEACAM1, human CEACAM6, human CEACAM7, human CEACAM8 and *Macaca fascicularis* CEACAM6 proteins. In some embodiments, the antibody does not bind to, or does not significantly cross-react with the extracellular domain of the aforementioned human and *Macaca fascicularis* CEACAM proteins other than CEACAM5.

"Affinity" is defined, in theory, by the equilibrium association between the whole antibody and the antigen. It can be experimentally assessed by a variety of known methods, such as measuring association and dissociation rates with surface plasmon resonance or measuring the EC_{50} (or apparent K_D) in an immunochemical assay (ELISA, FACS). In these assays, the

EC_{50} is the concentration of the antibody which induces a response halfway between the baseline and maximum after some specified exposure time on a defined concentration of antigen by ELISA (enzyme-linked immuno-sorbent assay) or cell expressing the antigen by FACS (Fluorescence Activated Cell Sorting).

A monoclonal antibody binding to an antigen 1 (Ag1) is "cross-reactive" to an antigen 2 (Ag2) when the EC_{50}s are in a similar range for both antigens. In the present application, a monoclonal antibody binding to Ag1 is cross-reactive to Ag2 when its affinity for Ag2 is within 10-fold or less (for instance within 5-fold) from its affinity of Ag1, affinities being measured with the same method for both antigens.

A monoclonal antibody binding to Ag1 is "not significantly cross-reactive" to Ag2 when the affinities are very different for the two antigens. Affinity for Ag2 may not be measurable if the binding response is too low. In the present application, a monoclonal antibody binding to Ag1 is not significantly cross-reactive to Ag2, when the binding response of the monoclonal antibody to Ag2 is less than 5% of the binding response of the same monoclonal antibody to Ag1 in the same experimental setting and at the same antibody concentration. In practice, the antibody concentration used can be the EC_{50} or the concentration required to reach the saturation plateau obtained with Ag1. A monoclonal antibody "binds specifically" to (or "is specific for") Ag1 when it is not significantly cross-reactive to Ag2.

In some embodiments, an antibody according to the invention has an affinity for *Macaca fascicularis* CEACAM5 which is within 10-fold or less (for instance within 5-fold) from its affinity for human CEACAM5. Thus, the antibody according to the invention may be used in toxicological studies performed in monkeys because the toxicity profile observed in monkeys would be relevant to anticipate potential adverse effects in humans.

In some embodiments, the antibody of the invention has an affinity for human CEACAM5 or *Macaca fascicularis* CEACAM5, or both, which is ≤ 10nM; for instance, the antibody of the invention may have an affinity for human CEACAM5 which is between 1 and 10 nM, such as an affinity for human CEACAM5 of about 6 nM.

Affinity for human CEACAM5 or for *Macaca fascicularis* CEACAM5 may be determined e.g. as the EC50 value in an ELISA using soluble recombinant CEACAM5 as capture antigen.

Alternatively, for the antibody of the invention, an apparent dissociation constant (apparent KD) may be determined by FACS analysis e.g. on tumor cell line MKN45 (DSMZ, ACC 409).

Additionally, antibodies according to the invention have been shown to be able to detect CEACAM5 expression by immunohistochemistry, e.g. in frozen and formalin-fixed and paraffin embedded (FFPE) tissue sections.

Any combination of the embodiments described herein above and below forms part of the invention.

In some embodiments, the antibody according to the invention is a conventional antibody, such as a conventional monoclonal antibody, or an antibody fragment, a bispecific or multispecific antibody.

In some embodiments, the antibody according to the invention comprises or consists of an IgG, or a fragment thereof.

In some embodiments, the antibody of the invention may be e.g. a murine antibody, a chimeric antibody, a humanized antibody, or a human antibody. Numerous methods for humanization of an antibody sequence are known in the art; see e.g. the review by Almagro & Fransson (2008) Front Biosci. 13: 1619-1633. One commonly used method is CDR grafting, or antibody reshaping, which involves grafting of the CDR sequences of a donor antibody, generally a mouse antibody, into the framework scaffold of a human antibody of different specificity. Since CDR grafting may reduce the binding specificity and affinity, and thus the biological activity, of a CDR grafted non-human antibody, back mutations may be introduced at selected positions of the CDR grafted antibody in order to retain the binding specificity and affinity of the parent antibody. Identification of positions for possible back mutations can be performed using information available in the literature and in antibody databases. Amino acid residues that are candidates for back mutations are typically those that are located at the surface of an antibody molecule, while residues that are buried or that have a low degree of surface exposure will not normally be altered. An alternative humanization technique to CDR grafting and back mutation is resurfacing, in which non-surface exposed residues of non-human origin are retained, while surface residues are altered to human residues. Another alternative technique is known as "guided selection" (Jespers et al. (1994) Biotechnology 12, 899) and can be used to derive from a murine antibody a fully human antibody conserving the epitope and binding charateristics of the parental antibody.

For chimeric antibodies, humanization typically involves modification of the framework regions of the variable region sequences.

Amino acid residues that are part of a CDR will typically not be altered in connection with humanization, although in certain cases it may be desirable to alter individual CDR amino acid residues, for example to remove a glycosylation site, a deamidation site or an undesired cysteine residue. N-linked glycosylation occurs by attachment of an oligosaccharide chain to an asparagine residue in the tripeptide sequence Asn-X-Ser or Asn-X-Thr, where X may be any amino acid except Pro. Removal of an N-glycosylation site may be achieved by mutating either the Asn or the Ser/Thr residue to a different residue, for instance by way of conservative

substitution. Deamidation of asparagine and glutamine residues can occur depending on factors such as pH and surface exposure. Asparagine residues are particularly susceptible to deamidation, primarily when present in the sequence Asn-Gly, and to a lesser extent in other dipeptide sequences such as Asn-Ala. When such a deamidation site, for instance Asn-Gly, is present in a CDR sequence, it may therefore be desirable to remove the site, typically by conservative substitution to remove one of the implicated residues. Substitution in a CDR sequence to remove one of the implicated residues is also intended to be encompassed by the present invention.

In a humanized antibody or fragment thereof, the variable domains of heavy and light chains may comprise human acceptor framework regions. A humanized antibody may further comprise human constant heavy and light chain domains, where present.

In some embodiments, the antibody according to the invention may be an antibody fragment (for instance a humanized antibody fragment) selected from the group consisting of Fv, Fab, F(ab')2, Fab', dsFv, (dsFv)2, scFv, sc(Fv)2, and diabodies.

In some embodiments, the antibody according to the invention may be a bispecific or multispecific antibody formed from antibody fragments, at least one antibody fragment being a fragment of an antibody according to the present invention. Multispecific antibodies are polyvalent protein complexes as described for instance in EP 2 050 764 A1 or US 2005/0003403 A1.

Bispecific or multispecific antibodies according to the invention can have specificity for (a) the human and *Macaca fascicularis* CEACAM5 proteins and (b) at least one other antigen. In some embodiments, the at least one other antigen is not a human or *Macaca fascicularis* CEACAM family member. In other embodiments, the at least one other antigen may be an epitope on human or *Macaca fascicularis* CEACAM5 other than the epitope targeted by mAb1.

The antibodies of the invention can be produced by any technique known in the art. Antibodies according to the invention can be used e.g. in an isolated (e.g. purified) form or contained in a vector, such as a membrane or lipid vesicle (e.g. a liposome).

Nucleic acids and host cells of the invention

A further aspect of the invention relates to an isolated nucleic acid comprising or consisting of a nucleic acid sequence encoding an antibody of the invention as defined above.

Typically, said nucleic acid is a DNA or RNA molecule, which may be included in any suitable vector, such as a plasmid, cosmid, episome, artificial chromosome, phage or a viral vector.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence.

Accordingly, a further aspect of the invention relates to a vector comprising a nucleic acid of the invention as defined above.

Such vectors may comprise regulatory elements, such as a promoter, enhancer, terminator and the like, to cause or direct expression of said polypeptide upon administration to a subject. Examples of promoters and enhancers used in the expression vector for an animal cell include early promoter and enhancer of SV40 (Mizukami T. et al. 1987), LTR promoter and enhancer of Moloney mouse leukemia virus (Kuwana Y et al. 1987), promoter (Mason JO et al. 1985) and enhancer (Gillies SD et al. 1983) of immunoglobulin H chain and the like.

Any expression vector for animal cells can be used, so long as a gene encoding the human antibody C region can be inserted and expressed. Examples of suitable vectors include pAGE107 (Miyaji H et al. 1990), pAGE103 (Mizukami T et al. 1987), pHSG274 (Brady G et al. 1984), pKCR (O'Hare K et al. 1981), pSG1 beta d2-4-(Miyaji H et al. 1990) and the like.

Other examples of plasmids include replicating plasmids comprising an origin of replication, or integrative plasmids, such as for instance pUC, pcDNA, pBR, and the like.

Other examples of viral vectors include adenoviral, retroviral, herpes virus and AAV vectors. Such recombinant viruses may be produced by techniques known in the art, such as by transfecting packaging cells or by transient transfection with helper plasmids or viruses. Typical examples of virus packaging cells include PA317 cells, PsiCRIP cells, GPenv+ cells, 293 cells, etc. Detailed protocols for producing such replication-defective recombinant viruses may be found for instance in WO 95/14785, WO 96/22378, US 5,882,877, US 6,013,516, US 4,861,719, US 5,278,056 and WO 94/19478.

A further object of the present invention relates to a host cell which has been transfected, infected or transformed by a nucleic acid and/or a vector according to the invention.

The term "transformation" means the introduction of a "foreign" (i.e. extrinsic) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. A host cell that receives and expresses introduced DNA or RNA bas been "transformed".

The nucleic acids of the invention may be used to produce an antibody of the invention in a suitable expression system. The term "expression system" means a host cell and compatible

vector under suitable conditions, e.g. for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors. Other examples of host cells include, without limitation, prokaryotic cells (such as bacteria) and eukaryotic cells (such as yeast cells, mammalian cells, insect cells, plant cells, etc.). Specific examples include *E. coli*, *Kluyveromyces* or *Saccharomyces* yeasts, mammalian cell lines (e.g., Vero cells, CHO cells, 3T3 cells, COS cells, etc.) as well as primary or established mammalian cell cultures (e.g., produced from lymphoblasts, fibroblasts, embryonic cells, epithelial cells, nervous cells, adipocytes, etc.). Examples also include mouse SP2/0-Ag14 cell (ATCC CRL1581), mouse P3X63-Ag8.653 cell (ATCC CRL1580), CHO cell in which a dihydrofolate reductase gene (hereinafter referred to as "DHFR gene") is defective (Urlaub G et al; 1980), rat YB2/3HL.P2.G11.16Ag.20 cell (ATCC CRL1662, hereinafter referred to as “YB2/0 cell"), and the like. In some embodiments, the YB2/0 cell is used, since ADCC activity of chimeric or humanized antibodies is enhanced when expressed in this cell.

For expression of a humanized antibody, the expression vector may be either of a type in which a gene encoding an antibody heavy chain and a gene encoding an antibody light chain exists on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression levels of antibody H and L chains in animal cells, a humanized antibody expression vector is of the tandem type Shitara K et al. J Immunol Methods. 1994 Jan. 3;167(1-2):271-8). Examples of tandem type humanized antibody expression vector include pKANTEX93 (WO 97/10354), pEE18 and the like.

The present invention also relates to a method of producing a recombinant host cell expressing an antibody according to the invention, said method comprising the steps consisting of : (i) introducing in vitro or ex vivo a recombinant nucleic acid or a vector as described above into a competent host cell, (ii) culturing in vitro or ex vivo the recombinant host cell obtained and (iii), optionally, selecting the cells which express and/or secrete said antibody.

Such recombinant host cells can be used for the production of antibodies of the invention.

Methods of producing antibodies of the invention

Antibodies of the invention may be produced by any technique known in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination.

Knowing the amino acid sequence of a desired antibody, one skilled in the art can readily produce said antibodies or immunoglobulin chains using standard techniques for production

of polypeptides. For instance, they can be synthesized using well-known solid phase methods using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions. Alternatively, antibodies and immunoglobulin chains of the invention can be produced by recombinant DNA techniques, as is well-known in the art. For example, these polypeptides (e.g. antibodies) can be obtained as DNA expression products after incorporation of DNA sequences encoding the desired polypeptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired polypeptide, from which they can be later isolated using well-known techniques.

For instance, the present invention provides the following DNA sequences encoding the antibody mAb1:

mAb1 heavy chain nucleotide sequence

wherein mVk signal peptide is underlined,
start and stop codons are *in italics*,
VH region sequence in **boldface**, and
CDRs are indicated with double underlining:

*ATG*GAGACCGACACCCTGCTGCTGTGGGTGCTGCTGCTGTGGGTGCCCGGGTCGACC
GGT**GAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCT**
GTCCCTCACCTGCACTGTCTCTGATGGCTCCGTCAGCAGGGGTGGTTACTACTTGACC
TGGATCCGCCAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGT
GGGAGCACCTACTTCAACCCGTCCCTCAGGAGTCGGGTTACCATGTCAGTAGACACG
TCTAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTG
TATTACTGTGCGAGAGGGATAGCAGTGGCTCCCTTTGACTACTGGGGCCAGGGAACC
CTGGTCACCGTCTCTTCAGCTAGCACCAAGGGCCCCAGCGTGTTCCCCCTGGCCCCCA
GCAGCAAGTCCACAAGCGGAGGAACAGCCGCCCTGGGCTGCCTGGTGAAGGACTACT
TCCCCGAGCCCGTGACCGTGTCCTGGAACAGCGGAGCCCTGACCTCCGGCGTGCACA
CCTTCCCCGCCGTGCTGCAGAGCAGCGGCCTGTACAGCCTGAGCAGCGTGGTGACAG
TGCCAAGCAGCAGCCTGGGAACCCAGACCTACATCTGCAACGTGAACCACAAGCCCAG
CAACACCAAGGTGGACAAGAGAGTGGAGCCCAAGAGCTGCGACAAGACCCATACCTGT
CCACCCTGCCCAGCCCCCCCAGTGGCCGGACCCTCCGTGTTCCTGTTCCCCCCCAAGC
CCAAGGACACCCTGATGATCAGCAGGACCCCCGAGGTGACCTGCGTGGTGGTGGACG
TGAGCCACGAGGACCCAGAGGTGAAGTTCAATTGGTATGTGGACGGCGTGGAGGTGCA
CAACGCCAAGACCAAGCCCAGAGAGGAACAGTACAACAGCACCTACAGGGTGGTGTCC
GTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGAATACAAGTGCAAGGTCT
CCAACAAGGCCCTGCCCTCCAGCATCGAGAAAACCATCAGCAAGGCCAAGGGCCAGCC
ACGGGAGCCCCAGGTGTACACACTGCCCCCATCTCGGGAAGAAATGACCAAGAACCAG
GTGTCCCTGACCTGTCTGGTGAAGGGCTTTTACCCCAGCGACATCGCCGTGGAGTGGG

AGAGCAACGGCCAGCCCGAGAACAACTACAAGACCACCCCCCCTGTGCTGGACAGCGA
CGGCAGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGTCCAGGTGGCAGCAGGGC
AACGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCACAACCACTACACACAGAAGA
GCCTGAGCCTGTCCCCCGGC*TGA*
(SEQ ID NO: 15)

mAb1 light chain nucleotide sequence

wherein uPA signal peptide is underlined,
start and stop codons are *in italics,*
VL region sequence in **boldface**, and
CDRs are indicated with double underlining:

*ATG*AGGGCCCTGCTGGCTAGACTGCTGCTGTGCGTGCTGGTCGTGTCCGACAGCAAGG
GC**GAAATCGTACTCACGCAGTCTCCAGCCACCCTGTCTGTGTCTCCAGGGGAAAGAG**
CCACCCTCTCCTGCAGGACCAGTCAGAGTGTTCGCAGCAACTTAGCCTGGTACCAGC
AGAAGCCTGGCCAGGCTCCCAGGCTCCTCATCTATGCTGCATCCACCAGGGCCACTG
GTATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGAGTTCACTCTCACCATCA
GCAGCCTGCAGTCTGAAGATTTTGCAGTTTATTACTGTCAGCAGTATACTAACTGGCC
ATTCACTTTCGGCCCTGGGACCAAAGTGGACATCAAACGTACGGTGGCTGCACCATCT
GTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTG
CCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCC
TCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTA
CAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTAC
GCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGG
GAGAGTGT*TGATAG*

(SEQ ID NO: 16)

The present invention also provides the following DNA sequences encoding the antibodies mAb1-M, mAb2-M and mAb3-M, mAb6-M, mAb7-M:

SEQ ID NO 37: HC mAb1-M & mAb2-M (HC-LALA-YTE) (nucleotide)

Signal peptide is underlined

start and stop codons are *in italics*,
VH region sequence in **boldface**, and
CDRs are indicated with double underlining:

*ATG*AAGCTGCCTGTCAGACTGCTGGTGCTGATGTTCTGGATTCCCGCCTCTCTGTCC**GA**
GGTCCAGCTGCAAGAATCTGGCCCTGGACTGGTCAAGCCCTCTCAGACCCTGTCTCT
GACCTGCACCGTGTCTGACGGCTCTGTTTCTAGAGGCGGCTACTACCTGACCTGGATC
AGACAGCATCCTGGCAAGGGCCTCGAGTGGATCGGCTACATCTACTACTCCGGCTCC

ACCTACTTCAACCCCAGCCTGAGATCCAGAGTGACCATGTCCGTGGACACCTCCAAG
AACCAGTTCTCCCTGAAGCTGTCCTCCGTGACCGCTGCTGATACCGCCGTGTACTACT
GCGCCAGAGGAATCGCCGTGGCTCCCTTTGATTATTGGGGCCAGGGCACCCTGGTCA
CCGTGTCCTCTGCTTCTACCAAGGGACCCAGCGTGTTCCCTCTGGCTCCTTCCAGCAAG
TCTACCTCTGGCGGAACAGCTGCTCTGGGCTGCCTGGTCAAGGACTACTTTCCTGAGC
CTGTGACCGTGTCTTGGAACTCTGGCGCTCTGACATCCGGCGTGCACACCTTTCCAGCT
GTGCTGCAATCCTCCGGCCTGTACTCTCTGAGCAGCGTCGTGACCGTGCCTTCTAGCT
CTCTGGGCACCCAGACCTACATCTGCAATGTGAACCACAAGCCTTCCAACACCAAGGTG
GACAAGAGAGTGGAACCCAAGTCCTGCGACAAGACCCACACCTGTCCTCCATGTCCTG
CTCCAGAAGCTGCTGGCGGCCCTTCCGTGTTTCTGTTCCCTCCAAAGCCTAAGGACACC
CTGTACATCACCCGCGAGCCTGAAGTGACCTGCGTGGTGGTGGATGTGTCTCACGAGG
ACCCCGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAACGCCAAGAC
CAAGCCTAGAGAGGAACAGTACAACAGCACCTACAGAGTGGTGTCCGTGCTGACCGTG
CTGCACCAGGATTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGGCCC
TGCCTGCTCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCTAGGGAACCCCA
GGTTTACACCCTGCCTCCAAGCCGGGAAGAGATGACCAAGAATCAGGTGTCCCTGACC
TGTCTCGTGAAGGGCTTCTACCCCTCCGATATCGCTGTGGAATGGGAGAGCAATGGCC
AGCCAGAGAACAACTACAAGACAACCCCTCCTGTGCTGGACTCCGACGGCTCATTCTTC
CTGTACTCCAAGCTGACAGTGGACAAGTCCAGATGGCAGCAGGGCAACGTGTTCTCCT
GCTCCGTGATGCACGAGGCCCTGCACAATCACTACACCCAGAAGAGTCTGTCCCTGTC
TCCTGGC*TGATGA*

SEQ ID NO 38: HC mAb3-M (HC-K222R-LALA-YTE) (nucleotide)

Signal peptide is underlined

start and stop codons are *in italics*,
VH region sequence in **boldface**, and
CDRs are indicated with double underlining:

*ATG*AAGCTGCCTGTCAGACTGCTGGTGCTGATGTTCTGGATTCCCGCCTCTCTGTCC**GA**
GGTCCAGCTGCAAGAATCTGGCCCTGGACTGGTCAAGCCCTCTCAGACCCTGTCTCT
GACCTGCACCGTGTCTGACGGCTCTGTTTCTAGAGGCGGCTACTACCTGACCTGGATC
AGACAGCATCCTGGCAAGGGCCTCGAGTGGATCGGCTACATCTACTACTCCGGCTCC
ACCTACTTCAACCCCAGCCTGAGATCCAGAGTGACCATGTCCGTGGACACCTCCAAG
AACCAGTTCTCCCTGAAGCTGTCCTCCGTGACCGCTGCTGATACCGCCGTGTACTACT
GCGCCAGAGGAATCGCCGTGGCTCCCTTTGATTATTGGGGCCAGGGCACCCTGGTCA
CCGTGTCCTCTGCTTCTACCAAGGGACCCAGCGTGTTCCCTCTGGCTCCTTCCAGCAAG
TCTACCTCTGGCGGAACAGCTGCTCTGGGCTGCCTGGTCAAGGACTACTTTCCTGAGC
CTGTGACCGTGTCTTGGAACTCTGGCGCTCTGACATCCGGCGTGCACACCTTTCCAGCT
GTGCTGCAATCCTCCGGCCTGTACTCTCTGAGCAGCGTCGTGACCGTGCCTTCTAGCT
CTCTGGGCACCCAGACCTACATCTGCAATGTGAACCACAAGCCTTCCAACACCAAGGTG
GACAAGAGAGTGGAACCCAAGTCCTGCGACAGAACCCACACCTGTCCTCCATGTCCTG
CTCCAGAAGCTGCTGGCGGCCCTTCCGTGTTTCTGTTCCCTCCAAAGCCTAAGGACACC
CTGTACATCACCCGCGAGCCTGAAGTGACCTGCGTGGTGGTGGATGTGTCTCACGAGG
ACCCCGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAACGCCAAGAC
CAAGCCTAGAGAGGAACAGTACAACAGCACCTACAGAGTGGTGTCCGTGCTGACCGTG
CTGCACCAGGATTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGGCCC
TGCCTGCTCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCTAGGGAACCCCA
GGTTTACACCCTGCCTCCAAGCCGGGAAGAGATGACCAAGAATCAGGTGTCCCTGACC
TGTCTCGTGAAGGGCTTCTACCCCTCCGATATCGCTGTGGAATGGGAGAGCAATGGCC
AGCCAGAGAACAACTACAAGACCACACCTCCTGTGCTGGACTCCGACGGCTCATTCTTC
CTGTACTCCAAGCTGACAGTGGACAAGTCCAGATGGCAGCAGGGCAACGTGTTCTCCT

GCTCCGTGATGCACGAGGCCCTGCACAATCACTACACCCAGAAGAGTCTGTCCCTGTC
TCCTGGC*TGATGA*

SEQ ID NO 39: LC mAb2-M, mAb3-M and mAb7-M (LC-Tag) (nucleotide)

Signal peptide underlined

start and stop codons are *in italics*,
VL region sequence in **boldface**, and
CDRs are indicated with double underlining:

*ATG*AAGCTGCCTGTCAGACTGCTGGTGCTGATGTTCTGGATTCCCGCCAGCCTGTCC**G**
AGATCGTGCTGACTCAATCTCCCGCCACACTGTCTGTGTCTCCCGGCGAAAGAGCTAC
CCTGTCTTGCCGGACATCTCAGTCCGTGCGGTCTAACCTGGCCTGGTATCAGCAGAAG
CCTGGACAGGCTCCTCGGCTGCTGATCTACGCTGCTTCTACCAGAGCCACAGGCATC
CCCGCCAGATTTTCTGGCTCTGGATCTGGCACCGAGTTTACCCTGACCATCTCCAGCC
TGCAGTCCGAGGATTTCGCCGTGTACTACTGCCAGCAGTATACCAACTGGCCCTTCAC
CTTCGGACCCGGCACAAAGGTGGACATCAAGAGAACCGTGGCCGCTCCTTCCGTGTT
CATCTTCCCACCTTCCGACGAGCAGCTGAAGTCCGGCACAGCTTCTGTCGTGTGCCTG
CTGAACAACTTCTACCCTCGGGAAGCCAAGGTGCAGTGGAAAGTGGATAACGCCCTGC
AGAGCGGCAACTCCCAAGAGTCTGTGACCGAGCAGGACTCCAAGGACAGCACCTACAG
CCTGAGCAGCACCCTGACACTGTCCAAGGCCGACTACGAGAAGCACAAGGTGTACGCC
TGCGAAGTGACCCATCAGGGCCTGTCTAGCCCTGTGACCAAGTCTTTCAACAGAGGCG
AGTGCGGCGGCACCTTGCAGTCTCCACCT*TGATAA*

SEQ ID NO 52: HC mAb6-M & HC mAb7-M (nucleotide)

Signal peptide underlined

start and stop codons are *in italics*,
VL region sequence in **boldface**, and
CDRs are indicated with double underlining:

*ATG*AAGCTGCCTGTCAGACTGCTGGTGCTGATGTTCTGGATTCCCGCCTCTCTGTCC**GA**
GGTCCAGCTGCAAGAATCTGGCCCTGGACTGGTCAAGCCCTCTCAGACCCTGTCTCT
GACCTGCACCGTGTCTGACGGCTCTGTTTCTAGAGGCGGCTACTACCTGACCTGGATC
AGACAGCATCCTGGCAAGGGCCTCGAGTGGATCGGCTACATCTACTACTCCGGCTCC
ACCTACTTCAACCCCAGCCTGAGATCCAGAGTGACCATGTCCGTGGACACCTCCAAG
AACCAGTTCTCCCTGAAGCTGTCCTCCGTGACCGCTGCTGATACCGCCGTGTACTACT
GCGCCAGAGGAATCGCCGTGGCTCCCTTTGATTATTGGGGCCAGGGCACCCTGGTCA
CCGTGTCCTCTGCTTCTACCAAGGGACCCAGCGTGTTCCCTCTGGCTCCTTCCAGCAAG
TCTACCTCTGGCGGAACAGCTGCTCTGGGCTGCCTGGTCAAGGACTACTTTCCTGAGC
CTGTGACCGTGTCTTGGAACTCTGGCGCTCTGACATCCGGCGTGCACACCTTTCCAGCT
GTGCTGCAATCCTCCGGCCTGTACTCTCTGAGCAGCGTCGTGACCGTGCCTTCTAGCT
CTCTGGGCACCCAGACCTACATCTGCAATGTGAACCACAAGCCTTCCAACACCAAGGTG
GACAAGAGAGTGGAACCCAAGTCCTGCGACAAGACCCACACCTGTCCTCCATGTCCTG
CTCCAGAAGCTGCTGGCGGCCCTTCCGTGTTTCTGTTCCCTCCAAAGCCTAAGGACACC
CTGATGATCTCTCGGACCCCTGAAGTGACCTGCGTGGTGGTGGATGTGTCTCACGAGG
ACCCAGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAACGCCAAGAC
CAAGCCTAGAGAGGAACAGTACAACAGCACCTACAGAGTGGTGTCCGTGCTGACCGTG
CTGCACCAGGATTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGGCCC
TGCCTGCTCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCTAGGGAACCCCA
GGTTTACACCCTGCCTCCAAGCCGGGAAGAGATGACCAAGAATCAGGTGTCCCTGACC

TGTCTCGTGAAGGGCTTCTACCCCTCCGATATCGCTGTGGAATGGGAGAGCAATGGCC
AGCCAGAGAACAACTACAAGACAACCCCTCCTGTGCTGGACTCCGACGGCTCATTCTTC
CTGTACTCCAAGCTGACAGTGGACAAGTCCAGATGGCAGCAGGGCAACGTGTTCTCCT
GCTCCGTGATGCACGAGGCCCTGCACAATCACTACACACAGAAGAGTCTGTCCCTGTCT
CCTGGC*TGATGA*

SEQ ID NO 53: LC mAb1-M & LC mAb6-M (nucleotide)

Signal peptide underlined

start and stop codons are *in italics*,
VL region sequence in **boldface**, and
CDRs are indicated with double underlining:

*ATG*GAGACCGACACCCTGCTGCTGTGGGTGCTGCTGCTGTGGGTGCCCGGGTCGACC
GGT**GAAATCGTACTCACGCAGTCTCCAGCCACCCTGTCTGTGTCTCCAGGGGAAAGA**
GCCACCCTCTCCTGCAGGACCAGTCAGAGTGTTCGCAGCAACTTAGCCTGGTACCAG
CAGAAGCCTGGCCAGGCTCCCAGGCTCCTCATCTATGCTGCATCCACCAGGGCCACT
GGTATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGAGTTCACTCTCACCATC
AGCAGCCTGCAGTCTGAAGATTTTGCAGTTTATTACTGTCAGCAGTATACTAACTGGC
CATTCACTTTCGGCCCTGGGACCAAAGTGGACATCAAACGTACGGTGGCAGCACCATC
TGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTG
CCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCC
TCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTA
CAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTAC
GCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGG
GAGAGTGT*TGATAG*

CD20 Isotype controls

SEQ ID NO 40: HC mAb5-M (antiCD20-HC)

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTS
YNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTV
SAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE**AA**GG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG
NVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO 41: LC mAb5-M (antiCD20-LC)

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFS
GSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPSDEQL
KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY
EKHKVYACEVTHQGLSSPVTKSFNRGEC**GGTLQSPP**

SEQ ID NO 42: HC mAb5-M (antiCD20-HC) (nucleotide)

Signal peptide underlined

start and stop codons are *in italics*

*ATG*AAGCTGCCTGTCAGACTGCTGGTGCTGATGTTCTGGATTCCCGCCAGCCTGTCTCA
GGTCCAGTTGCAACAACCTGGCGCCGAGCTTGTGAAGCCTGGCGCTTCTGTGAAGATG
TCCTGCAAGGCCTCCGGCTACACCTTCACCAGCTACAACATGCACTGGGTCAAGCAGA
CCCCTGGCAGAGGCCTGGAATGGATCGGCGCTATCTATCCTGGCAACGGCGACACCTC
CTACAACCAGAAGTTCAAGGGCAAAGCTACCCTGACCGCCGACAAGTCCTCTTCCACC
GCTTACATGCAGCTGTCCAGCCTGACCTCTGAGGACTCCGCCGTGTACTACTGCGCCC
GGTCTACCTATTATGGCGGCGACTGGTACTTCAACGTGTGGGGCGCTGGCACCACCGT
GACAGTTTCTGCCGCTTCTACCAAGGGACCCAGCGTGTTCCCTCTGGCTCCTTCCAGCA
AGTCTACCTCTGGCGGAACAGCTGCTCTGGGCTGCCTGGTCAAGGACTACTTTCCTGA
GCCTGTGACCGTGTCCTGGAACTCTGGCGCTCTGACATCTGGCGTGCACACCTTTCCA
GCTGTGCTGCAGTCCTCCGGCCTGTACTCTCTGTCCTCTGTCGTGACCGTGCCTTCCAG
CTCTCTGGGAACCCAGACCTACATCTGCAATGTGAACCACAAGCCTTCCAACACCAAGG
TGGACAAGAGAGTGGAACCCAAGTCCTGCGACAAGACCCACACCTGTCCTCCATGTCC
TGCTCCAGAAGCTGCTGGCGGCCCTTCCGTGTTTCTGTTCCCTCCAAAGCCTAAGGACA
CCCTGATGATCTCTCGGACCCCTGAAGTGACCTGCGTGGTGGTGGATGTGTCTCACGA
GGACCCAGAAGTGAAGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAACGCCAAG
ACCAAGCCTAGAGAGGAACAGTACAACTCCACCTACAGAGTGGTGTCCGTGCTGACCG
TGCTGCACCAGGATTGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGGC
CCTGCCTGCTCCTATCGAAAAGACCATCTCCAAGGCCAAGGGCCAGCCTAGGGAACCC
CAGGTTTACACCCTGCCTCCAAGCCGGGAAGAGATGACCAAGAACCAGGTGTCCCTGA
CCTGCCTCGTGAAGGGCTTCTACCCTTCCGATATCGCCGTGGAATGGGAGAGCAATGG
CCAGCCTGAGAACAACTACAAGACAACCCCTCCTGTGCTGGACTCCGACGGCTCATTCT
TCCTGTACTCCAAGCTGACAGTGGACAAGTCCAGATGGCAGCAGGGCAACGTGTTCTC
CTGCTCCGTGATGCACGAGGCCCTGCACAATCACTACACACAGAAGTCCCTGTCTCTGT
CCCCTGGCAAG*TGATGA*

SEQ ID NO 43: LC mAb5-M (antiCD20-LC) (nucleotide)

Signal peptide underlined

start and stop codons are *in italics*

*ATG*AAGCTGCCTGTCAGACTGCTGGTGCTGATGTTCTGGATTCCCGCCTCTCTGTCCCA
GATCGTGCTGTCTCAGTCTCCCGCCATCCTGAGTGCTAGCCCTGGCGAGAAAGTGACA
ATGACCTGCAGAGCCTCCTCCTCCGTGTCCTACATCCACTGGTTCCAGCAGAAGCCTG
GCAGCTCCCCTAAGCCTTGGATCTACGCCACCTCCAATCTGGCCTCTGGCGTGCCAGT
CAGATTCTCCGGATCTGGCTCCGGCACCTCCTACAGCCTGACCATCTCTAGAGTGGAA
GCCGAGGATGCCGCCACCTACTACTGTCAGCAGTGGACCAGCAATCCTCCTACCTTTG
GCGGAGGCACCAAGCTGGAAATCAAGCGGACAGTGGCCGCTCCTTCCGTGTTCATCTT
CCCACCTTCCGACGAGCAGCTGAAGTCTGGCACAGCCTCTGTCGTGTGCCTGCTGAAC
AACTTCTACCCTCGGGAAGCCAAGGTGCAGTGGAAGGTGGACAACGCTCTGCAGTCCG
GCAACTCCCAAGAGTCTGTGACCGAGCAGGACTCCAAGGACTCTACCTACTCTCTGAG
CAGCACCCTGACACTGTCCAAGGCCGACTACGAGAAGCACAAGGTGTACGCCTGCGAA
GTGACCCATCAGGGCCTGTCTAGCCCTGTGACCAAGTCTTTCAACAGAGGCGAGTGTG
GCGGCACCCTGCAGTCTCCACCT*TGATAA*

SEQ ID NO 64: rituximab HC

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTS
YNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTV
SAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGGP
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST
YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO 65: rituximab LC

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFS
GSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPSDEQL
KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY
EKHKVYACEVTHQGLSSPVTKSFNRGEC

The invention further relates to a method of producing an antibody of the invention, which method comprises the steps consisting of: (i) culturing a transformed host cell according to the invention; (ii) expressing the antibody; and (iii) recovering the expressed antibody.

Antibodies of the invention can be suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

In some embodiments, a humanized chimeric antibody of the present invention can be produced by obtaining nucleic acid sequences encoding humanized VL and VH regions as previously described, constructing a human chimeric antibody expression vector by inserting them into an expression vector for animal cell having genes encoding human antibody CH and human antibody CL, and expressing the coding sequence by introducing the expression vector into an animal cell.

As the CH domain of a human chimeric antibody, any region which belongs to human immunoglobulin heavy chains may be used, for instance those of IgG class are suitable and any one of subclasses belonging to IgG class, such as IgG1, IgG2, IgG3 and IgG4, can be used. Also, as the CL of a human chimeric antibody, any region which belongs to human immunoglobulin light chains may be used, and those of kappa class or lambda class can be used.

Methods for producing humanized or chimeric antibodies may involve conventional recombinant DNA and gene transfection techniques are well known in the art (see e.g. Morrison SL. et al. (1984) and patent documents US5,202,238; and US5,204, 244).

Methods for producing humanized antibodies based on conventional recombinant DNA and gene transfection techniques are well known in the art (see, e. g., Riechmann L. et al. 1988; Neuberger MS. et al. 1985). Antibodies can be humanized using a variety of techniques known in the art including, for example, the technique disclosed in the application WO2009/032661, CDR-grafting (EP 239,400; PCT publication WO91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan EA (1991); Studnicka GM et al. (1994); Roguska MA. et al. (1994)), and chain shuffling (U.S. Pat. No.5,565,332). The general recombinant DNA technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).

A Fab of the present invention can be obtained by treating an antibody of the invention (e.g. an IgG) with a protease, such as papaine. Also, the Fab can be produced by inserting DNA sequences encoding both chains of the Fab of the antibody into a vector for prokaryotic expression, or for eukaryotic expression, and introducing the vector into prokaryotic or eukaryotic cells (as appropriate) to express the Fab.

A F(ab')2 of the present invention can be obtained treating an antibody of the invention (e.g. an IgG) with a protease, pepsin. Also, the F(ab')2 can be produced by binding a Fab' described below via a thioether bond or a disulfide bond.

A Fab' of the present invention can be obtained by treating F(ab')2 of the invention with a reducing agent, such as dithiothreitol. Also, the Fab' can be produced by inserting DNA sequences encoding Fab' chains of the antibody into a vector for prokaryotic expression, or a vector for eukaryotic expression, and introducing the vector into prokaryotic or eukaryotic cells (as appropriate) to perform its expression.

A scFv of the present invention can be produced by taking sequences of the CDRs or VH and VL domains as previously described for the antibody of the invention, then constructing a DNA encoding a scFv fragment, inserting the DNA into a prokaryotic or eukaryotic expression vector, and then introducing the expression vector into prokaryotic or eukaryotic cells (as appropriate) to express the scFv. To generate a humanized scFv fragment, a well-known technology called CDR grafting may be used, which involves selecting the complementary determining regions (CDRs) according to the invention, and grafting them onto a human scFv fragment framework of known three dimensional structure (see, e. g., W098/45322; WO 87/02671; US5,859,205; US5,585,089; US4,816,567; EP0173494).

Modification of the antibodies of the invention

Amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody.

Modifications and changes may be made in the structure of the antibodies of the present invention, and in the DNA sequences encoding them, and still result in a functional antibody or polypeptide with desirable characteristics.

In making the changes in the amino sequences of polypeptide, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index for the interactive biologic function of a protein is generally understood in the art. It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8) ; phenylalanine (+2.8); cysteine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

A further aspect of the present invention also encompasses function-conservative variants of the polypeptides of the present invention.

For example, certain amino acids may be substituted by other amino acids in a protein structure without appreciable loss of activity. Since the interactive capacity and nature of a protein define its biological functional activity, certain amino acid substitutions can be made in a protein sequence, and of course in its encoding DNA sequence, while nevertheless obtaining a protein with like properties. It is thus contemplated that various changes may be made in the antibody sequences of the invention, or corresponding DNA sequences which encode said polypeptides, without appreciable loss of their biological activity.

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e. still obtain a biological functionally equivalent protein. It is also possible to use well-established technologies, such as alanine-scanning approaches, to identify, in an antibody or polypeptide of the invention, all the amino acids that can be substituted without significant loss of binding to the antigen. Such residues can be qualified as neutral, since they are not involved in antigen binding or in maintaining the structure of the antibody. One or more of these neutral

positions can be substituted by alanine or by another amino acid can without changing the main characteristics of the antibody or polypeptide of the invention.

Neutral positions can be seen as positions where any amino acid substitution could be incorporated. Indeed, in the principle of alanine-scanning, alanine is chosen since it this residue does not carry specific structural or chemical features. It is generally admitted that if an anlanine can be substituted for a specific amino acid without changing the properties of a protein, many other, if not all amino acid substitutions are likely to be also neutral. In the opposite case where alanine is the wild-type amino acid, if a specific substitution can be shown as neutral, it is likely that other substitutions would also be neutral.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take any of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

It may be also desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody, or e.g. to alter the binding to Fc receptors. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing inter-chain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and/or antibody-dependent cellular cytotoxicity (ADCC) (Caron PC. et al. 1992; and Shopes B. 1992). In some embodiments, an antibody of the invention may be an antibody with a modified amino acid sequence that results in reduced or eliminated binding to most Fcγ receptors, which can reduce uptake and toxicity in normal cells and tissues expressing such receptors, e.g. macrophages, liver sinusoidal cells etc.. An example for such an antibody is one including substitutions of two leucine (L) residues to alanine (A) at position 234 and 235 (i.e. LALA); this double substitution has been demonstrated to reduce Fc binding to FcγRs and consequently to decrease ADCC as well to reduce complement binding/activation. Another example for such an antibody is one including the substitution P329G in addition to the LALA double substitution (i.e. PG-LALA; see e.g. Schlothauer et al., Novel human IgG1 and IgG4 Fc-engineered antibodies with completely abolished immune effector functions, Protein Engineering, Design and Selection, Volume 29, Issue 10, October 2016, Pages 457–466). In some embodiments, an antibody of the invention may thus be an antibody having an amino acid sequence that (i) contains e.g. the LALA or the

PG-LALA set of substitutions and (ii) is otherwise identical to the amino acid sequence of one of the antibodies of the invention described herein above with reference to the respective SEQ ID NOs.

Another type of amino acid modification of the antibody of the invention may be useful for altering the original glycosylation pattern of the antibody, i.e. by deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody. The presence of either of the tripeptide sequences asparagine-X-serine, and asparagine-X-threonine, where X is any amino acid except proline, creates a potential glycosylation site. Addition or deletion of glycosylation sites to the antibody can conveniently be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites).

Another type of modification involves the removal of sequences identified, either in silico or experimentally, as potentially resulting in degradation products or heterogeneity of antibody preparations. As examples, deamidation of asparagine and glutamine residues can occur depending on factors such as pH and surface exposure. Asparagine residues are particularly susceptible to deamidation, primarily when present in the sequence Asn-Gly, and to a lesser extent in other dipeptide sequences such as Asn-Ala. When such a deamidation site, in particular Asn-Gly, is present in an antibody or polypeptide, it may therefore be considered to remove the site, typically by conservative substitution to remove one of the implicated residues. Such substitutions in a sequence to remove one or more of the implicated residues are also intended to be encompassed by the present invention.

Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N-or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, orhydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. For example, such methods are described in WO87/05330.

Removal of carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Sojahr H. et al. (1987) and by Edge, AS. et al. (1981). Enzymatic cleavage of carbohydrate

moieties on antibodies can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura, NR. et al. (1987).

Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of non-proteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, e.g. in the manner set forth in US Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Other amino acid sequence modifications known in the art may also be applied to an antibody of the invention.

Immunoconjugates of the invention

The present invention provides immunoconjugates, also referred to herein as antibody-drug conjugates or, more briefly, conjugates. As used herein, all these terms have the same meaning and are interchangeable. Suitable methods for preparing immunoconjugates are known in the art. The immunoconjugates of the invention may be prepared by in vitro methods, e.g. as described herein.

The present invention provides an immunoconjugate comprising an antibody of the invention (such as e.g. mAb1, or an antibody with the same six CDRs as mAb1) covalently linked via a linker to at least one growth inhibitory agent.

The term "growth inhibitory agent" (also referred to as an "anti-proliferative agent") refers to a molecule or compound or composition which inhibits growth of a cell, such as a tumor cell, in vitro and/or in vivo.

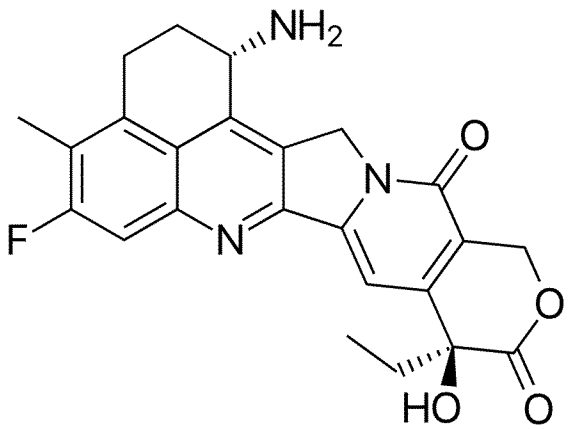
In some embodiments, the growth inhibitory agent is a cytotoxic drug (also referred to as a cytotoxic agent). In some embodiments, the growth inhibitory agent is a radioactive moiety.

The term "cytotoxic drug" as used herein refers to a substance that directly or indirectly inhibits or prevents the function of cells and/or causes destruction of the cells. The term "cytotoxic drug" includes e.g. chemotherapeutic agents, enzymes, antibiotics, toxins such as small molecule toxins or enzymatically active toxins, toxoids, vincas, taxanes, maytansinoids or maytansinoid analogs, tomaymycin or pyrrolobenzodiazepine derivatives, cryptophycin derivatives, leptomycin derivatives, auristatin or dolastatin analogs, prodrugs, topoisomerase I inhibitors, topoisomerase II inhibitors, DNA alkylating agents, anti-tubulin agents, CC-1065 and CC-1065 analogs.

Topoisomerase I inhibitors are molecules or compounds that inhibit the human enzyme topoisomerase I which is involved in altering the topology of DNA by catalyzing the transient breaking and rejoining of a single strand of DNA. Topoisomerase I inhibitors are highly toxic to dividing cells e.g. of a mammal. Examples of suitable topoisomerase I inhibitors include

camptothecin (CPT) and analogs thereof such as topotecan, irinotecan, silatecan, cositecan, exatecan, lurtotecan, gimatecan, belotecan and rubitecan.

In some embodiments, the immunoconjugates of the invention comprise the cytotoxic drug exatecan as the growth inhibitory agent. Exatecan has the chemical name (1*S*,9*S*)-1-Amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10*H*,13*H*-benzo(de)pyrano(3',4':6,7)indolizino(1,2-b)quinoline-10,13-dione. Exatecan is represented by the following structural **formula (I)**:



(I)

In further embodiments of the invention, other CPT analogs and other cytotoxic drugs may be used, e.g. as listed above. Examples of some cytotoxic drugs and of methods of conjugation are further given in the application WO2008/010101 which is incorporated by reference.

The term "radioactive moiety" refers to a chemical entity (such as a molecule, compound or composition) that comprises or consists of a radioactive isotope suitable for treating cancer, such as At^{211}, Bi^{212}, Er^{169}, I^{131}, I^{125}, Y^{90}, In^{111}, P^{32}, Re^{186}, Re^{188}, Sm^{153}, Sr^{89}, or radioactive isotopes of Lu. Such radioisotopes generally emit mainly beta-radiation. In some embodiments, the radioactive isotope is an alpha-emitter isotope, for example Thorium 227 which emits alpha-radiation. Immunoconjugates can be prepared e.g. as described in the application WO2004/091668.

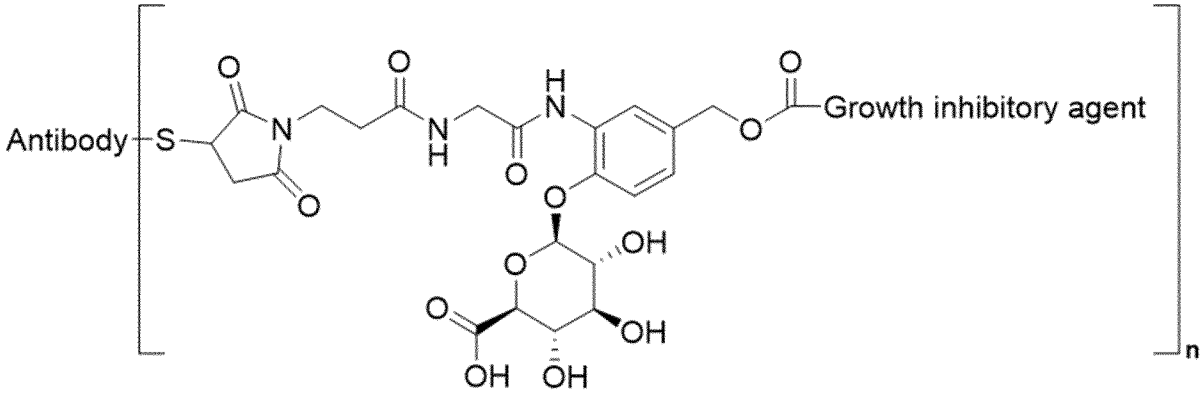
In an immunoconjugate of the present invention, an antibody of the present invention is covalently linked via a linker to the at least one growth inhibitory agent. "Linker", as used herein, means a chemical moiety comprising a covalent bond and/or any chain of atoms that covalently attaches the growth inhibitory agent to the antibody. Linkers are well known in the art and include e.g. disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Conjugation of an antibody of the invention

with cytotoxic drugs or other growth inhibitory agents may be performed e.g. using a variety of bifunctional protein coupling agents including but not limited to N-succinimidyl pyridyldithiobutyrate (SPDB), butanoic acid 4-[(5-nitro-2-pyridinyl)dithio]-2,5-dioxo-1-pyrrolidinyl ester (nitro-SPDB), 4-(Pyridin-2-yldisulfanyl)-2-sulfo-butyric acid (sulfo-SPDB), N-succinimidyl (2-pyridyldithio) propionate (SPDP), succinimidyl (N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)-hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al (1987). Carbon labeled 1-isothiocyanatobenzyl methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to an antibody (WO 94/11026).

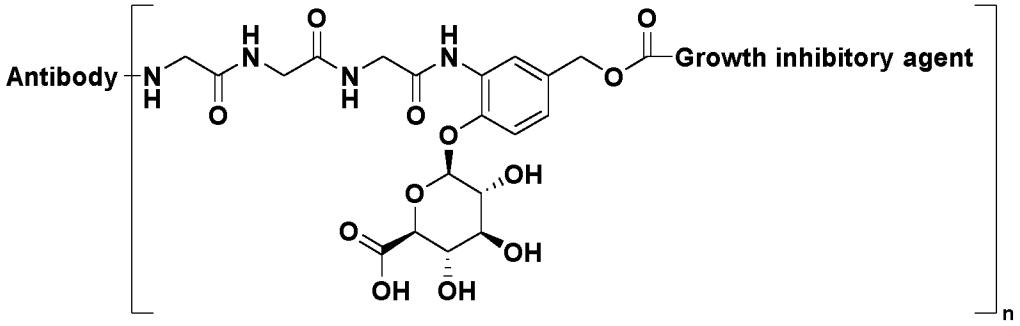
In embodiments of the present invention, the linker may be a "cleavable linker", which may facilitate release of the cytotoxic drug or other growth inhibitory agent inside of or in the vicinity of a cell, e.g. a tumor cell. In some embodiments, the linker is a linker cleavable in an endosome of a mammalian cell. For example, an acid-labile linker, a peptidase-sensitive linker, an esterase labile linker, a photolabile linker or a disulfide-containing linker (see e.g. U.S. Patent No. 5,208,020) may be used.

When referring to a structural formula representing an immunoconjugate, the following nomenclature is also used herein: a growth inhibitory agent and a linker, taken together, are also referred to as a [(linker)–(growth inhibitory agent)] moiety; for instance, an exatecan molecule and a linker, taken together, are also referred to as a [(linker)–(exatecan)] moiety.

In some specific embodiments of the present invention, the linker is a linker cleavable by the human enzyme glucuronidase. For example, an immunoconjugate of the present invention may thus have the following **formula (II)** or **formula (IIA)**, which include a linker cleavable by glucuronidase:



(II),



(IIA),

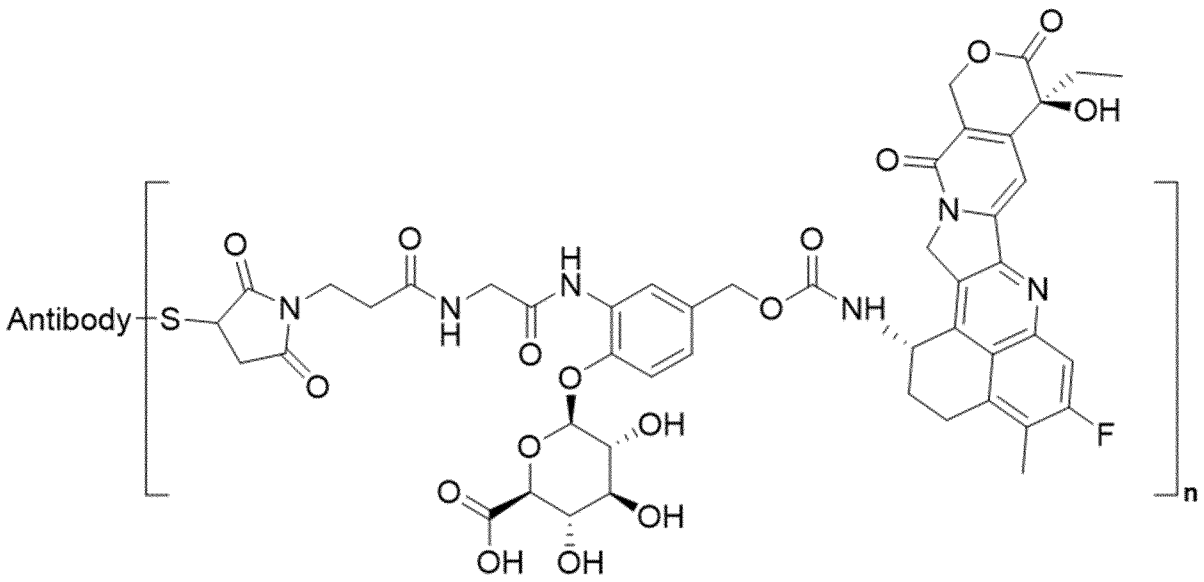
wherein the antibody is the antibody of the invention, wherein S is a sulfur atom of the antibody, and wherein n is a number of [(linker)–(growth inhibitory agent)] moieties covalently linked to the antibody. The number n may be e.g. between 1 and 10; in more specific embodiments comprising formula (II) above, n is between 7 and 8; in even more specific embodiments using formula (II) above, n is between 7.5 and 8.0 (i.e. about 8). In embodiments using formula IIA above, n is preferably between 3 and 4 and most preferably between 3.5 and 4.0 (i.e. about 4). In some embodiments, S is a sulfur atom of a cysteine of the antibody. In some embodiments, the antibody is mAb1-M, mAb2-M, mAb3-M, mAb6-M or mAb7-M.

The number n is also referred to as "drug-to-antibody ratio" (or "DAR"); this number n is always to be understood as an average number for any given (preparation of an) immunoconjugate.

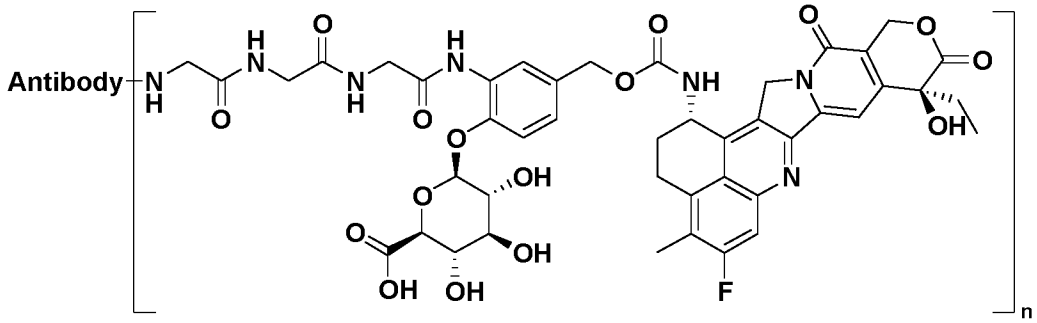
In the above formula (II), the chemical structure between the sulfur atom of the antibody and the growth inhibitory agent is a linker. One of these linkers is also contained in each of the formulae (IV) to (VIIIA) depicted further below.

In any one of the embodiments with linkers cleavable by glucuronidase, as described above, the growth inhibitory agent may be exatecan, for example.

Accordingly, in some embodiments, the present invention provides an immunoconjugate comprising an antibody according to the invention covalently linked via a linker to exatecan, wherein the conjugate has the following **formula (IV) or formula (IVA)**:



(IV),



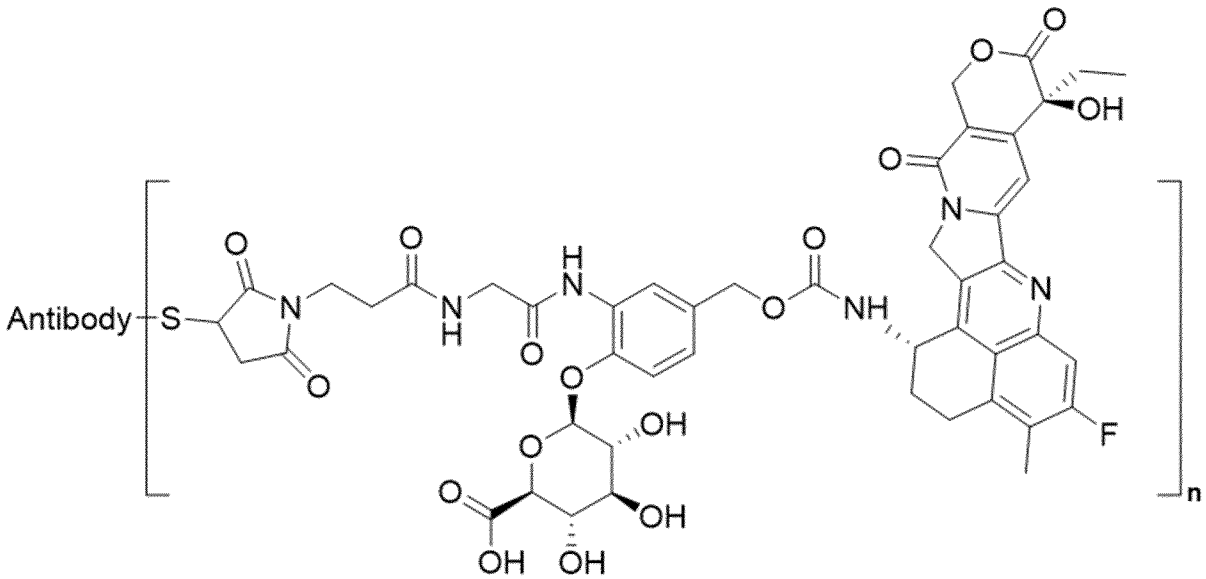
(IVA),

wherein S is a sulfur atom of the antibody, and wherein n is a number of [(linker)–(exatecan)] moieties covalently linked to the antibody. The number n (also referred to as the DAR) may be e.g. between 1 and 10; in more specific embodiments which comprise formula IV, n is between 7 and 8; in even more specific embodiments based on formula IV, n is between 7.5 and 8.0 (i.e. about 8). In embodiments using formula IVA above, n is preferably between 3 and 4 and most preferably between 3.5 and 4.0 (i.e. about 4). In some embodiments, the antibody is mAb1-M, mAb2-M, mAb3-M, mAb6-M or mAb7-M.

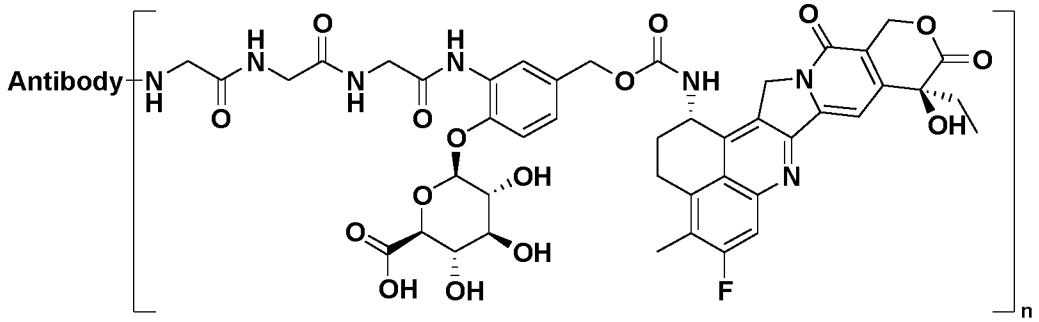
In some embodiments, in an immunoconjugate of the invention such as the exatecan conjugates with glucuronidase-cleavable linkers as described above, the linker is covalently attached to the antibody at a sulfur atom of a cysteine residue of the antibody. For example, this cysteine residue of the antibody may be one of the cysteine residues capable of forming an interchain disulfide bond (also referred to herein as an interchain disulfide bridge). As there

are four interchain disulfide bonds in an IgG1 antibody, involving a total of eight cysteine residues, attachment of the linker to the antibody at a sulfur atom of such cysteine residues provides that the DAR may be up to 8 and, in such cases, the DAR is typically between 7 and 8, such as between 7.5 and 8.0 (i.e. about 8), provided that the antibody is an IgG1 or has the same number of interchain disulfide bonds as an IgG1.

Accordingly, in some embodiments, the present invention provides an immunoconjugate comprising an antibody according to the invention covalently linked via a linker to exatecan, wherein the conjugate has the following **formula (VI) or formula (VIA)**:



(VI),



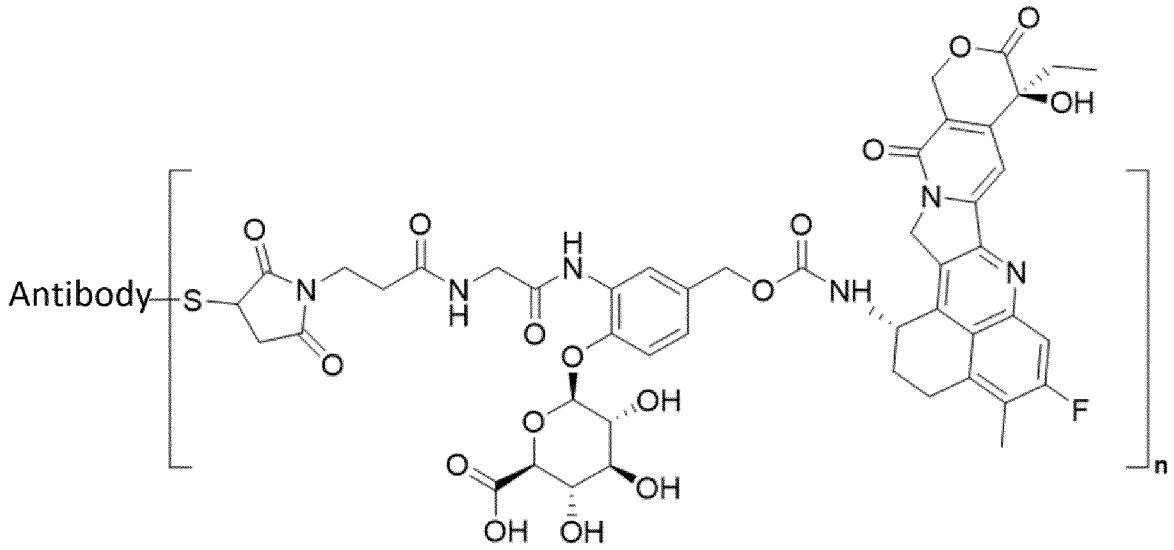
(VIA),

wherein S is a sulfur atom of a cysteine of the antibody, and wherein n is a number of [(linker)–(exatecan)] moieties covalently linked to the antibody. The number n (also referred to as the DAR) may be e.g. between 1 and 10; in more specific embodiments based on formula VI above, n is between 7 and 8; in even more specific embodiments using formula VI, n is between

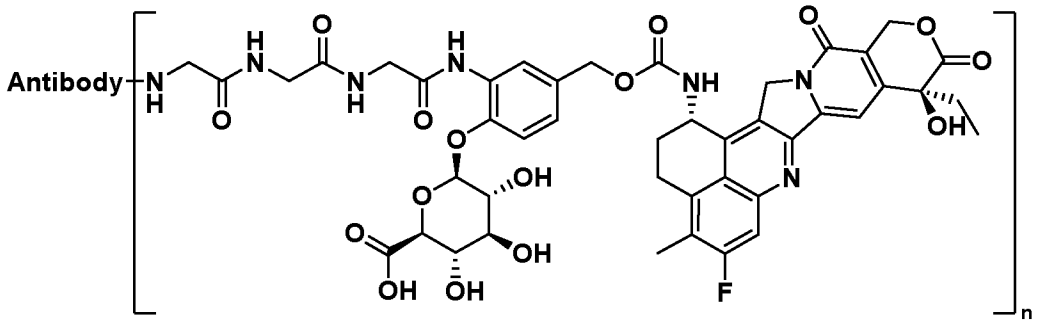
7.5 and 8.5 (preferably 8). In embodiments using formula VIA above, n is preferably between 3 and 5 and more preferably between 3.5 and 4.5 and most preferably 4.

In any of the immunoconjugates described above, any antibody of the invention (as described herein above and below) may be used. In some embodiments, the immunoconjugate of the invention comprises mAb1-M, mAb2-M, mAb3-M, mAb6-M or mAb7-M as the antibody.

Accordingly, in some embodiments, the present invention provides an immunoconjugate comprising an antibody according to the invention (preferably selected from the group consisting of mAb1-M, mAb2-M, mAb3-M, mAb6-M and mAb7-M) covalently linked via a linker to exatecan, wherein the conjugate has the following **formula (VIII) or formula (VIIIA)**:



(VIII),



(VIIIA),

wherein S is a sulfur atom of a cysteine of the antibody (preferably mAb1-M, mAb2-M, mAb3-M, mAb6-M or mAb7-M), and wherein n is a number of [(linker)–(exatecan)] moieties covalently

linked to the antibody (preferably mAb1-M, mAb2-M, mAb3-M, mAb6-M or mAb7-M). The number n (also referred to as the DAR) may be e.g. between 1 and 10; in more specific embodiments, n is between 7 and 8; in even more specific embodiments, n is between 7.5 and 8.0 (i.e. about 8). In some embodiments, S is a sulfur atom of a cysteine of the antibody (preferably mAb1-M, mAb2-M, mAb3-M, mAb6-M or mAb7-M) capable of forming an interchain disulfide bridge and the DAR is about 8. An example of such an immunoconjugate (namely "ADC1") is further described in the Examples. Preferred immunoconjugates of the invention are listed below:

Immunoconjugate name	**conjugate has the following formula**	**Antibody in conjugate**	**DAR**
A1	VIII	anti-CEACAM5 mAb LALA mutation YTE mutation	between 7.5 and 8.0
A2	VIIIA	anti-CEACAM5 mAb LALA mutation YTE mutation MTG Tag	between 3.5 and 4.5
A3	VIIIA	anti-CEACAM5 mAb LALA mutation YTE mutation K222R mutation (MTG optimization) MTG Tag	between 3.5 and 4.5
A4	VIII	anti-CEACAM5 mAb LALA mutation	between 7.5 and 8.0
A5	VIIIA	anti-CEACAM5 mAb LALA mutation MTG Tag	between 3.5 and 4.5
A6	VIII	mAb1-M	8
A7	VIIIA	mAb2-M	4
A8	VIIIA	mAb3-M	4
A9	VIII	mAb6-M	8
A10	VIIIA	mAb7-M	4

In a further preferred embodiment, the invention provides an antibody-drug conjugate, wherein the drug is exatecan and wherein the antibody-drug conjugate comprises any one of the following (i) through (v):

(i) an anti-CEACAM5 monoclonal antibody that comprises a light chain that comprises the amino acid sequence of SEQ ID NO:14 and a heavy chain that comprises the amino acid sequence of SEQ ID NO:34 and wherein exatecan is linked to said antibody via the linker-type and conjugation type shown for ADC1-M in Table 4; or

(ii) an anti-CEACAM5 monoclonal antibody that comprises a light chain that comprises the amino acid sequence of SEQ ID NO:36 and a heavy chain that comprises the amino acid sequence of SEQ ID NO:34 and wherein exatecan is linked to said antibody via the linker-type and conjugation type shown for ADC2-M in Table 4; or

(iii) an anti-CEACAM5 monoclonal antibody that comprises a light chain that comprises the amino acid sequence of SEQ ID NO:36 and a heavy chain that comprises the amino acid sequence of SEQ ID NO:35 and wherein exatecan is linked to said antibody via the linker-type and conjugation type shown for ADC3-M in Table 4; or

(iv) an anti-CEACAM5 monoclonal antibody that comprises a light chain that comprises the amino acid sequence of SEQ ID NO:14 and a heavy chain that comprises the amino acid sequence of SEQ ID NO:51 and wherein exatecan is linked to said antibody via the linker-type and conjugation type shown for ADC6-M in Table 4; or

(v) an anti-CEACAM5 monoclonal antibody that comprises a light chain that comprises the amino acid sequence of SEQ ID NO:36 and a heavy chain that comprises the amino acid sequence of SEQ ID NO:51 and wherein exatecan is linked to said antibody via the linker-type and conjugation type shown for ADC7-M in Table 4.

In a further preferred embodiment the invention provides an antibody-drug conjugate selected from ADC1-M, ADC2-M, ADC3-M, ADC6-M and ADC7-M having all the characteristics for the respective ADC as outlined in Table 4, including the respective DAR as shown in Table 4.

In other embodiments of the present invention, the linker may be a "non-cleavable linker" (for example an SMCC linker). Release of the growth inhibitory agent from the antibody can occur upon lysosomal degradation of the antibody.

In other embodiments of the invention, the immunoconjugate may be a fusion protein comprising an antibody of the invention and a cytotoxic or growth inhibitory polypeptide (as the growth inhibitory agent); such fusion proteins may be made by recombinant techniques or by peptide synthesis, i.e. methods well known in the art. A molecule of encoding DNA may comprise respective regions encoding the two portions of the conjugate (antibody and cytotoxic

or growth inhibitory polypeptide, respectively) either adjacent to one another or separated by a region encoding a linker peptide.

The antibodies of the present invention may also be used in directed enzyme prodrug therapy such as antibody-directed enzyme prodrug therapy by conjugating the antibodies to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active cytotoxic drug (see, for example, WO 88/07378 and U.S. Patent No. 4,975,278). The enzyme component of an immunoconjugate useful for ADEPT may include any enzyme capable of acting on a prodrug in such a way as to convert it into its more active, cytotoxic form. Enzymes that are useful in this context include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic fluorocytosine into the anticancer drug 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as O-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; P-lactamase useful for converting drugs derivatized with P- lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. The enzymes can be covalently bound to the antibodies of the invention by techniques well known in the art, such as the use of the linkers discussed above.

Suitable methods for preparing an immunoconjugate of the invention are well known in the art (see e.g. Hermanson G. T., Bioconjugate Techniques, Third Edition, 2013, Academic Press). For instance, methods of conjugating a cytotoxic drug to an antibody via a linker that attaches covalently to cysteine residues of interchain disulfide bridges of the antibody are well known.

In general, an immunoconjugate of the present invention can be obtained e.g. by a process comprising the steps of:

(i) preparing a compound comprising the linker and the growth inhibitory agent (e.g. cytotoxic drug), also referred to herein as a “drug-linker compound”;

(ii) bringing into contact an optionally buffered aqueous solution of an antibody according to the invention with a solution of the drug-linker compound;

(iii) then optionally separating the conjugate which was formed in (ii) from the unreacted antibody and/or drug-linker compound.

The aqueous solution of antibody can be buffered with buffers such as e.g. histidine, potassium phosphate, acetate, citrate or N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes buffer). The buffer may be chosen depending upon the nature of the antibody. The drug-linker compound can be dissolved e.g. in an organic polar solvent such as dimethyl sulfoxide (DMSO) or dimethylacetamide (DMA).

For conjugation to the cysteine residues of an antibody, the antibody is subjected to reduction (e.g. using TCEP) before step (ii). Suitable reduction conditions to reduce only the interchain disulfide bonds are known in the art.

The reaction temperature for conjugation is usually between 20 and 40°C. The reaction time can vary and is typically from 1 to 24 hours. The reaction between the antibody and the drug-linker compound can be monitored by size exclusion chromatography (SEC) with a refractometric and/or UV detector. If the conjugate yield is too low, the reaction time can be extended.

A number of different chromatography methods can be used by the person skilled in the art in order to perform the separation of step (iii): the conjugate can be purified e.g. by SEC, adsorption chromatography (such as ion exchange chromatography, IEC), hydrophobic interaction chromatography (HIC), affinity chromatography, mixed-support chromatography such as hydroxyapatite chromatography, or high performance liquid chromatography (HPLC) such as reverse-phase HPLC. Purification by dialysis or filtration or diafiltration can also be used.

After step (ii) and/or (iii), the conjugate-containing solution can be subjected to an additional step (iv) of purification e.g. by chromatography, ultrafiltration and/or diafiltration. Such an additional step of purification e.g. by chromatography, ultrafiltration and/or diafiltration can also be performed with the antibody-containing solution after the reduction reaction, in cases where reduction is performed prior to conjugation.

The conjugate is recovered at the end of such a process in an aqueous solution. The drug-to-antibody ratio (DAR) is a number that can vary with the nature of the antibody and of the drug-linker compound used along with the experimental conditions used for the conjugation (such as the ratio (drug-linker compound)/(antibody), the reaction time, the nature of the solvent and of the cosolvent if any). Thus, the contact between the antibody and the drug-linker compound can lead to a mixture comprising several conjugates differing from one another by different drug-to-antibody ratios. The DAR that is determined is thus an average value.

Performing conjugation at the cysteine residues of interchain disulfide bridges using an antibody that has four interchain disulfide bridges (e.g. mAb1 or any IgG1 antibody) – which is a method well known in the art – offers the advantage that a relatively homogeneous DAR of

about 8 can be achieved by choosing reaction conditions that allow conjugation to proceed to completion (or at least close to completion).

An exemplary method which can be used to determine the DAR consists of measuring spectrophotometrically the ratio of the absorbance at of a solution of purified conjugate at λ_D and 280 nm. 280 nm is a wavelength generally used for measuring protein concentration, such as antibody concentration. The wavelength λ_D is selected so as to allow discriminating the drug from the antibody, i.e. as readily known to the skilled person, λ_D is a wavelength at which the drug has a high absorbance and λ_D is sufficiently remote from 280 nm to avoid substantial overlap in the absorbance peaks of the drug and antibody. For instance, λ_D may be selected as being 370 nm for exatecan (or for camptothecin or other camptothecin analogs), or 252 nm for maytansinoid molecules.

A method of DAR calculation may be derived e.g. from Antony S. Dimitrov (ed), LLC, 2009, Therapeutic Antibodies and Protocols, vol 525, 445, Springer Science: The absorbances for the conjugate at λ_D ($A_{\lambda D}$) and at 280 nm (A_{280}) are measured either on the monomeric peak of the size exclusion chromatography (SEC) analysis (allowing to calculate the "DAR(SEC)" parameter) or using a classic spectrophotometer apparatus (allowing to calculate the "DAR(UV)" parameter). The absorbances can be expressed as follows:

$A_{\lambda D} = (C_D \times \varepsilon_{D\lambda D}) + (C_A \times \varepsilon_{A\lambda D})$

$A_{280} = (C_D \times \varepsilon_{D280}) + (C_A \times \varepsilon_{A280})$

wherein :

• C_D and C_A are respectively the concentrations in the solution of the drug and of the antibody

• $\varepsilon_{D\lambda D}$ and ε_{D280} are respectively the molar extinction coefficients of the drug at λ_D and 280 nm

• $\varepsilon_{A\lambda D}$ and ε_{A280} are respectively the molar extinction coefficients of the antibody at λ_D and 280 nm.

Resolution of these two equations with two unknowns leads to the following equations:

$C_D = [(\varepsilon_{A280} \times A_{\lambda D}) - (\varepsilon_{A\lambda D} \times A_{280})] / [(\varepsilon_{D\lambda D} \times \varepsilon_{A280}) - (\varepsilon_{A\lambda D} \times \varepsilon_{D280})]$

$C_A = [A_{280} - (C_D \times \varepsilon_{D280})] / \varepsilon_{A280}$

The average DAR is then calculated from the ratio of the drug concentration to that of the antibody: $DAR = C_D / C_A$.

An alternative method for preparing an immunoconjugate of the invention is described in the following. This method can in particular be used for antibodies that comprise comprises an amino acid sequence selected from the group consisting of GGTLQSPP, LLQGA,

GGLLQGPP, TLQSG, TLQSPP and TLQSA in at least one of its light chain constant regions (CL) and/or in at least one of its heavy chain constant regions (CH).Thus, a further aspect of the invention relates to a method for producing an antibody-linker-conjugate comprising the steps:

(1) providing an antibody that comprises an amino acid sequence selected from the group consisting of GGTLQSPP, LLQGA, GGLLQGPP, TLQSG, TLQSPP and TLQSA and more preferably the amino acid sequence TLQSPP or GGTLQSPP (most preferably the sequence GGTLQSPP) in at least one and preferably both of its light chain constant regions (CL) and/or in at least one and preferably both of its heavy chain constant regions (CH);

(2) mixing together in a reaction buffer at least the following components:

(a) said antibody provided in step (1);

(b) a microbial transglutaminase preferably a transglutaminase comprising the amino acid sequence **MGSGSGSGTGEEKRSYAETHRLTADDVDDINALNESAPAASSAGPS**FRAPDSDERVTPPAEPLDRMPDPYRPSYGRAETIVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNSGQYPTNRLAFAFFDEDKYKNELKNGRPRSGETRAEFEGRVAKDSFDEAKGFQRARDVASVMNKALENAHDEGAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKDRNGGNHDPSKMKAVIYSKHFWSGQDRSGSSDKRKYGDPEAFRPDRGTGLVDMSRDRNIPRSPTSPGESFVNFDYGWFGAQTEADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSDGYSDFDRGAYVVTFVPKSWNTAPDKVTQGWP (SEQ ID NO: 44); or FRAPDSDERVTPPAEPLDRMPDPYRPSYGRAETIVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNSGQYPTNRLAFAFFDEDKYKNELKNGRPRSGETRAEFEGRVAKDSFDEAKGFQRARDVASVMNKALENAHDEGAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKDRNGGNHDPSKMKAVIYSKHFWSGQDRSGSSDKRKYGDPEAFRPDRGTGLVDMSRDRNIPRSPTSPGESFVNFDYGWFGAQTEADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSDGYSDFDRGAYVVTFVPKSWNTAPDKVTQGWPLE (SEQ ID NO: 47); or FRAPDSDERVTPPAEPLDRMPDPYRPSYGRAETIVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNSGQYPTNRLAFAFFDEDKYKNELKNGRPRSGETRAEFEGRVAKDSFDEAKGFQRARDVASVMNKALENAHDEGAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKDRNGGNHDPSKMKAVIYSKHFWSGQDRSGSSDKRKYGDPEAFRPDRGTGLVDMSRDRNIPRSPTSPGESFVNFDYGWFGAQTEADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSDGYSDFDRGAYVVTFVPKSWNTAPDKVTQGWP (SEQ ID NO: 45); or

DSDERVTPPAEPLDRMPDPYRPSYGRAETIVNNYIRKWQQVYSHRDGRKQQMTE
EQREWLSYGCVGVTWVNSGQYPTNRLAFAFFDEDKYKNELKNGRPRSGETRAEF
EGRVAKDSFDEAKGFQRARDVASVMNKALENAHDEGAYLDNLKKELANGNDALR
NEDARSPFYSALRNTPSFKDRNGGNHDPSKMKAVIYSKHFWSGQDRSGSSDKRK
YGDPEAFRPDRGTGLVDMSRDRNIPRSPTSPGESFVNFDYGWFGAQTEADADKT
VWTHGNHYHAPNGSLGAMHVYESKFRNWSDGYSDFDRGAYVVTFVPKSWNTAP
DKVTQGWPLE (SEQ ID NO: 48); or

DSDERVTPPAEPLDRMPDPYRPSYGRAETIVNNYIRKWQQVYSHRDGRKQQMTE
EQREWLSYGCVGVTWVNSGQYPTNRLAFAFFDEDKYKNELKNGRPRSGETRAEF
EGRVAKDSFDEAKGFQRARDVASVMNKALENAHDEGAYLDNLKKELANGNDALR
NEDARSPFYSALRNTPSFKDRNGGNHDPSKMKAVIYSKHFWSGQDRSGSSDKRK
YGDPEAFRPDRGTGLVDMSRDRNIPRSPTSPGESFVNFDYGWFGAQTEADADKT
VWTHGNHYHAPNGSLGAMHVYESKFRNWSDGYSDFDRGAYVVTFVPKSWNTAP
DKVTQGWP (SEQ ID NO: 49); and

(c) a linker that comprises an H_2N-moiety capable of reacting with the antibody from step (1) in the presence of said transglutaminase and wherein the linker is preferably a drug-linker where said linker is covalently attached to a drug;

(3) separating the antibody-linker-conjugate produced in step (2) from unreacted linker and from said transglutaminase preferably by subjecting said mixture from step (2) to a size-exclusion chromatography.

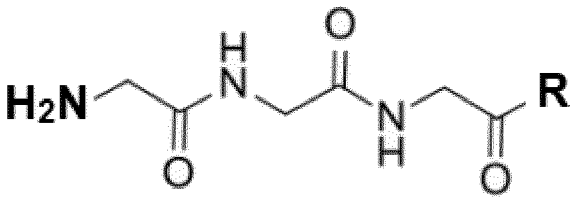
In one embodiment, the transglutaminase is encoded by the polynucleotide
ATGGGTAGCGGTAGCGGTTCAGGCACCGGTGAAGAAAAACGTAGCTATGCAGAAACC
CATCGTCTGACCGCAGATGATGTTGATGATATTAATGCACTGAATGAAAGCGCACCGG
CAGCAAGCAGCGCAGGTCCGAGCTTTCGTGCACCGGATAGTGATGAACGTGTTACCCC
TCCGGCAGAACCGCTGGATCGTATGCCGGATCCGTATCGTCCGAGCTATGGTCGTGCC
GAAACCATTGTTAATAACTATATTCGTAAGTGGCAGCAGGTTTATAGCCATCGTGATGGT
CGTAAACAGCAGATGACCGAAGAACAGCGTGAATGGCTGAGTTATGGTTGTGTTGGTGT
TACCTGGGTTAATAGCGGTCAGTATCCGACCAATCGTCTGGCATTTGCATTTTTCGATGA
GGACAAATACAAGAACGAGCTGAAAAATGGTCGTCCGCGTAGCGGTGAAACCCGTGCA
GAATTTGAAGGTCGTGTTGCAAAAGATAGCTTTGATGAAGCAAAAGGTTTTCAGCGTGC
ACGTGATGTTGCAAGCGTTATGAATAAAGCACTGGAAAATGCACATGATGAAGGTGCCT
ATCTGGACAACCTGAAAAAAGAACTGGCAAATGGTAATGATGCCCTGCGTAATGAAGAT
GCACGTAGCCCGTTTTATAGCGCACTGCGTAATACCCCGAGCTTTAAAGATCGTAATGG
TGGTAATCATGACCCGAGCAAAATGAAAGCAGTGATCTATAGCAAACACTTTTGGAGCG
GTCAGGATCGTAGTGGTAGCAGCGATAAACGTAAATATGGTGATCCGGAAGCATTTCGT
CCGGATCGTGGCACCGGTCTGGTTGATATGAGCCGTGATCGTAATATTCCGCGTAGTC

CGACCAGTCCGGGTGAAAGCTTTGTTAATTTTGATTATGGTTGGTTTGGCGCACAGACC
GAAGCAGATGCAGATAAAACCGTTTGGACCCATGGCAATCATTATCATGCACCGAATGG
TAGCCTGGGTGCAATGCATGTTTATGAAAGCAAATTTCGCAATTGGAGCGACGGCTATA
GCGATTTTGATCGTGGTGCCTATGTTGTTACCTTTGTTCCGAAAAGCTGGAATACCGCTC
CGGATAAAGTTACCCAAGGTTGGCCG*TAA* (SEQ ID NO: 46).

In a preferred embodiment of the method, said reaction buffer is 7 % DMSO, 24 mM HEPES, pH 7.0.

Preferably, the antibody used in the method of the invention further comprises a LLQGA and/or a GGLLQGPP sequence in at least one of its light chain constant regions (CL) and/or in at least one of its heavy chain constant regions (CH).

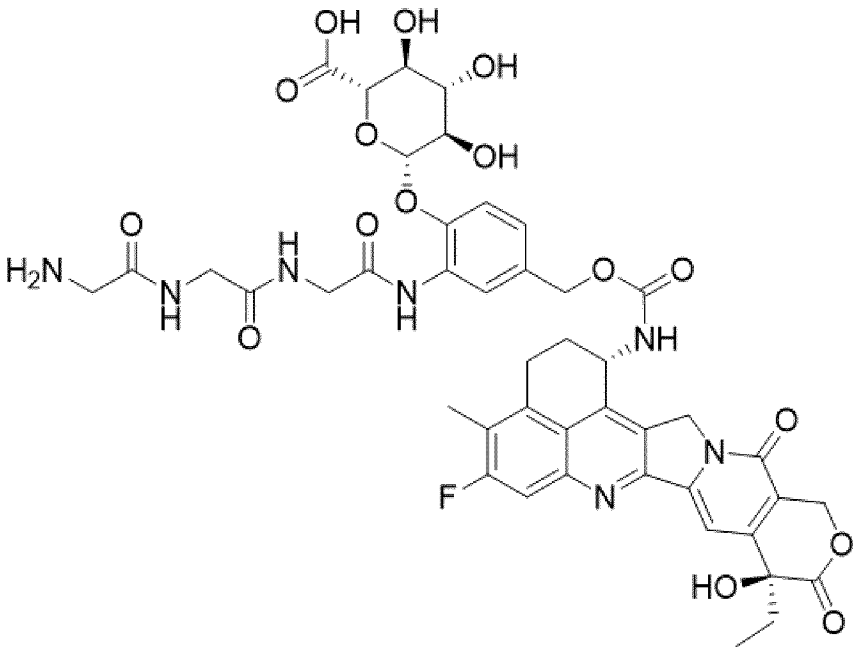
In the method of the invention, said linker is a linker having the formula



;

wherein R is the remainder of the linker and may optionally also comprise a drug, whereby the drug is preferably exatecan. In more preferred embodiments of the method of the invention the linker is NH2-GGG-beta-glucuronide.

In a preferred embodiment of the method of the invention in step (2) the mixture comprises the following drug-linker:



In a further embodiment of the method of the invention, the mixture in step (2) comprises 5 molar equivalents of linker or drug-linker, respectively, per conjugation site, wherein a conjugation site is a sequence LLQGA, GGLLQGPP, GGTLQSPP, TLQSG, TLQSPP or TLQSA comprised in the light chain constant regions (CL) and/or in the heavy chain constant region (CH) of said antibody.

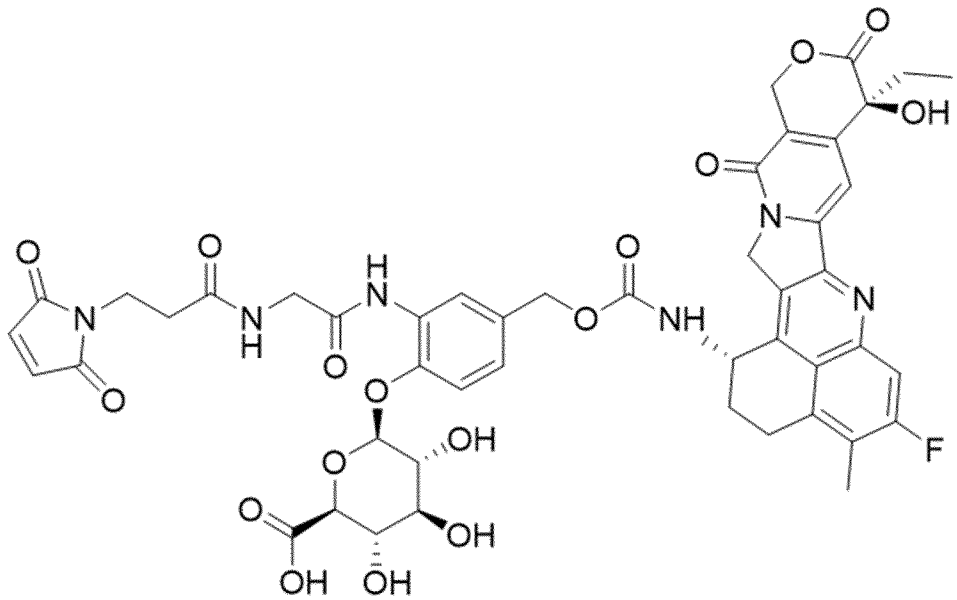
In a further embodiment of the method of the invention, the antibody is an anti-CEACAM5 antibody of the invention as described herein and/or the drug is a growth inhibitory agent as defined herein.

A further aspect of the invention relates to an antibody-linker conjugate producible according to the method of the invention, wherein the linker is preferably a linker or drug linker as described herein in the context of the inventive ADCs.

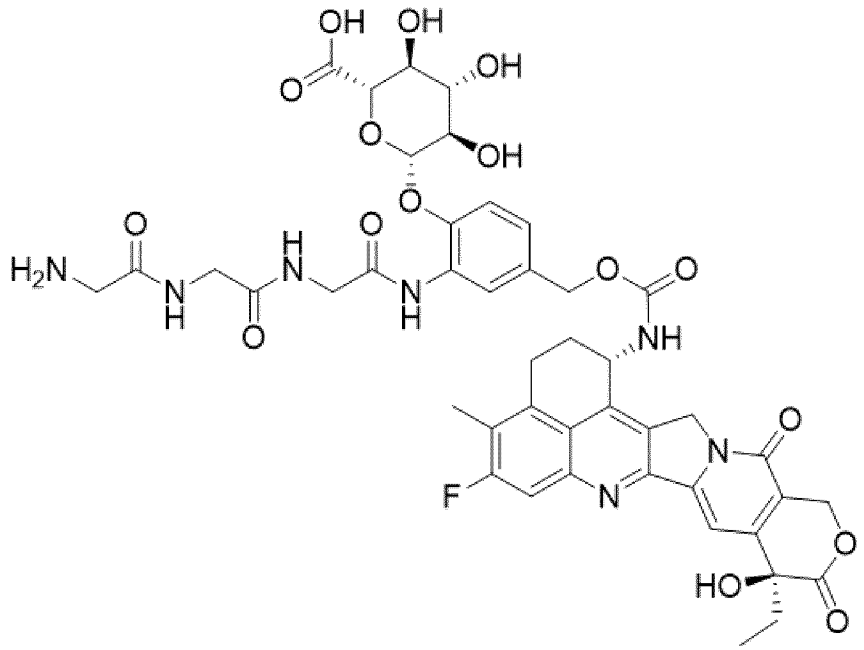
Exemplary methods for preparing an immunoconjugate of the invention are described in the Examples.

Drug-linker compounds

The present invention also provides compounds comprising a linker and a growth inhibitory agent (e.g. a cytotoxic drug), also referred to herein as “drug-linker compounds”. For instance, the present invention provides a compound of the following **formula (X) or formula (XA)**:



(X),



(XA),

or a physiologically acceptable salt thereof; the compound according to formula X is also referred to herein as “drug-linker compound 1”, “compound DL1” or “DL1” and the compound according to formula XA is also referred to herein as “drug-linker compound 1-M”, “compound DL1-M” or “DL1-M”.

These drug-linker compounds may be used to prepare immunoconjugates of the invention as described herein above and below.

The drug-linker compounds of the invention (e.g. those of **formula (X) or (XA)** depicted above) may be prepared by chemical synthesis, for instance as described in the Examples further below.

Pharmaceutical compositions

The antibodies or immunoconjugates of the invention may be combined with pharmaceutically acceptable carriers, diluents and/or excipients, and optionally with sustained-release matrices including but not limited to the classes of biodegradable polymers, non-biodegradable polymers, lipids or sugars, to form pharmaceutical compositions.

Thus, another aspect of the invention relates to a pharmaceutical composition comprising an antibody or an immunoconjugate of the invention and a pharmaceutically acceptable carrier, diluent and/or excipient.

"Pharmaceutical" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other unwanted reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier, diluent or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

As used herein, "pharmaceutically acceptable carriers" include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, and the like that are physiologically compatible. Examples of suitable carriers, diluents and/or excipients include, but are not limited to, one or more of water, amino acids, saline, phosphate buffered saline, buffer phosphate, acetate, citrate, succinate; amino acids and derivates such as histidine, arginine, glycine, proline, glycylglycine; inorganic salts such as NaCl or calcium chloride; sugars or polyalcohols such as dextrose, glycerol, ethanol, sucrose, trehalose, mannitol; surfactants such as polysorbate 80, polysorbate 20, poloxamer 188; and the like, as well as combination thereof. In many cases, it will be useful to include isotonic agents, such as sugars, polyalcohols, or sodium chloride in a pharmaceutical composition, and the formulation may also contain an antioxidant such as tryptamine and/or a stabilizing agent such as Tween 20.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and gender of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous or intraocular administration and the like.

In an embodiment, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation for injection. These may be isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical composition can be administrated through drug combination devices.

The doses used for the administration can be adapted as a function of various parameters, and for instance as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

To prepare pharmaceutical compositions, an effective amount of the antibody or immunoconjugate of the invention may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions; in all such cases, the form must be sterile and injectable with the appropriate device or system for delivery without degradation, and it must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions of active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

An antibody or immunoconjugate of the invention can be formulated into a pharmaceutical composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, or mandelic acid, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, glycine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In some cases, it may be desirable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compounds in the required amount in the appropriate solvent with any of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions can be prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of

preparation include vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more concentrated, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions can be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution can be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The antibody or immunoconjugate of the invention may be formulated within a therapeutic mixture to comprise e.g. about 0.01 to 100 milligrams per dose or so.

In addition to the antibody or immunoconjugate formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include e.g. tablets or other solids for oral administration, time release capsules, and any other form currently used.

In some embodiments, the use of liposomes and/or nanoparticles is contemplated for the introduction of polypeptides into host cells. The formation and use of liposomes and/or nanoparticles are known to those of skill in the art.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) are generally designed using polymers able to be degraded in vivo. Biodegradable polyalkyl-cyanoacrylate nanoparticles, or biodegradable polylactide or polylactide coglycolide

nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be easily made by those of skill in the art.

Liposomes can be formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 µm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 A, containing an aqueous solution in the core. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations.

Besides the above-mentioned examples, further pharmaceutical forms such as nanoparticles, microparticles and -capsules, implants (e.g. lipid implants), or self-solidifying or -emulsifying systems, are also contemplated.

Therapeutic methods and uses

The inventors have found that an antibody of the invention (e.g. mAb1) is able to internalize as part of the CEACAM5-antibody complex after binding. Furthermore, they have shown that such an antibody, conjugated to a cytotoxic drug (exatecan), mediates a cytotoxic effect on tumor cells in vitro. The inventors have also shown that these immunoconjugates of the invention induce a marked anti-tumor activity in vivo e.g. in murine xenograft models of human colorectal carcinoma derived from a patient, when used at a dose of 10 mg/kg, with a single injection. In fact, the immunoconjugates of the invention show broad activity in a large set of in vitro and in vivo models. Cytotoxic potency correlates well with target (CEACAM5) expression and is much lower in target-negative cells. In several cell-line-derived xenograft (CDX) and patient-derived xenograft (PDX) models of different cancer types a very good antitumor activity was demonstrated. The immunoconjugates were well tolerated in a non-human primate dose-range finding study with a side effect profile which is typical for a topoisomerase-I inhibitor chemotherapy. The preclinical data indicate a good therapeutic window for later clinical testing. The antibodies, immunoconjugates and pharmaceutical compositions of the invention may thus be useful for treating cancer.

Accordingly, the present invention provides the antibody, immunoconjugate or pharmaceutical composition of the invention for use as a medicament. For instance, the invention provides the antibody, immunoconjugate or pharmaceutical composition of the invention for use in the treatment of cancer. The invention further provides a method of treating cancer, comprising administering the antibody, immunoconjugate or pharmaceutical composition of the invention to a subject in need thereof.

The cancer to be treated with antibodies, immunoconjugates, or pharmaceutical compositions of the invention is preferably a cancer expressing CEACAM5, more preferably a cancer

overexpressing CEACAM5 as compared to normal (i.e. non-tumoral) cells of the same tissue origin. Expression of CEACAM5 by cells may be readily assayed for instance by using an antibody according to the invention (or a commercially available anti-CEACAM5 antibody), for instance as described in the following section "Diagnostic uses", and e.g. by an immunohistochemical method.

In some embodiments, the cancer to be treated with antibodies, immunoconjugates, or pharmaceutical compositions of the invention is a colorectal cancer, non-small-cell lung carcinoma, pancreatic cancer, gastric cancer, cervical cancer, esophageal cancer (e.g. esophageal adenocarcinoma), cholangiocarcinoma, breast cancer, prostate cancer, ovarian cancer, urothelial cancer, bladder cancer, or cancer of the stomach, uterus, endometrium, thyroid, or skin. In some specific embodiments, the cancer to be treated with antibodies, immunoconjugates, or pharmaceutical compositions of the invention is colorectal cancer, gastric cancer, non-small cell lung cancer, pancreatic cancer, esophageal cancer or prostate cancer.

The antibodies or immunoconjugates of the invention may be used in cancer therapy alone or in combination with any suitable growth inhibitory agent.

The antibodies of the invention may be conjugated (linked) to a growth inhibitory agent, as described above. Antibodies of the invention may thus be useful for targeting said growth inhibitory agent to cancerous cells expressing or over-expressing CEACAM5 on their surface.

It is also well known that therapeutic monoclonal antibodies can lead to the depletion of cells bearing the antigen specifically recognized by the antibody. This depletion can be mediated through at least three mechanisms: antibody mediated cellular cytotoxicity (ADCC), complement dependent lysis, and direct inhibition of tumor growth through signals mediated by the antigen targeted by the antibody.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which antibodies bound to Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell. To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed.

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system to antibodies which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g. as described in

Gazzano-Santoro et al. (Journal of Immunological Methods. 1997 Mar;202(2):163-171) may be performed.

In some embodiments, an antibody of the invention may be an antibody with a modified amino acid sequence that results in reduced or eliminated binding to most Fcγ receptors, which can reduce uptake and toxicity in normal cells and tissues expressing such receptors, e.g. macrophages, liver sinusoidal cells etc..

An aspect of the invention relates to a method of treating cancer, comprising administering a therapeutically effective amount of the antibody, immunoconjugate or pharmaceutical composition of the invention to a subject in need thereof.

In the context of the invention, the term "treating" or "treatment", as used herein, means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such disorder or condition. By the term "treating cancer" as used herein is meant the inhibition of the growth of malignant cells of a tumor and/or the progression of metastases from said tumor. Such treatment can also lead to the regression of tumor growth, i.e., the decrease in size of a measurable tumor. For instance, such treatment can lead to the complete regression of the tumor or metastasis.

In the context of the therapeutic applications of the present invention, the term “subject” or "patient” or “subject in need thereof” or “patient in need thereof” refers to a subject (e.g. a human or non-human mammal) affected or likely to be affected by a tumor. For instance, said patient may be a patient who has been determined to be susceptible to a therapeutic agent targeting CEACAM5, in particular to an antibody or immunoconjugate according to the invention, for instance according to a method as described herein below.

By a "therapeutically effective amount" is meant a sufficient amount to treat said cancer disease at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the antibodies, immunoconjugates and pharmaceutical compositions (collectively referred to as the “therapeutic agent”) of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific therapeutic agent employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific therapeutic agent employed; the duration of the treatment; drugs used in combination or coincidental with the specific therapeutic agent employed; and like factors well known in the medical arts. For example, it is well known within the skill of the art to start doses

of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

The antibody, immunoconjugate or pharmaceutical composition of the invention may also be used for inhibiting the progression of metastases of a cancer.

Antibodies, immunoconjugates or pharmaceutical compositions of the invention may also be used in combination with any other therapeutic intervention for treating a cancer (e.g. adjuvant therapy) and/or for reducing the growth of a metastatic cancer. For instance, the other therapeutic intervention for such combination may be a standard-of-care (SOC) therapeutic agent for the cancer to be treated.

Efficacy of the treatment with an antibody or immunoconjugate or pharmaceutical composition according to the invention may be readily assayed in vivo, for instance in a mouse model of cancer and by measuring e.g. changes in tumor volume between treated and control groups, % tumor regression, partial regression or complete regression.

Diagnostic uses

CEACAM5 has been reported to be highly expressed on the surface of cancer cells such as e.g. colorectal, gastric, lung, and pancreatic tumor cells, and expression in normal tissues is limited to a few normal epithelial cells such as colon and esophagus epithelial cells.

Therefore, CEACAM5 constitutes a cancer marker and has the potential to be used e.g. to indicate the effectiveness of an anti-cancer therapy or to detect recurrence of the disease.

In an embodiment, the antibody of the invention can be used as component of an assay in the context of a therapy targeting CEACAM5 expressing tumors, in order to determine susceptibility of the patient to the therapeutic agent, monitor the effectiveness of the anti-cancer therapy or detect recurrence of the disease after treatment. In some embodiments, the same antibody of the invention can be used both as component of the therapeutic agent and as component of the diagnostic assay.

Thus, a further aspect of the invention relates to a use of an antibody according to the invention for detecting CEACAM5 expression *ex vivo* in a biological sample from a subject. Another aspect of the invention relates to the use of an antibody of the invention for detecting CEACAM5 expression *in vivo* in a subject. When used for detection of CEACAM5, the antibody may be labelled with a detectable molecule such as e.g. a fluorophore or an enzyme.

Detection of CEACAM5 may be intended for e.g.

a) diagnosing the presence of a cancer in a subject, or

b) determining susceptibility of a patient having cancer to a therapeutic agent targeting CEACAM5, in particular an antibody or immunoconjugate according to the invention, or

c) monitoring effectiveness of an anti-CEACAM5 cancer therapy or detecting a cancer relapse after anti-CEACAM5 cancer therapy, in particular wherein said therapy is therapy with an antibody or immunoconjugate according to the invention;

by detecting expression of the surface protein CEACAM5 on tumor cells.

In embodiments, the antibody is intended for an in vitro or ex vivo diagnostic use. For example, CEACAM5 may be detected using an antibody of the invention in vitro or ex vivo in a biological sample obtained from a subject. Use according to the invention may also be an in vivo use. For example, an antibody according to the invention can be administered to the subject and antibody-cell complexes can be detected and/or quantified, whereby the detection of said complexes is indicative of a cancer.

The invention further relates to an in vitro or ex vivo method of detecting the presence of a cancer in a subject, comprising the steps of:

(a) contacting a biological sample from a subject with an antibody according to the invention, in particular in conditions suitable for the antibody to form complexes with said biological sample;

(b) measuring the level of antibody bound to said biological sample; and

(c) detecting the presence of a cancer by comparing the measured level of bound antibody with a control, an increased level of bound antibody compared to control being indicative of a cancer.

The invention also relates to an in vitro or ex vivo method of determining susceptibility of a patient having cancer to a therapeutic agent targeting CEACAM5, in particular an antibody or immunoconjugate according to the invention, which method comprises the steps of:

(a) contacting a biological sample from a patient having cancer with an antibody according to the invention, in particular in conditions suitable for the antibody to form complexes with said biological sample;

(b) measuring the level of antibody bound to said biological sample; and

(c) comparing the measured level of bound antibody to said biological sample with the level of antibody bound to a control;

wherein an increased level of bound antibody to said biological sample compared to control is indicative of a patient susceptible to a therapeutic agent targeting CEACAM5.

In the above methods, said control can be a normal, non-cancerous biological sample of the same type, or a reference value determined as representative of the antibody binding level in a normal biological sample of the same type.

In an embodiment, the antibodies of the invention are useful for diagnosing a CEACAM5 expressing cancer, such as a colorectal cancer, gastric cancer, non-small cell lung cancer, pancreatic cancer, esophageal cancer, prostate cancer or other solid tumors expressing CEACAM5.

The invention further relates to an in vitro or ex vivo method of monitoring effectiveness of anti-CEACAM5 cancer therapy, comprising the steps of:

(a) contacting a biological sample from a subject undergoing anti-CEACAM5 cancer therapy with an antibody according to the invention, in particular in conditions suitable for the antibody to form complexes with said biological sample;

(b) measuring the level of antibody bound to said biological sample; and

(c) comparing the measured level of bound antibody with the level of antibody bound to a control;

wherein a decreased level of bound antibody to said biological sample compared to control is indicative of effectiveness of said anti-CEACAM5 cancer therapy. In said method, an increased level of bound antibody to said biological sample compared to control would be indicative of ineffectiveness of said anti-CEACAM5 cancer therapy. In an embodiment of this method of monitoring effectiveness, said control is a biological sample of the same type as the biological sample submitted to analysis, but which was obtained from the subject at an earlier time point during the course of the anti-CEACAM5 cancer therapy.

The invention further relates to an in vitro or ex vivo method of detecting cancer relapse after anti-CEACAM5 cancer therapy, comprising the steps of:

(a) contacting a biological sample from a subject, the subject having completed anti-CEACAM5 cancer therapy, with an antibody according to the invention, in particular in conditions suitable for the antibody to form complexes with said biological sample;

(b) measuring the level of antibody bound to said biological sample; and

(c) comparing the measured level of bound antibody with the level of antibody bound to a control;

wherein an increased level of bound antibody to said biological sample compared to control is indicative of cancer relapse after anti-CEACAM5 cancer therapy. Said control may be, in particular, a biological sample of the same type as the biological sample submitted to analysis,

but which was obtained from the subject previously, namely upon or after completion of the anti-CEACAM5 cancer therapy.

Said anti-CEACAM5 cancer therapy is e.g. a therapy using an antibody or immunoconjugate according to the invention. Said anti-CEACAM5 cancer therapy targets a CEACAM5 expressing cancer, such as a colorectal cancer, gastric cancer, non-small cell lung cancer, pancreatic cancer, esophageal cancer, prostate cancer or other solid tumors expressing CEACAM5.

In some embodiments, antibodies of the invention may be labelled with a detectable molecule or substance, such as a fluorescent molecule or fluorophore, a radioactive molecule, an enzyme or any other labels known in the art that provide (either directly or indirectly) a signal.

As used herein, the term "labeled", with regard to the antibody according to the invention, is intended to encompass direct labeling of the antibody by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the polypeptide, as well as indirect labeling of the polypeptide by reactivity with a detectable substance.

An antibody of the invention may be labelled with a radioactive molecule by any method known to the art. For example, radioactive molecules include but are not limited to radioactive atoms for scintigraphic studies such as I^{123}, I^{124}, In^{111}, Re^{186}, Re^{188}, Tc^{99}. Antibodies of the invention may also be labelled with a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as iodine-123, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

A "biological sample" encompasses a variety of sample types obtained from a subject that can be used in a diagnostic or monitoring assay. Biological samples include but are not limited to blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. Therefore, biological samples encompass clinical samples, cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples, such as tumor samples.

In some embodiments, the biological sample may be a formalin-fixed and paraffin-embedded (FFPE) tissue sample.

The invention also relates to an in vivo method of detecting the presence of a cancer in a subject, comprising the steps of:

a) administering an antibody according to the invention to a patient, wherein the antibody is labelled with a detectable molecule;

b) detecting localization of said antibody in the patient by imaging, e.g. by detecting the detectable molecule.

In said method, the cancer may be a CEACAM5 expressing cancer, such as a colorectal cancer, gastric cancer, non-small cell lung cancer, pancreatic cancer, esophageal cancer, prostate cancer or other solid tumors expressing CEACAM5.

Antibodies of the invention may also be useful for staging of cancer (e.g., in radioimaging). They may be used alone or in combination with other cancer markers.

The terms "detection" or "detected" as used herein include qualitative and/or quantitative detection (i.e. measuring levels) with or without reference to a control.

In the context of the invention, the term "diagnosing", as used herein, means the determination of the nature of a medical condition, intended to identify a pathology which affects the subject, based on a number of collected data.

Kits

Finally, the invention also provides kits comprising at least one antibody or immunoconjugate of the invention. Kits containing antibodies of the invention can find use in detecting the surface protein CEACAM5, or in therapeutic or diagnostic assays. Kits of the invention can contain an antibody coupled to a solid support, e.g., a tissue culture plate or beads (e.g., sepharose beads). Kits can be provided which contain antibodies for detection and quantification of the surface protein CEACAM5 in vitro, e.g. in an ELISA or a Western blot. Such an antibody useful for detection may be provided with a label such as a fluorescent or radiolabel.

BRIEF DESCRIPTION OF THE SEQUENCES

Amino acid sequences:

SEQ ID NO: 1	Human CEACAM5 protein sequence according to GenBank accession number AAA51967.1
SEQ ID NO: 2	*Macaca fascicularis* CEACAM5 protein sequence (NCBI Reference Sequence XP_005589491.1)
SEQ ID NO: 3	CDR1-H of mAb1
SEQ ID NO: 4	CDR2-H of mAb1
SEQ ID NO: 5	CDR3-H of mAb1
SEQ ID NO: 6	CDR1-L of mAb1
SEQ ID NO: 7	CDR2-L of mAb1

SEQ ID NO: 8 CDR3-L of mAb1

SEQ ID NO: 9 VH of mAb1

SEQ ID NO: 10 VL of mAb1

SEQ ID NO: 11 CH of mAb1

SEQ ID NO: 12 CL of mAb1, of mAb1-M and of mAb6-M

SEQ ID NO: 13 HC of mAb1

SEQ ID NO: 14 LC of mAb1, of mAb1-M and of mAb6-M

Nucleic acid sequences:

SEQ ID NO: 15 DNA sequence encoding HC of mAb1

SEQ ID NO: 16 DNA sequence encoding LC of mAb1

Amino acid sequences:

SEQ ID NO: 17 HC of antibody hu8G4

SEQ ID NO: 18 LC of antibody hu8G4

SEQ ID NO: 19 HC of optimized antibody Variant 1

SEQ ID NO: 20 LC of optimized antibody Variant 1

SEQ ID NO: 21 LC of optimized antibody Variant 2

SEQ ID NO: 22 LC of optimized antibody Variant 4

SEQ ID NO: 23 LC of optimized antibody Variant 5

SEQ ID NO: 24 HC of optimized antibody Variant 6

SEQ ID NO: 25 HC of huMab2-3 (allotype)

SEQ ID NO: 26 LC of huMab2-3

SEQ ID NO: 27 HC of hmn-14

SEQ ID NO: 28 LC of hmn-14

SEQ ID NO: 29 HC of rb8G4

SEQ ID NO: 30 LC of rb8G4

SEQ ID NO: 31 CH of mAb1-M & mAb2-M (CH-LALA-YTE)

SEQ ID NO: 32 CH of mAb3-M (CH-K222R-LALA-YTE)

SEQ ID NO: 33 CL of mAb2-M, of mAb3-M and of mAb7-M (CL-Tag)

SEQ ID NO: 34 HC of mAb1-M and of mAb2-M (HC-LALA-YTE)

SEQ ID NO: 35 HC of mAb3-M (HC-K222R-LALA-YTE)

SEQ ID NO: 36 LC of mAb2-M, of mAb3-M and of mAb7-M (LC-Tag)

SEQ ID NO: 40 HC of mAb5-M (antiCD20-HC)

SEQ ID NO: 41 LC of mAb5-M (antiCD20-LC)

SEQ ID NO: 44 Transglutaminase

SEQ ID NO: 45 Transglutaminase (activated, version1)

SEQ ID NO: 47 Transglutaminase (activated, version2)

SEQ ID NO: 48 Transglutaminase (activated, version3)

SEQ ID NO: 49 Transglutaminase (activated, version4)

SEQ ID NO: 50 CH of mAb6-M and of mAb7-M (CH-LALA)

SEQ ID NO: 51 HC of mAb6-M and of mAb7-M (HC-LALA)

SEQ ID NO: 54 Framework region FR1

SEQ ID NO: 55 Framework region FR2

SEQ ID NO: 56 Framework region FR3

SEQ ID NO: 57 Framework region FR4

SEQ ID NO: 58 Framework region FR5

SEQ ID NO: 59 Framework region FR6

SEQ ID NO: 60 Framework region FR7

SEQ ID NO: 61 Framework region FR8

Nucleic acid sequences:

SEQ ID NO: 37 DNA sequence encoding HC of mAb1-M and of mAb2-M (HC-LALA-YTE)

SEQ ID NO: 38 DNA sequence encoding HC of mAb3-M (LC-K222R-LALA-YTE)

SEQ ID NO: 39 DNA sequence encoding LC of mAb2-M, of mAb3-M and of mAb7-M (LC-Tag)

SEQ ID NO: 42 DNA sequence encoding HC of mAb5-M (antiCD20-HC)

SEQ ID NO: 43 DNA sequence encoding LC of mAb5-M (antiCD20-LC)

SEQ ID NO: 46 DNA sequence encoding Transglutaminase

SEQ ID NO: 52 DNA sequence encoding HC of mAb6-M and of mAb7-M (HC-LALA)

SEQ ID NO: 53 DNA sequence encoding LC of mAb1-M and of mAb6-M

Amino acid sequences:

SEQ ID NO: 62 labetuzumab heavy chain

SEQ ID NO: 63 labetuzumab light chain

SEQ ID NO: 64 rituximab HC

SEQ ID NO: 65 rituximab LC

EXAMPLES

Example 1: Anti-CEACAM5 antibodies

1.1 Immunization of transgenic rats and isolation of hybridomas

To generate monoclonal antibodies to human CEACAM5 protein (Carcinoembryonic antigen-related cell adhesion molecule 5; CD66e), human immunoglobulin gene transgenic rats (OmniRat™) were obtained from CHARLES RIVER LABORATORIES INTERNATIONAL INC. (WILMINGTON, MA). 5 animals were immunized 4 times with CEACAM5 cDNA (encoding amino acids 35-675 of the human CEACAM5 protein sequence with UniProt ID no. P06731; the sequence of P06731 is identical to SEQ ID NO: 1 except for the substitution of E398 of SEQ ID NO: 1 by K398) cloned into an Aldevron proprietary immunization vector (pB8-CEA-hum-MC) and was transiently transfected into the OMT Rats cells using a Gene gun.

Anti-CEACAM5 titers were evaluated by a cell-based ELISA (CELISA) assay using cells that express CEACAM5 on their cell membrane (titer results presented below). The immunized animal serum was taken at day 31 of the immunization protocol, after 4 rounds of genetic material immunization (IS31d-4). Sera, diluted in PBS + 3% FBS, were tested by flow cytometry on mammalian cells transiently transfected with the CEACAM5 cDNA cloned into an Aldevron proprietary expression vector (pB1-CEA-hum-MC). A goat anti-rat IgG R-phycoerythrin conjugate (Southern Biotech, #3030-09) was used as a secondary antibody at 10 µg/ml.

All animals were sacrificed and lymphocytes from lymph-nodes were pooled and cryo-preserved for future use. Cells were fused with the Ag8 mouse myeloma cell line to create viable hybridomas. Hybridoma cells from this fusion were then transferred to ten 96well plates.

1.2 CEACAM5 specificity

Hybridoma supernatants were screened using a cell-based ELISA (CELISA) assay for the detection of ant-CEACAM5 antibodies that did not bind CEACAM1 (BGP), CEACAM3 (CGM1a), CEACAM4 (CGM7), CEACAM6 (NCA) and CEACAM8 (NCA-95). A goat anti-rat IgG R-phycoerythrin conjugate (Southern Biotech, #3030-09) was used as a secondary antibody at 10 µg/ml.

Clones that showed specificity to human CEACAM5 and not to its related proteins were transferred to one 96well plate and the hybridoma supernatant was evaluated for specificity and cross reactivity in ELISA assay. In this assay the 8G4 hybridoma clone and its subclones showed specificity to human CEACAM5 and cross reactivity to *Macaca fascicularis* CEACAM5.

1.3 Detection of antibody sequence and cloning

Total RNA was prepared from each hybridoma clone according to the RNeasy 96 Protocol, Qiagen. Subsequently total RNA was transcribed into cDNA using Random Hexamers and SuperScript©III.

The resultant cDNA was quality-controlled by qPCR and VH and Vk were amplified by PCR. The PCR products were purified using AMpure XP PCR clean-up kit in combination with a KingFisher instrument.

The VH and Vk genes of 8G4 subclones were cloned into destination vectors hi00_pTT5_VH_ccdB and hh00_pTT5_Vk_ccdB, respectively, using the procedure of homologous recombination (so called „Lucigen-Cloning"). The reaction mixes were transformed in One Shot® Mach1™-T1R Chemically Competent *E. coli*. Correctly recombined clones were confirmed by Sanger sequencing.

1.4 Humanization and biochemical characterization of hits, and candidate selection

8G4 and other clones were reformatted and expressed as human IgG1 molecules. They were assessed by SDS-PAGE, size exclusion chromatography (SEC), selectivity, affinity, cell binding and potency. Based on the results, one humanized candidate antibody, designated as hu8G4, was selected for amino acid sequence optimization to improve manufacturability and affinity.

The amino acid sequence of the humanized candidate antibody hu8G4 is as follows:

Heavy chain:

EVQLVESGPGLVKPSQTLSLTCTVSDGSVSRGGYYLTWIRQHPGKGLEWIGYIYYSGSTYF
NPSLRSRLTMSVDTSKNQFSLKLSSVTAADTAVYYCARGIAVAPFDYWGQGTLVTVSSAST
KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS
LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLF
PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS
VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLT
CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 17)

Light chain:

ETTLTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF
SGSGSGTEFTLTIGSLQSEDFAVYFCQQYTNWPFTFGPGTKVEIKRTVAAPSVFIFPPSDEQ
LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFN RGEC (SEQ ID NO: 18)

1.5 Biophysical Improvement Strategy for hu8G4 leading *inter alia* to mAb1

An assessment of the variable region sequences of hu8G4 identified six non-germline amino acid residues in the light chain framework and two non-germline amino acid residues in the heavy chain framework. An assessment of amino acids and sequence motifs potentially prone to post-translational modification, such as deamidation motifs, surface-accessible methionines, and free cysteines, did not identify any amino acid residues with increased liability. Several designed antibody sequences were generated in which certain amino acids were replaced with the germline-associated amino acid at that position. The different VH and VL optimization designs were then co-expressed in HEK 293 6E cells as Fab and full IgG1 molecules, purified and tested (see e.g. the optimized Variants 1-10 below).

The amino acid sequences of 10 optimized antibody variants in full IgG1 format were as follows:

Variant 1 (VH1.00/VL1.00)

HC:

EVQLQESGPGLVKPSQTLSLTCTVSDGSVSRGGYYLTWIRQHPGKGLEWIGYIYYSGSTYF
NPSLRSRLTMSVDTSKNQFSLKLSSVTAADTAVYYCARGIAVAPFDYWGQGTLVTVSSAST
KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS
LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLF
PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS
VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLT
CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 19)

LC:

ETTLTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF
SGSGSGTEFTLTIGSLQSEDFAVYFCQQYTNWPFTFGPGTKVEIKRTVAAPSVFIFPPSDEQ
LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 20)

Variant 2 (VH1.00/VL1.01)

HC: SEQ ID NO: 19

LC:

EIVLTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF
SGSGSGTEFTLTISSLQSEDFAVYFCQQYTNWPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQ

LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 21)

Variant 3 (VH1.00/VL1.02)

HC: SEQ ID NO: 19

LC: SEQ ID NO: 14

Variant 4 (VH1.00/VL1.03)

HC: SEQ ID NO: 19

LC:

EIVMTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF SGSGSGTEFTLTISSLQSEDFAVYFCQQYTNWPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 22)

Variant 5 (VH1.00/VL1.04)

HC: SEQ ID NO: 19

LC:

EIVMTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF SGSGSGTEFTLTISSLQSEDFAVYYCQQYTNWPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 23)

Variant 6 (VH1.02/VL1.00)

HC:

EVQLQESGPG LVKPSQTLSL TCTVSDGSVS RGGYYLTWIR QHPGKGLEWI GYIYYSGSTY FNPSLRSRVT MSVDTSKNQF SLKLSSVTAA DTAVYYCARG IAVAPFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY ICNVNHKPSN TKVDKRVEPK SCDKTHTCPP CPAPELLGGP SVFLFPPKPK DTLMISRTPE VTCVVVDVSH EDPEVKFNWY VDGVEVHNAK TKPREEQYNS TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIEKTISK AKGQPREPQV YTLPPSREEM TKNQVSLTCL VKGFYPSDIA VEWESNGQPE NNYKTTPPVL DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM HEALHNHYTQ KSLSLSPGK (SEQ ID NO: 24)

LC: SEQ ID NO: 20

Variant 7 (VH1.02/VL1.01)

HC: SEQ ID NO: 24

LC: SEQ ID NO: 21

Variant 8 (VH1.02/VL1.02)

HC: SEQ ID NO: 24

LC: SEQ ID NO: 14

Variant 9 (VH1.02/VL1.03)

HC: SEQ ID NO: 24

LC: SEQ ID NO: 22

Variant 10 (VH1.02/VL1.04)

HC: SEQ ID NO: 24

LC: SEQ ID NO: 23

All of the optimized Variants 1 to 10 performed similarly well in terms of maintenance of quality as assayed by percent aggregate by size exclusion chromatography, maintained stability based on Fluorescence Monitored Thermal Unfolding (FMTU), retained binding to MKN-45 cancer cell line and maintained selectivity toward the target. Variant 8 (i.e. the variant including VH1.02 and VL1.02) was selected for further development as an optimized variant with a sequence particularly similar to germline.

Further sequence optimization of the selected Variant 8 was then performed *inter alia* in order to reduce IgG Fc effector functions. Compared to the parent clone, the resulting final sequence-optimized (so) clone, designated so8G4 (also referred to as mAb1 herein), shows improved affinity and improved manufacturability and, also, shows reduced or no binding to FcγRI, FcγRIIa, FcγRIIIa, FcγRIIIa/complex, C1q, FcγRIIb and FcγRIIIb, while maintaining the affinity to CEACAM5 and FcRn. The amino acid sequence of this final sequence-optimized antibody so8G4 (also referred to as mAb1 herein) is as follows:

Heavy chain (HC): SEQ ID NO: 13 (as defined herein above)

Light chain (LC): SEQ ID NO: 14 (as defined herein above)

1.6 In-vitro characterization of mAb1

Antibody mAb1 was characterized with *in vitro* assays for several properties including: binding affinity, selectivity, and internalization.

1.6.1 Binding Affinity

- To determine the binding affinity of soluble antibody analyte to captured target protein CEACAM5 (human or cynomolgus monkey *Macaca fascicularis*). The following experimental conditions were used on Octet Red instrument:
- Biosensor coated with StreptAvidin.
- Biotinylated target protein concentration (where ECD stands for extracellular domain):
    - human_CEACAM5_ECD-his-biotin R&D Systems (biotinylated using routine methods) were captured at 2.5 µg/ml for 900 seconds at 1000rpm.
    - Recombinant *Macaca fascicularis* CEACAM5_ECD-His-biotin obtained from Syngene (biotinylated using routine methods) were captured at 5 µg/ml for 900 seconds at 1000rpm.
- Analyte Antibody Concentrations: 200, 100, 50, 25, 12.5, 6.25, 0 nM.
- Binding affinity KD (equilibrium dissociation constant) values were determined with Octet Evaluation software from the measured binding kinetics association (ka) and dissociation (kd) rate constants, where KD = kd/ka
- Antibody was used in Fab format

Results for Fab generated from mAb1:

Binding affinity KD for human CEACAM5 was 6.3 ± 1.98 nM.

Binding affinity KD for cynomolgus_monkey-CEACAM5 was 14.1 ± 2.53 nM

1.6.2. Selectivity

a) Species and domains

Selectivity determination for mAb1 was done by titrating the antibody from 4nM to 0.25pM and applying it on 1µg/ml bound recombinant human (rh) CEACAM5 ECD or its domains N-A1-B1, A2-B2, A3-B3 or bound recombinant *Macaca fascicularis* (mf) CEACAM5 ECD, all obtained from Syngene, in an ELISA assay. Results are shown in Fig. 1 and summarized below:

Binding EC50 to rhCEACAM5 is 153.4pM.

Binding EC50 to rhA2-B2 domain is 166.9pM.

Binding EC50 to mfCEACAM5 is 324.3pM.

No binding to rhN-A1-B1 or rhA3-B3 or BSA (bovine serum albumin, serving as negative control) was detected.

b) Different CEACAM proteins

Selectivity determination for Fab of mAb1 to human CEACAM5 and other human CEACAM family members was done in ELISA assay. Proteins were coated on 96-well assay plates: huCEACAM5-His6 (R&D Systems # 4128-CM), huCEACAM6-His6 (3934-CM R&D Systems and recombinant protein obtained from Syngene), huCEACAM1-His6 (2244-CM R&D Systems), huCEACAM3-His6 (C449 Novoprotein), huCEACAM7-His6 (C926 Novoprotein), huCEACAM8-His6 (C583 Novoprotein), huPSG1-His6 (CC66 Novoprotein), each protein was coating the plate at 12 nM concentration.

Results:

Fab of mAb1 bound human CEACAM5 (EC50 of 3.04 nM), but did not bind the other human CEACAM family members in ELISA assay even when using 1000 nM Fab of mAb1 which is a more than 300-fold higher concentration than the EC50 for binding to human CEACAM5.

Fab of mAb1 also did not bind to unrelated protein (BSA) in ELISA assay, at all concentrations tested.

1.6.3 Cellular binding of mAb1

The antibody's ability to bind its target protein on cells was determined by titrating the antibody on cells that express the target (e.g. human CEACAM5) and measuring the fluorescence MFI of the cells. Model cells for antibody binding comparison were the MKN45 cell line expressing human CEACAM5 as well as a CHO cell line expressing mfCEACAM5. Titration was done with 10-point x4 dilution, curve starting concentration 2000 nM in assay buffer (PBSx1 containing 1% BSA).

Exemplary data:

mAb1 binding to human CEACAM5-expressing MKN-45 cell line with EC50 = 10.62 ± 1.6 nM.

mAb1 binding to mfCEACAM5-expressing CHO cell line with EC50 = 4.8 ± 0.6 nM.

1.6.4 Cellular binding comparison to known antibodies

Cellular binding of our lead antibody mAb1 was compared to ADC-related known antibodies huMab2-3 (as in the known ADC SAR408701) and hMN14 (also referred to as hmn-14 herein) (as in the known ADC labetuzumab govitecan or IMMU-130) on MKN45 cell line which expresses CEACAM5.

The following amino acid sequences were used for the above-mentioned known antibodies in the experiments described herein below:

huMab2-3:

HC (allotype):
EVQLQESGPGLVKPGGSLSLSCAASGFVFSSYDMSWVRQTPERGLEWVAYISSGGGITYA
PSTVKGRFTVSRDNAKNTLYLQMNSLTSEDTAVYYCAAHYFGSSGPFAYWGQGTLVTVSS
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSV
FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQ
VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF
SCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 25)

LC:
DIQMTQSPASLSASVGDRVTITCRASENIFSYLAWYQQKPGKSPKLLVYNTRTLAEGVPSRF
SGSGSGTDFSLTISSLQPEDFATYYCQHHYGTPFTFGSGTKLEIKRTVAAPSVFIFPPSDEQL
KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY
EKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 26)

hmn-14:

HC:
EVQLVESGGGVVQPGRSLRLSCSASGFDFTTYWMSWVRQAPGKGLEWIGEIHPDSSTINY
APSLKDRFTISRDNAKNTLFLQMDSLRPEDTGVYFCASLYFGFPWFAYWGQGTPVTVSSA
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGP
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
QGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 27)

LC:
DIQLTQSPSSLSASVGDRVTITCKASQDVGTSVAWYQQKPGKAPKLLIYWTSTRHTGVPS

RFSGSGSGTDFTFTISSLQPEDIATYYCQQYSLYRSFGQGTKVEIKRTVAAPSVFIFPPS

DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL

SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 28)

Rituximab was included in the comparison as a control. Results for antibodies binding to the cells are shown in Fig. 2 and summarized below:

mAb1 (so8G4) = 8.3 nM

huMab2-3 = 6 nM

hmn-14 = 11.8 nM

Average of several experiments (EC50):

mAb1 (so8G4) = 10.4 nM ± 1.6 nM (n=12)

huMab2-3 = 4.9 nM ± 1.6 nM (n=3)

hmn-14 = 16 nM (n=2)

Cellular binding of anti CEACAM5 antibodies to MKN45 cell line show that the binding of mAb1 and the known antibodies is similar.

1.6.5 Internalization Assay using Cell Discoverer

A relevant characteristic of an ADC is its internalization into a target-expressing cell and lysosomes, and thus internalization is a relevant property of antibodies to be used as part of ADCs. Antibody internalization rate into the late endosome and lysosome (low pH vesicles) can be monitored by directly labeling the antibodies with a pH-sensitive dye (pHrodo) which emits strong fluorescence at a pH lower than 6.0 upon excitation. This fluorescence can be imaged in the Cell-Discoverer7 (Zeiss) and internalization rate can be calculated.

To analyze the internalization rates of several antibodies, MKN45 cells were seeded in a 96well, dark, flat clear bottom plate (Cellvis) at 25,000 cells/well. Cells were cultivated over night with 100 µl/well of RMPI-1640 + 10% FBS (Thermo). Cell media was removed, and cells were stained with 100 µl of 10 µg/ml Hoechst dye diluted in PBS x 1 for 15 min at room temperature (RT) in the dark. Cells were then washed twice with PBS x 1.

Anti-CEACAM5 human IgG antibodies (so8G4 (i.e. mAb1), humab2-3, hmn-14), and an anti-MerTK antibody (Merck) were directly labeled with pHrodo, were diluted to a concentration of

100 nM in warm RPMI1640 + 10% FBS without phenol red and were added to their respective wells. Plate was incubated in the Cell Discoverer at 37°C, 5% CO_2, for 20 hours, and images were acquired every 20 minutes, as further described below.

Internalization of pHrodo labeled antibodies into the late endosomes and lysosomes of cells was imaged by Cell-Discoverer7 (Zeiss) using fluorescence at excitation of 567nm and emission detector of 592/25nm. Cell nuclei were labeled with Hoechst and imaged at excitation of 385nm and emission detector of 425/30nm.

Fluorescence of each well was recorded every 20 min for 20 h. Analysis of Sum Fluorescence Intensity per cell (SFI) was calculated by the Zen Software (ZEN3.1) and Excel analysis of linear regression.

Results are shown in Fig. 3 and Fig. 4 and the slope of the linear part of the curves (see Fig. 4) is also summarized in the table below:

Sample	Slope of Curve	R^2
so8G4	28519	99.41
so8G4	29844	99.66
so8G4	28513	99.92
humab2-3	19154	99.42
humab2-3	17398	99.19
humab2-3	20201	99.68
hmn-14	24350	98.61
hmn-14	18754	99.56
hmn-14	23701	99.58

Conclusions:

1. so8G4 (mAb1) has a higher average binding rate (28958±766) than humab2-3 (18917±1416) and hmn-14 (22268±3060).

2. so8G4 (mAb1) also has a higher internalization intensity compared to humab2-3 and hmn-14.

3. mAb1-M, mAb2-M, mAb3-M, mAb6-M and mAb7-M are expected to show internalization properties corresponding to mAb1.

1.7 Exemplary method of producing mAb1 e.g. for use in conjugation to drug-linker compounds

Anti-CEACAM5 antibody mAb1 was produced in recombinant CHO-K1Sv cell line. Cell cultures were conducted in batch mode in a 200 l single-use bioreactor. Cells were grown in proprietary CHO fed-batch growth media supplemented with glucose at 37° C. The cultures were fed with a mixture of proprietary feed components on days 3, 5, 7 and 10 post inoculation.

Crude conditioned media from the bioreactor runs were clarified using 3x1.1 m^2 Millistak+ Pod DOHC (Millipore MD0HC10FS1) and 1.1 m^2 Millistak+ Pod XOHC (Millipore #MX0HC01 FS1) filters, followed by terminal filtration with a Millipore Opticap XL3 0.5 / 0.2 µm filter (Millipore #KHGES03HH3).

Following clarification, the antibody mAb1 was purified using a standard antibody purification process consisting of Protein A capture step and ion exchange chromatographic steps. The anti-CEACAM5 antibody mAb1 served as an intermediate for generation of ADC molecules.

1.8 Expression and purification of human/rabbit chimeric variant of mAb1 and its use in immunohistochemistry (IHC) on formaldehyde fixed and paraffin embedded cell lines and human tumor tissues

A human/rabbit chimeric variant of mAb1 was generated by routine recombinant methods. The human/rabit chimeric variant of mAb1 (also referred to as “rb8G4” herein) had the following amino acid sequence:

Heavy chain

EVQLQESGPGLVKPSQTLSLTCTVSDGSVSRGGYYLTWIRQHPGKGLEWIGYIYYSGSTYF
NPSLRSRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARGIAVAPFDYWGQGTLVTVSSQPK
APSVFPLAPCCGDTPSSTVTLGCLVKGYLPEPVTVTWNSGTLTNGVRTFPSVRQSSGLYSL
SSVVSVTSSSQPVTCNVAHPATNTKVDKTVAPSTCSKPTCPPPELLGGPSVFIFPPKPKDTL
MISRTPEVTCVVVDVSEDDPEVQFTWYINNEQVRTARPPLREQQFNSTIRVVSTLPIAHEDW
LRGKEFKCKVHNKALPAPIEKTISKARGQPLEPKVYTMGPPREELSSRSVSLTCMINGFYPS
DISVEWEKNGKAEDNYKTTPAVLDSDGSYFLYSKLSVPTSEWQRGDVFTCSVMHEALHNH
YTQKSISRSPGK (SEQ ID NO: 29)

Light chain

EIVLTQSPATLSVSPGERATLSCRTSQSVRSNLAWYQQKPGQAPRLLIYAASTRATGIPARF SGSGSGTEFTLTISSLQSEDFAVYYCQQYTNWPFTFGPGTKVDIKRDPVAPSVLLFPPSKEE LTTGTATIVCVANKFYPSDITVTWKVDGTTQQSGIENSKTPQSPEDNTYSLSSTLSLTSAQYN SHSVYTCEVVQGSASPIVQSFNRGDC (SEQ ID NO: 30)

Antibody rb8G4 was expressed in HEK cells (Expi 293 suspension cells) by transient transfection and purified using MabSelect SuRe and citrate buffers. rb8G4 was then used for IHC on formaldehyde fixed and paraffin embedded cell lines and human tumor tissues:

Material and methods

Cell lines and tissues

Human cancer cell lines were cultivated from the Merck cell bank, fixed in 4% buffered formaldehyde, and embedded in paraffin (FFPE). The paraffin embedded cell lines were arranged into cell line microarrays (CMAs) (Zytomed). FFPE tissue sections of a tissue microarray (TMA) with human organs were from amsbio (FDA Standard Tissue Array, T8234701). FFPE human tumor samples were provided by BioIVT and Indivumed GmbH.

Methods

For the IHC staining with the anti-CEACAM-5 antibody rb8G4, sections of 4 µm from formaldehyde fixed paraffin embedded (FFPE) cancer cell line microarrays (CMAs) and human tumor tissues were mounted on charged slides (SuperFrost Ultra Plus, Thermo Fisher Scientific or TOMO, Matsunami). The staining procedure was performed using a Discovery XT (Roche Diagnostics) staining platform. Following deparaffinization, the sections were heated for epitope retrieval in Tris-EDTA buffer pH 8 (CC1, Roche Diagnostics). The sections were incubated with the primary monoclonal antibody rb8G4 diluted to 0.5 or 0.7 µg/ml in phosphate-buffered saline (PBS) or antibody diluent buffer (DCS). The clone DA1E (rabbit monoclonal IgG, NEB) served as isotype control antibody. The primary antibodies were followed by the HQ anti-rabbit IgG detection kit (Roche Diagnostics). Slides were counterstained with hematoxylin, washed in tap water, dehydrated, and mounted on glass coverslips in Entellan Neu (VWR) permanent mounting media.

CMAs and the TMA with human organ tissue were stained and scanned with the NanoZoomer (Hamamatsu) with a resolution of 0.46 µm/pixel. Human tumor sections were stained and scanned using an AxioScan.Z1 (Zeiss) instrument with a resolution of 0.44 µm/pixel. The scans of the CMAs were analyzed with the image analysis software HALO (Indica Labs, USA). For the determination of the amount of antigen present, positive brown stained area was calculated as percent area of the viable tissue area. Staining (arbitrary units) is calculated as

Antibody staining (AU) = %positive tissue area * Average optic density (OD ranges from 0 to 1)

of the brown colour. The maximum value of the antibody staining is 100 = 100% of the tissue area is black (the grey scale OD value is 1).

CEACAM-5 mRNA data of cancer cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE; Broad Institute of MIT & Harvard).

Results

Validation on cancer cell lines and human normal tissue

The antibody rb8G4 showed on FFPE cancer cell lines a signal in the cytoplasm and the plasma membrane (Fig. 5).

The specificity of the antibody rb8G4 on FFPE tissue/cells was shown by comparing the staining signal on 104 cancer cell lines with the mRNA expression (CCLE dataset) of these cell lines. The resulting Pearson correlation coefficient of r=0.88 supports the conclusion that the antibody rb8G4 (also referred to as SO8G4AB323) detects a CEACAM-5 epitope in FFPE tissues/cells (Fig. 6). This cancer cell line microarray and individually selected positive and negative cell lines served as control matrices in staining runs with human normal and tumor tissue.

The staining with the antibody rb8G4 in normal human tissue (Fig. 7) is in agreement with the CEACAM-5 mRNA expression (Fig. 8) (Source: http://www.proteinatlas.org/ENSG00000105388-CEACAM5/tissue), further supporting the specificity of the antibody for CEACAM-5.

Human tumor tissue

The antibody rb8G4 stained positive in several human tumor indications, as shown in colorectal cancer (Fig. 9), gastric cancer (Fig. 10), esophageal cancer (Fig. 11), and non-small cell lung cancer (Fig. 12). The signal is localized in the cytoplasm and at the plasma membrane.

1.9 Flow cytometry and western blot using mAb1 and rb8G4

Binding of mAb1, rb8G4 and a commercially available anti-CEACAM5 antibody to CEACAM5-positive and -negative cell lines was compared.

Method used: 5E5 to 1E6 cells were used for flow cytometry analyses using a BD FACSCanto II (BD Biosciences) in 5 mL polystyrene tubes. Staining with 10 µg/mL primary antibodies (mAb1, rb8G4, mouse monoclonal Agilent Dako #M7072 clone #IL7) and respective fluorescently labeled secondary antibodies (donkey anti-human IgG Jackson-Dianova #709-

116-149; donkey anti-mouse IgG Jackson ImmunoResearch #715-116-150, donkey anti-rabbit IgG Jackson-Dianova #711-116-152) were conducted in 50 µL 1% PBS/BSA for 20 to 30 min at 4 °C. Between and after staining steps, cells were washed thrice with 1% PBS/BSA and resuspended in 500 µL 1% PBS/BSA (including 0.2 µg/mL DAPI for live cell gating) for flow cytometry analyses. For data evaluation, FlowJo software (BD Biosciences) was used.

Results: mAb1 and rb8G4 showed binding corresponding to mRNA expression level data on CEACAM5-positive cell lines only (Table 1 below; MKN-45, NCI-H441). In contrast, for the commercial antibody, binding was weaker and limited to a CEACAM5-high cell line (Table 1 below; MKN-45). In conclusion, mAb1 and rb8G4 specifically detect CEACAM5-positive cancer cells and can be utilized as a detection agent.

CELL LINE	MAB1	RB8G4	AGILENT/DAKO M7072 CLONE IL7
MKN-45	23.2	36.2	3.8
NCI-H441	2.2	3.2	1.1
A549	1.1	1.1	1.0
MDA-MB-468	1.1	1.0	1.0

Table 1. Binding of different antibodies to CEACAM5-positive and CEACAM5-negative cell lines. Quotients of median fluorescent intensity (MFI) of respective antibodies divided by MFI of secondary antibody only controls are listed per cell line.

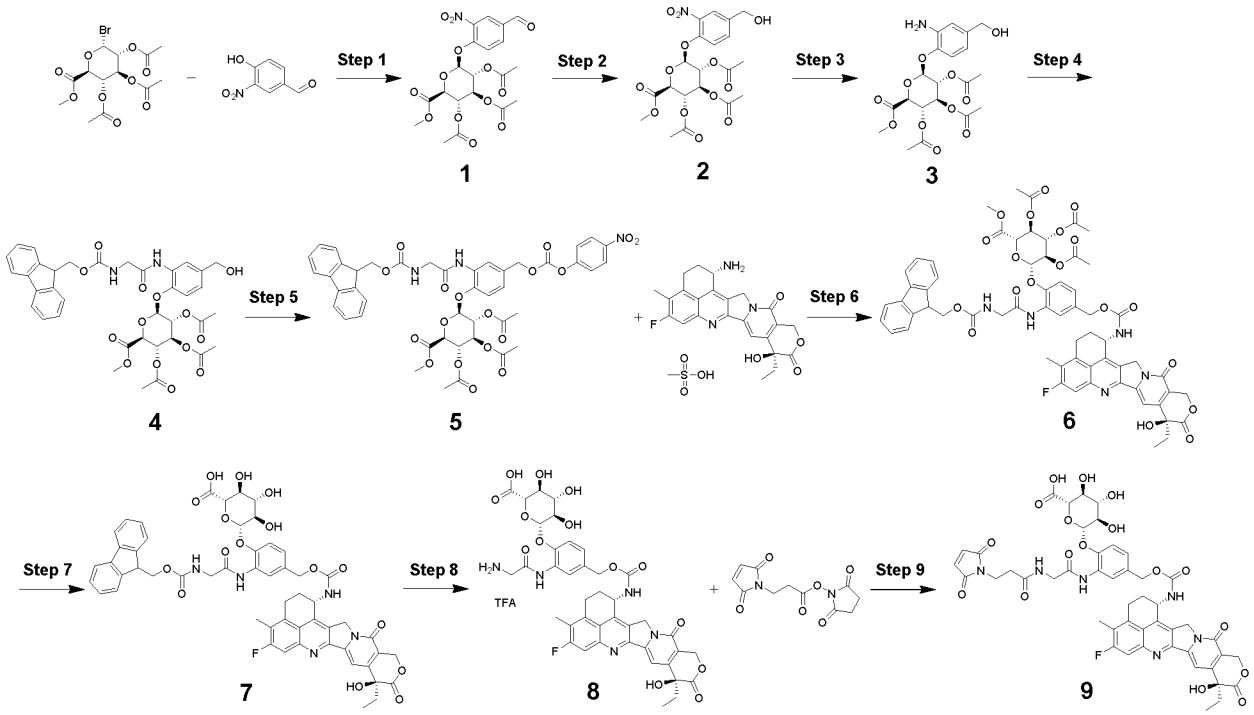
Binding of human mAb1 and rb8G4 to CEACAM5-positive and -negative cell line lysates was also investigated by Western Blots.

Method used: Western blots were performed according to standard protocols (Sambrook, J. & Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, Volume 1, CSHL Press). For SDS-PAGE followed by membrane wet blotting, 15 µg total protein of RIPA cell lysates quantified by BCA kit (Thermo Scientific, #23227) were loaded per lane. Criterion XT 4-12% gels (Bio-Rad, #3450125) using MOPS running buffer (Bio-Rad #1610788) were used in a Criterion electrophoresis cell (Bio-Rad, #1656001). Transfer of proteins was confirmed by Ponceau staining. Membranes were washed before and in-between staining with 0.5 µg/mL to 1 µg/mL primary (mAb1 or rb8G4) and secondary antibodies (anti-human IgG, Jackson ImmunoResearch #109-035-098 or anti-rabbit IgG, CellSignaling #7074) was conducted.

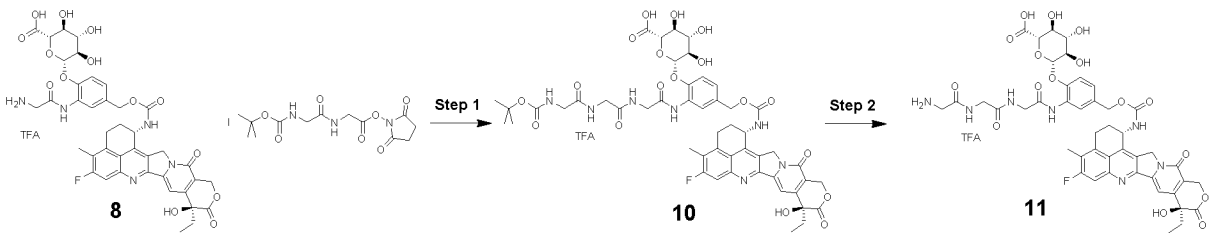
Stained membranes were visualized by ECL detection reagent using a Fusion FX imaging system (Vilber).

Results are shown in Fig. 13A and Fig. 13B: Both antibodies bound in a comparable pattern corresponding to the expected migration speed of highly glycosylated CEACAM5. CEACAM5 detection by mAb1 (Fig. 13A) and rb8G4 (Fig. 13B) was specific to CEACAM5-positive cell lines, and intensity correlated with mRNA expression levels. A secondary band observed with lower intensity corresponds to a potential second isoform previously described (Hatakeyama et al.: Novel protein isoforms of carcinoembryonic antigen are secreted from pancreatic, gastric and colorectal cancer cells. BMC Research Notes 2013 6:381).

Example 2: Synthesis of a drug-linker compound with glucuronide-based linker: Drug-linker compound 1 (DL1) and Drug-linker compound 1-M (DL1-M)



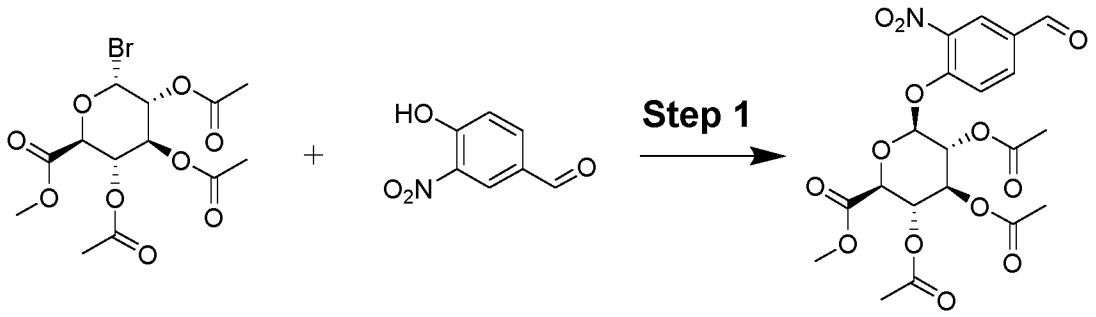
The synthetic route to compound **9** (also referred to herein as drug-linker compound 1 (DL1)).



The synthetic route to compound **11** (also referred to herein as drug-linker compound 1-M (**DL1-M**)

Protocol of chemical preparation to compound 9

Step 1: Compound **1**



1

To a stirred solution of (2S,3S,4S,5R,6R)-3,4,5-Triacetoxy-6-bromo-tetrahydro-pyran-2-carboxylic acid methyl ester (8.30 g; 20.90 mmol; 1.00 eq.) and 4-Hydroxy-3-nitro-benzaldehyde (5.24 g; 31.35 mmol; 1.50 eq.) in Acetonitrile (83.00 ml; 10.00 V) was added Silver(I) oxide (9.69 g; 41.80 mmol; 2.00 eq.). The reaction mixture was stirred at RT for 16 h. The reaction mixture was filtered through celite. The filtrate was concentrated under vacuum to get solid. The solid was dissolved in EtOAc and washed with 10% aqueous solution of $NaHCO_3$ to remove excess 4-Hydroxy-3-nitro-benzaldehyde. The organic layer was concentrated under vacuum to get compound **1** as sand colour solid.

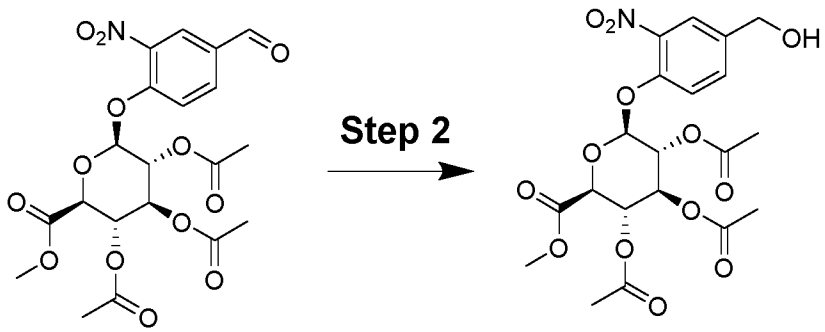
Yield: 9.0 g

Percentage Yield: 89.1%

Analytical data:

NMR: ^{1}H-NMR (400 MHz, DMSO-d6): 9.98 (s,1H), 8.46 (s, 1H),8.25-8.21 (m, 1H),7.64 (d, J = 11.60Hz, 1H), 5.94 (d, J= 10.00 Hz,1H),5.51-5.44 (m, 1H),5.20-5.09 (m, 2H),4.80 (d, J = 13.20 Hz, 1H), 3.64 (s,3H), 2.09 (s, 9H).

Step 2: Compound **2**



1 **2**

To a stirred solution of compound **1** (9.00 g; 18.62 mmol; 1.00 eq.) in Propan-2-ol (33.00 ml; 3.67 V) and $CHCl_3$ (167.00 ml; 18.56 V) were added silica gel 60-120 (3.60 g; 112.09 mmol;

6.02 eq.) followed by sodium borohydride (1.80 g; 46.55 mmol; 2.50 eq.). The reaction mixture was stirred for 1 h at RT. After completion, the reaction mixture was quenched with cooled H_2O and filtered through celite. The filtrate was extracted with Dichloromethane and dried over Na_2SO_4. The solvent was concentrated to get compound **2** as off-white powder.

Yield: 8.70 g

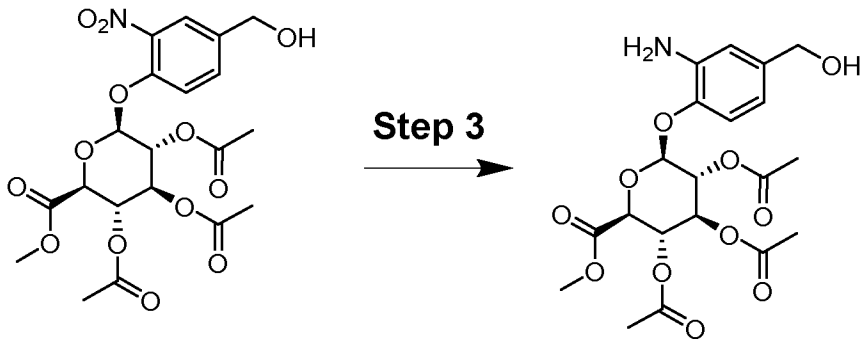
Percentage Yield: 92.4%

Analytical data

LCMS: Column: ATLANTIS dC18 (50x4.6mm) 5 µm; Mobile phase A: 0.1% HCOOH in H2O: ACN (95:5); B: ACN

RT (min): 2.05; M+H: 503.2, Purity: 96.6%

Step 3: Compound **3**



2 **3**

To a stirred solution of compound **2** (8.70 g; 17.21 mmol; 1.00 eq.) in ethyl acetate (100.00 ml; 11.49 V) and THF (100.00 ml; 11.49 V) was added Palladium on carbon (10% w/w) (2.50 g; 2.35 mmol; 0.14 eq.). The reaction mixture stirred for 3 h at RT under hydrogen atmosphere. After completion, the reaction mixture was filtered off through celite. The solvent was concentrated under vacuum to get compound **3** as off-white solid.

Yield: 8.5 g

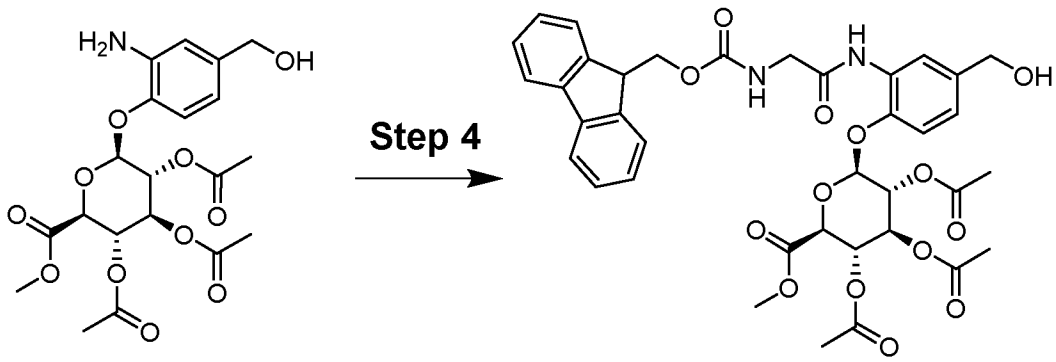
Percentage Yield: 100%

Analytical data:

LCMS: Column: ATLANTIS dC18 (50x4.6mm) 5 µm; Mobile phase A: 0.1% HCOOH in H2O: ACN (95:5); B: ACN

RT (min): 1.73; M+H: 456.10, Purity: 95.1%

Step 4: Compound **4**



3 **4**

To a stirred solution of compound **3** (10.00 g; 20.89 mmol; 1.00 eq.) and (9H-Fluoren-9-ylmethoxycarbonylamino)-acetic acid (7.60 g; 25.06 mmol; 1.20 eq.) in DCM (250.00 ml; 25.00 V) was added 2-Ethoxy-2H-quinoline-1-carboxylic acid ethyl ester (15.65 g; 62.66 mmol; 3.00 eq.) at 0 °C. The reaction mixture was stirred for 16h at RT. After completion, solvent was removed under reduced pressure to get a crude product. The crude product was purified by column chromatography (56% EtOAc:petroleum ether) to get compound with purity 80%. The compound was purified further by washings with30% EtOAc and pet ether to get compound **4** as white solid.

Yield: 8.5 g

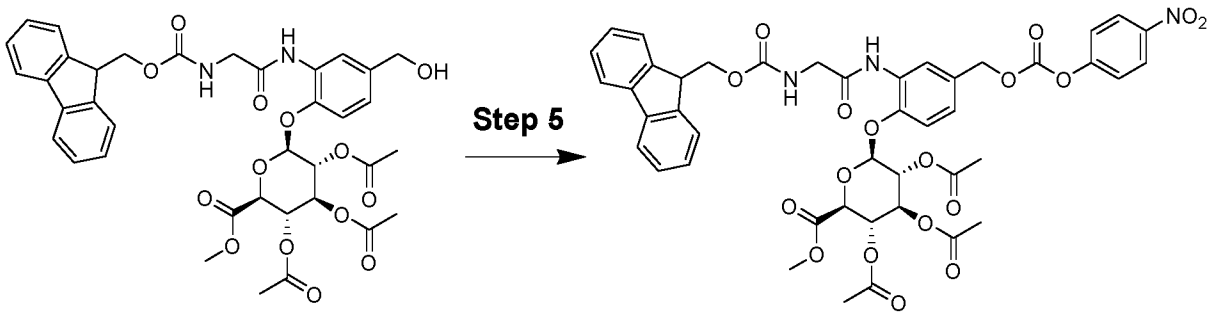
Percentage Yield: 50.7%

Analytical data:

LCMS: Column: ATLANTIS dC18 (50x4.6mm) 5 µm; Mobile phase A: 0.1% HCOOH in H_2O: ACN (95:5); B: ACN

RT (min): 3.03; M+H: 735.2, Purity: 81.9 %

Step 5: compound **5**



4 **5**

To a stirred solution of compound **4** (2.00 g; 2.49 mmol; 1.00 eq.) in THF (40.00 ml; 20.00 V) at 0 °C, were added Carbonic acid bis-(4-nitro-phenyl) ester (3.06 g; 9.97 mmol; 4.00 eq.) and

DIPEA (4.40 ml; 24.92 mmol; 10.00 eq.). The reaction mixture was stirred at RT for 12 h. After completion of the reaction, reaction mixture was concentrated under vacuum. The crude product was purified by column chromatography using silica gel (230-400) and pet ether / ethyl acetate as an eluent to afford compound **5** as pale yellow solid.

Yield: 2.0 g

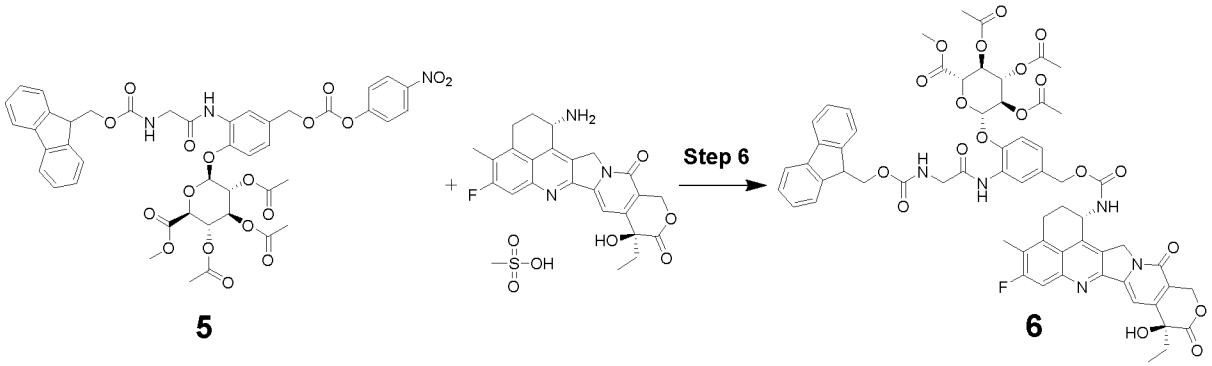
Percentage Yield: 84.6%

Analytical data:

LCMS: Column: X-Bridge C8(50X4.6) mm, 3.5µm; Mobile phase: A: 0.1% TFA in MilliQ water; B: ACN

RT (min): 3.24; M+H: 900.20, Purity: 94.9%

Step 6: Compound **6**



5 **6**

Compound **5** (1,369 g; 1,00 eq.) was dissolved in N,N-dimethylformamide (15,00 ml), Exatecan mesylate (679,7 mg; 1,00 eq.), 4-methylmorpholine for synthesis (0,422 ml; 3,00 eq.) and 1-Hydroxybenzotriazol (172,8 mg; 1,00 eq.) were added. The reaction mixture was stirred at room temperature for overnight. After the stirring time the reaction suspension was changed to a brown solution. The reaction was monitored by LC-MS, which showed a complete conversion of the starting material. The reaction mixture was purified via RP flash chromatography. The product containing fractions were combined, concentrated in vacuo and lyophilized overnight to afford compound **6** as an yellow solid.

Yield: 1.59 g

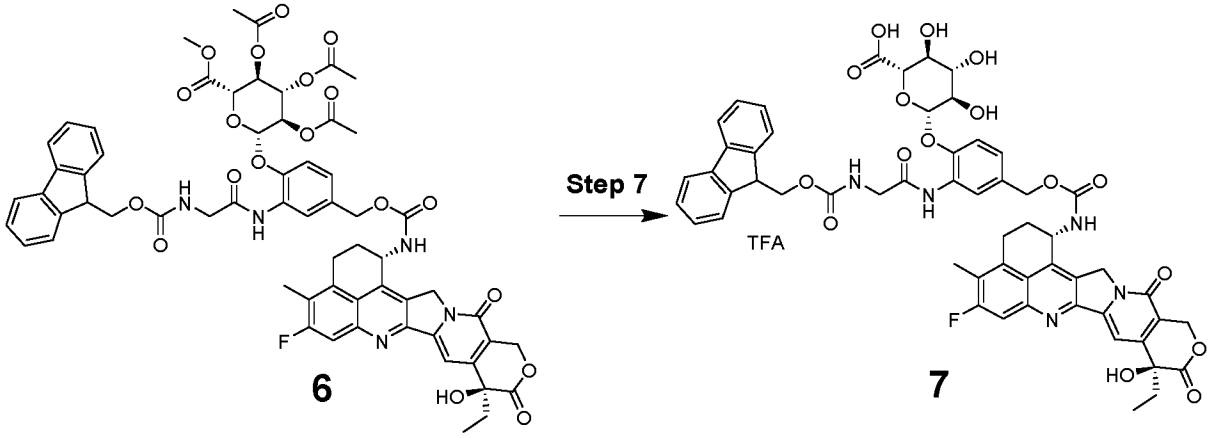
Percentage Yield: 87.5%

Analytical data:

LCMS: Column: Chromolith HR RP-18e (50-4,6 mm); Mobile phase A: 0.05% HCOOH in H_2O; B: 0.04% HCOOH and 1% H_2O in ACN; T: 40 °C; Flow: 3,3 ml/min; MS: 100-2000, amu positive; 1% ->100% B: 0 ->2,0min; 100% B: 2,0 ->2,5 min

RT (min): 1.95; M+H: 1196.40, Purity: 84.4%.

Step 7: Compound **7**



Compound **6** (1,586 g; 1,00 eq.) was dissolved in tetrahydrofuran (50,00 ml) and a solution (0.1M) of LiOH (contains Lithium hydroxide hydrate (281,77 mg; 6,00 eq.) in water (67,100 ml)) was added dropwise at 0°C. The pH value was checked during the addition. The pH should not exceed 10. The addition of the solution of LiOH was completed after 1.5 hours. The reaction was monitored by LC-MS, which showed a complete conversion of the starting material. The reaction was quenched with citric acid solution, pH adjusted to 5. The reaction mixture was concentrated under reduced pressure. The crude was purified by prep. HPLC. The product containing fractions were combined and lyophilized to afford Compound **7** as a dark yellow solid.

Yield: 728 mg

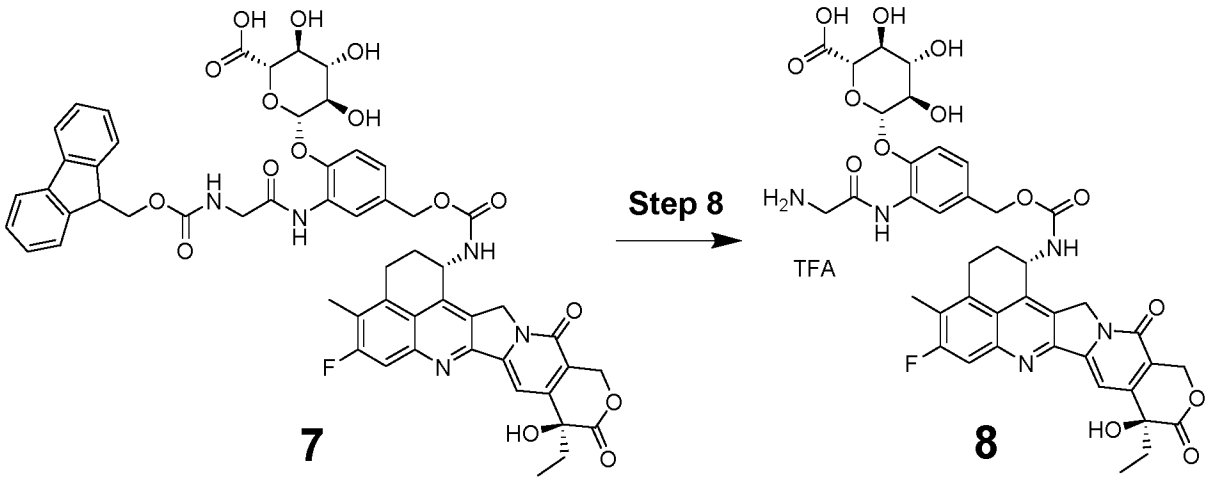
Percentage Yield: 54.8%

Analytical data:

LCMS: Column: Chromolith HR RP-18e (50-4,6 mm); Mobile phase A: 0.05% HCOOH in H_2O; B: 0.04% HCOOH and 1% H_2O in ACN; T: 40 °C; Flow: 3,3 ml/min; MS: 100-2000, amu positive; 1% ->100% B: 0 ->2,0min; 100% B: 2,0 ->2,5 min

RT (min): 1.68; M+H: 1056.30, Purity: 98.5%.

Step 8: Compound **8**



Compound **7** (728,000 mg; 1,00 eq.) was dissolved in N,N-dimethylformamide (20,00 ml). Piperidine (136,513 µl; 2,00 eq.) was added and the solution was stirred at RT for totally 4 hours. The reaction was monitored by LC-MS, which showed a complete conversion of the starting material. The reaction mixture was concentrated under reduced pressure and the crude product was purified by RP flash chromatography. The product containing fractions were combined, the solvent was removed partially and it was lyophilized overnight to afford compound **8** as an yellow solid.

Yield: 706 mg

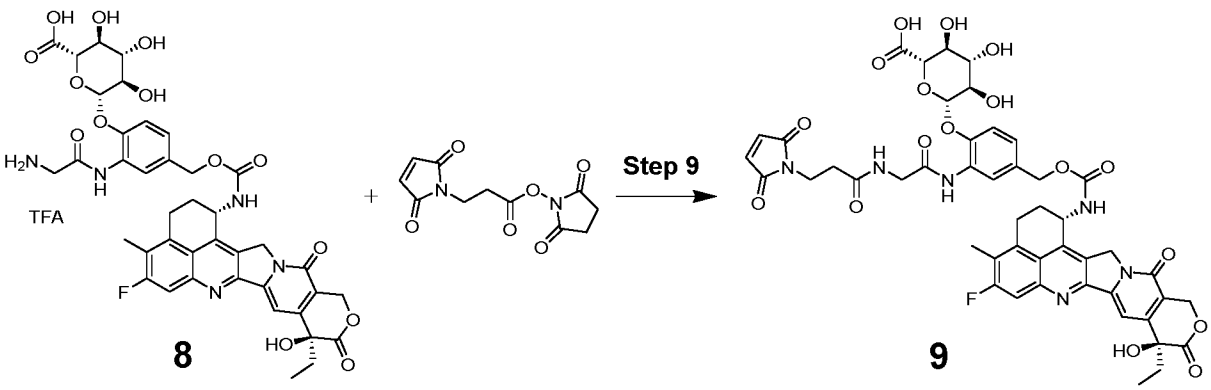
Percentage Yield: 100%

Analytical data:

LCMS: Column: Chromolith HR RP-18e (50-4,6 mm); Mobile phase A: 0.05% HCOOH in H_2O; B: 0.04% HCOOH and 1% H_2O in ACN; T: 40 °C; Flow: 3,3 ml/min; MS: 100-2000, amu positive; 1% ->100% B: 0 ->2,0min; 100% B: 2,0 ->2,5 min

RT (min): 1.22; M+H: 834.30, Purity: 97.6%.

Step 9: Compound **9**



To a solution of compound **8** (854 mg; 1,00 eq.) in dimethylformamid (30,00 ml) were added N-ethyldiisopropylamine (149,234 µl; 1,00 eq.) and 3-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-propionic acid 2,5-dioxo-pyrrolidin-1-yl ester (233,61 mg; 1,00 eq.). The reaction mixture was stirred at RT for 3 hours. The reaction was monitored by LC-MS, which showed a complete conversion of the starting material. The reaction mixture was concentrated under reduced pressure and the crude product was by RP flash chromatography. The product containing fractions were combined, concentrated and lyophilized to give the desired produce with a purity of 91%. This material was again purified by RP chromatography to give compound **9** as an yellow solid.

Yield: 580 mg

Percentage Yield: 60.1%

Analytical data:

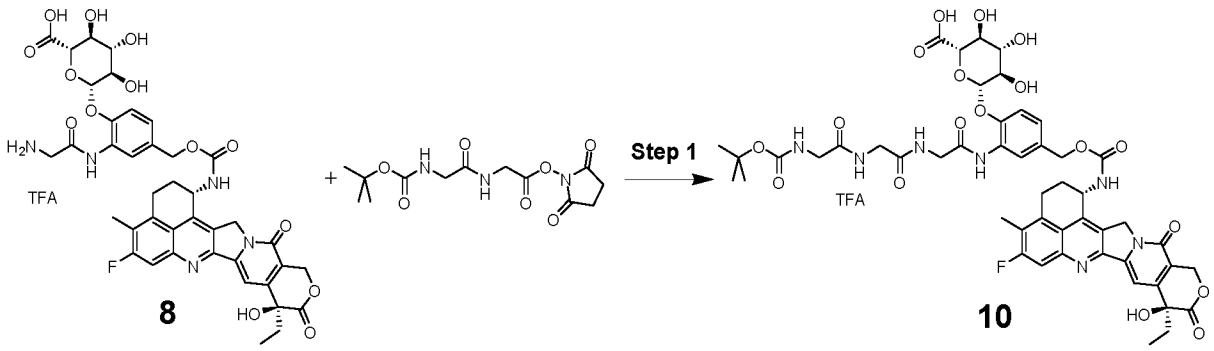
LCMS: Column: Chromolith HR RP-18e (50-4,6 mm); Mobile phase A: 0.05% HCOOH in H_2O; B: 0.04% HCOOH and 1% H_2O in ACN; T: 40 °C; Flow: 3,3 ml/min; MS: 100-2000, amu positive; 1% ->100% B: 0 ->2,0min; 100% B: 2,0 ->2,5 min

RT (min): 1.38; M+H: 985.30, Purity: 90% (the other 10% of isomer can be removed by HPLC)

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.10 – 12.44 (m, 1H), 9.08 (s, 1H), 8.32 (t, *J* = 5.8 Hz, 1H), 8.16 (s, 1H), 8.02 (d, *J* = 8.8 Hz, 1H), 7.76 (d, *J* = 10.9 Hz, 1H), 7.31 (s, 1H), 7.15 – 7.09 (m, 2H), 6.98 (s, 2H), 5.48 – 5.38 (m, 2H), 5.32 – 5.22 (m, 3H), 5.11 – 5.01 (m, 2H), 4.87 (d, *J* = 7.6 Hz, 1H), 3.92 – 3.88 (m, 1H), 3.89 – 3.84 (m, 2H), 3.65 – 3.61 (m, 2H), 3.46 – 3.41 (m, 1H), 3.42 – 3.37 (m, 1H), 3.38 – 3.31 (m, 1H), 3.28 – 3.20 (m, 1H), 3.15 – 3.07 (m, 1H), 2.48 – 2.44 (m, 2H), 2.38 (s, 3H), 2.24 – 2.13 (m, 2H), 1.94 – 1.80 (m, 2H), 0.88 (t, *J* = 7.3 Hz, 3H).

Protocol of chemical preparation to compound 11

Step 1: Compound **10**



To a solution of compound **8** (289.00 mg; 1.00 eq) in dimethyl formamide (10.00 ml) were added N-ethyl diisopropylamino (0.104 ml, 2.00 eq) and 2,5-dioxopyrrolidin-1-yl (tert-butoxycarbonyl)glycylglycinate (105.70 mg; 1.00 eq). The reaction mixture was stirred at room temperature for 1 hour and the reaction was mirrored by LC-MS. After

completion, the solvent was removed in vacuo and the crude product was purified by prep. HPLC over a Sunfire column. The fractions containing product were combined and lyophilized to afford compound 10 trifluoroacetic acid salt as a yellow solid.

Yield: 162.00 mg, 0.14 mmol.

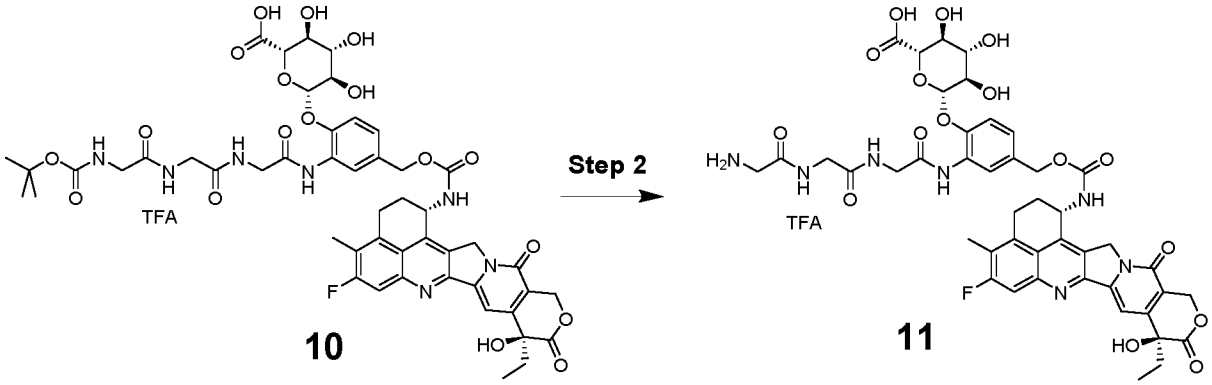
Percentage yield: 44.8 %

Analytical data:

LCMS: Column: Chromolith HR RP-18e (50-4,6 mm); Mobile phase A: 0.05% HCOOH in H2O; B: 0.04% HCOOH and 1% H2O in ACN; T: 40 °C; Flow: 3,3 ml/min; MS: 100-2000, amu positive; 1% ->100% B: 0 ->2,0min; 100% B: 2,0 ->2,5 min

RT (min): 1.41; M+H: 1048.1, Purity: 97.9%

Step 2: Compound **11**



To a suspension of compound **10** (162.00 mg; 1.00 eq) in dichloromethane (5.00 ml) was added 4.0 M hydrogen chloride solution in 1,4-dioxane (682.43 µl; 20.00 eq) and it was stirred at room temperature for 3.5 hours and the reaction was monitored by LC-MS. After completion, the reaction mixture was concentrated in vacuo and the crude product was purified by prep. HPLC. The fractions containing product were combined and lyophilized to afford compound **11** trifluoroacetic acid salt as a yellow solid.

Yield: 113 mg; 0.11 mmol.

Precent yield: 77.0 %

Analytical data:

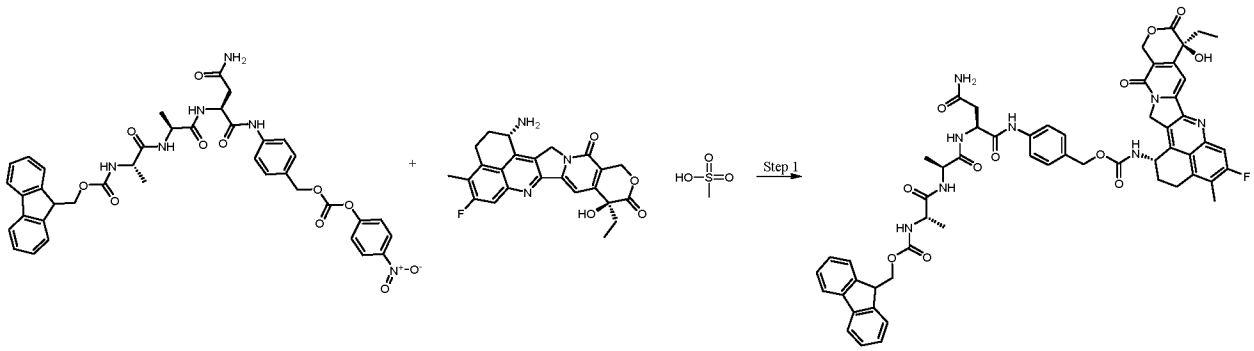
LCMS: Column: Chromolith HR RP-18e (50-4,6 mm); Mobile phase A: 0.05% HCOOH in H2O; B: 0.04% HCOOH and 1% H2O in ACN; T: 40 °C; Flow: 3,3 ml/min; MS: 100-2000, amu positive; 1% ->100% B: 0 ->2,0min; 100% B: 2,0 ->2,5 min

RT (min): 1.22; M+H: 948.0, M+2H: 474.8; Purity: 98.8%

^{1}H NMR (700 MHz, DMSO-d6) δ 12.93 – 12.79 (m, 1H), 9.14 (s, 1H), 8.61 (t, J = 5.8 Hz, 1H), 8.41 (t, J = 5.9 Hz, 1H), 8.14 (s, 1H), 8.05 (d, J = 8.8 Hz, 1H), 8.03 – 7.93 (m, 3H), 7.79 (d, J = 10.8 Hz, 1H), 7.31 (s, 1H), 7.13 (s, 2H), 6.58 – 6.47 (m, 1H), 5.80 – 5.61 (m, 1H), 5.48 – 5.40

(m, 2H), 5.31 – 5.24 (m, 3H), 5.08 (d, J = 12.2 Hz, 1H), 5.03 (d, J = 12.3 Hz, 1H), 4.91 (d, J = 7.7 Hz, 1H), 3.96 (d, J = 5.8 Hz, 2H), 3.95 – 3.86 (m, 3H), 3.62 (q, J = 5.8 Hz, 2H), 3.43 (t, J = 9.3 Hz, 1H), 3.41 (t, J = 8.5 Hz, 1H), 3.35 (t, J = 9.0 Hz, 1H), 3.27 – 3.20 (m, 1H), 3.15 – 3.09 (m, 1H), 2.38 (s, 3H), 2.23 – 2.13 (m, 2H), 1.93 – 1.81 (m, 2H), 0.88 (t, J = 7.3 Hz, 3H).

Example 3: Synthesis of a drug-linker compound with legumain-cleavable linker: Drug-linker compound 2 (DL2)



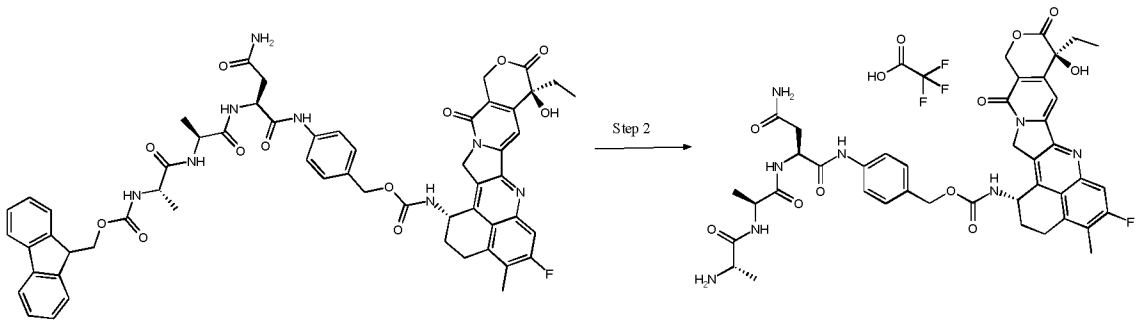
Step 1

{4-[(2S)-3-carbamoyl-2-[(2S)-2-[(2S)-2-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)propanamido]propanamido]propanamido]phenyl}methyl 4-nitrophenyl carbonate (400 mg; 0,52 mmol; 1,00 eq.) [commercially available from Levena Biopharma US] was dissolved in N,N-Dimethylformamide (5,00 ml). Exatecan mesylate (277,30 mg; 0,52 mmol; 1,00 eq.), N-Ethyldiisopropylamine (0,27 ml; 1,57 mmol; 3,00 eq.) and 1-Hydroxybenzotriazol (HOBT) (3,52 mg; 0,03 mmol; 0,05 eq.) were added. The reaction mixture was stirred at room temperature overnight. LC/MS indicated complete conversion.

The crude reaction mixture was purified via prep HPLC and lyophilized yielding 365mg (0.343 mmol) of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-(((S)-4-amino-1-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-1,4-dioxobutan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamate

LC/MS: [M+H] = 1064.2
Prep HPLC:

Column: sunfire prep c18 obd - 75.0 g (250 bar)
Solvent A : Wasser 0.1%TFA Solvent C :
Solvent B : Acetonitril 0.1%TFA



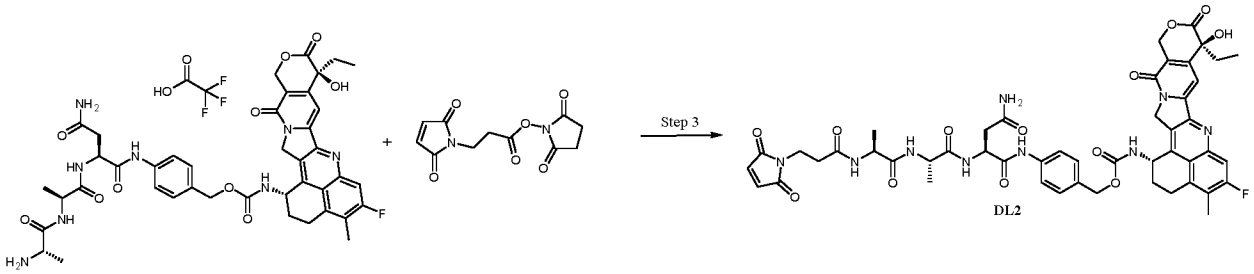
Step 2

(9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-(((S)-4-amino-1-((4-(((((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamoyl)oxy)methyl)phenyl)amino)-1,4-dioxobutan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamate (365 mg; 0,34 mmol; 1,00 eq.) was dissolved in N,N- (4,00 ml). Piperidine for synthesis (0,07 ml; 0,69 mmol; 2,00 eq.) was added and the reaction solution was stirred at rt for 1h.

The reaction mixture was purified via prep HPLC yielding 300mg (0.314 mmol) of 4-((S)-4-amino-2-((S)-2-((S)-2-aminopropanamido)propanamido)-4-oxobutanamido)benzyl ((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamate.

LC/MS: [M+H]: 841.3

Prep HPLC for purification:
RediSep Säule: C18 130g
SN: E0410A0D24BE1 Lot: 262118923W
Flowrate: 75 ml/min
Condition - Volumen: 390,0 ml
Eluent: A1 WATER 0.1%TFA
Eluent: B1 ACETONITRILE 0.1%TFA



Step 3

To a solution of 4-((S)-4-amino-2-((S)-2-((S)-2-aminopropanamido)propanamido)-4-oxobutanamido)benzyl ((1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl)carbamate (571mg; 0,60 mmol; 1,00 äq.) in N,N-Dimethylformamide (20ml) was added N-Ethyldiisopropylamine (203 µl; 1,20 mmol; 2,00 eq.) and N-Succinimidyl 3-maleimidopropionate (162 mg; 0,60 mmol; 1,00 eq.). The reaction mixture was then stirred for 10min and monitored via LC/MS.

The reaction mixture was purified via prep HPLC yielding 378mg (0.35 mmol) of **DL2**.

LC/MS: [M+H]: 992.4

Prep HPLC for purification:
RediSep column: C18 86g
SN: E0410A8B46130 Lot: 281729189W
Flowrate: 60 ml/min
Conditon - Volumen: 264,0 ml
Eluent 1: A1 WATER 0.1%TFA
Eluent 2: B1 ACETONITRILE 0.1%TFA

Analytics for sequence:
Method Info : A: H2O + 0,05% HCOOH | B: MeCN + 0,04% HCOOH + 1% H2O
T: 40 °C | Flow: 3,3 ml/min | MS: 100-2000 amu positive
Column: Chromolith HR RP-18e 50-4,6 mm
0% -> 100% B: 0 -> 2,0 min | 100% B: 2,0 -> 2,5 min

Example 4: Preparation of an immunoconjugate: a glucuronide-based conjugate of mAb1 (referred to as ADC1) and mAb1-M (the resulting immunoconjugate being referred to as ADC1-M), mAb4-M (the resulting immunoconjugate being referred to as ADC4-M) and mAb6-M (the resulting immunoconjugate being referred to as ADC6-M)

Although the following methods are described in the context of mAb1 (the resulting immunoconjugate being referred to as ADC1), the same method can also be used for mAb1-M (the resulting immunoconjugate being referred to as ADC1-M), mAb4-M (the resulting

immunoconjugate being referred to as ADC4-M) and for mAb6-M (the resulting immunoconjugate being referred to as ADC6-M).

4.1 Conjugation process

Antibody Preparation

The antibody mAb1 (as defined herein above) was thawed at 2 - 8°C up to 3 days prior to conjugation and stored at 2 - 8°C until use. The mAb (> 10 g) was equilibrated at room temperature on the day of conjugation prior to use. The mAb (9.6 mg/mL) was aliquoted (10.0 g, 1041.7 mL) and diluted to 5.59 mg/mL using conjugation buffer (200 mM Histidine, pH 6.5). The mAb solution was added into a 3 L Chemglass jacketed reactor and set to 25 ± 2°C while stirring at 50 rpm.

Reduction of Antibody

Added 7.0 mol eq. (9.7 mL) of 50 mM TCEP solution (50 mM TCEP in conjugation buffer) into the mAb solution vial and the reaction was allowed to proceed at 25 ± 2°C for 3 hours.

Conjugation

The drug-linker compound 1 (DL1) of formula **(X)** was weighed and dissolved in DMSO to prepare a 20 mM solution. 90% (148.6 mL) of the required DMSO was added to the reactor. Immediately after DMSO addition, 10.0 mol equivalents (38.2 mL) of 20 mM drug-linker solution was added to the reactor. Then, 10% (18.2 mL) of the remaining required DMSO was used to rinse the drug-linker vial to ensure total transfer. After final addition, the reaction was allowed to proceed at 25 ± 2°C for 1 hour. Total volume during conjugation was 1997.0 mL.

Note: Overall DMSO concentration of the reaction was 10% (v/v) (DMSO + Drug Linker solution).

Quench

Added 35 mol eq. (48.5 mL) of 50 mM NAC to the reactor and the reaction was allowed to proceed at 25 ± 2°C for 30 minutes.

Filtration

The filtered crude conjugate solution was transferred from the reactor and then filtered using a Millipak Gamma Gold 60 (MPGL06GH2) to give 1993.6 mL (Filter Load: 324.7 g/m2 [protein], 66.5 L/m2 [solution]) of filtered crude conjugate.

Diafiltration

The filtered crude conjugate solution was buffer exchanged (DV= 1993.6 mL) with a Pellicon 3 (30 kDa) Biomax membrane (1 x 0.11 m2, 300 LMH (550 mL/min), 16 psi TMP, actual loading 88.5 g/m2). Diafiltration buffer (10 mM Histidine, pH 5.5) was used to buffer exchange the crude conjugate for 16 diavolumes. After buffer exchanging, the solution was concentrated to ≥ 25 mg/mL, transferred into a bottle, and the membrane flushed with diafiltration buffer. Total volume recovered from UF/DF was 361.5 mL.

Formulation

The concentrated ADC (i.e. ADC1) was diluted to 20.0 mg/mL with 112.1 mL of diafiltration buffer (10 mM Histidine, pH 5.5). The resulting solution was diluted to 15.0 mg/mL with 157.6 mL of 4X formulation buffer (10 mM Histidine, 12% (w/v) Trehalose Dihydrate, 400 mM NaCl, pH 5.5) for a final target bulk drug substance (BDS) concentration of 15.0 mg/mL.

Filtration

The final formulated ADC was filtered using a 0.2 µm Millipak Gamma Gold 40 (MPGL04GH2) filter to yield 619.6 mL (Filter Load: 464.6 g/m2 [protein], 31.0 L/m2 [solution]) ADC1 BDS. The material was packaged into HDPE bottles and stored at ≤ -65°C.

4.2 Methods: Drug substance characterization: ADC1

Size exclusion chromatography (SEC) method

SEC Method Parameters

Wavelength 280 nm

Column Tosoh TSKgel 7.8 mm x 300 mm, 5 µm (P/N 0008541)

Mobile Phase 0.14 M Potassium Phosphate Monobasic

50 mM Sodium Phosphate Monobasic

0.06 M Potassium Phosphate Dibasic

0.25 M Potassium Chloride

5% IPA

Injection Volume 20 µL

Temperature 25°C

Flow rate 0.5 mL/min

Run Time 30 min

Typical SEC chromatogram showing the purity of the stock mAb, the conjugate post UF and the final BDS: Fig. 14.

For the BDS material shown above, 1.7% HMWS and a monomeric purity of 96.9% have been reported.

Reversed-Phase HPLC (RP HPLC) method

RP HPLC Method Parameters

Wavelength 280 nm

Column PLRP-S 1000 Å (50 x 2.1 mm, 8 µm) column, Agilent (P/N PL1912-1802)

Mobile Phase A 0.1% Formic Acid in Water with 0.01% TFA

Mobile Phase B 0.1% Formic Acid in ACN with 0.01% TFA

Gradient

Time (min)	MP A (%)	MP B (%)
0.00	80	20
3.00	80	20
20.00	0	100
22.00	80	20
26.00	0	100
4 min Equilabration to 80% A and 20% B		

Injection Volume 10 µL

Column Temperature 80°C

Flow rate 1.0 mL/min

Run Time 30 min

Sample Preparation Dilute sample to 2 mg/mL and add 40 µL to a micro centrifuge tube. Add 60 µL of the ~8 M Guanidine HCl, ~130 mM Tris, ~1mM EDTA, pH 7.6 buffer. Add 2 µL of 500 mM DTT and vortex to mix. Incubate sample for 30 ± 2 min at 37 ± 2°C.

Typical RP-HPLC chromatogram showing the separation of light and heavy chains: Fig. 15. The chromatogram shows an overlay of the stock mAb, the crude ADC and the final BDS.

For the ADC1 BDS material above, a DAR of 7.9 was reported.

Free-drug method

Free-drug method parameters

Wavelength 254 nm

Column Phenomenex Gemini, C18, 2 x 150 mm, 3 µm (P/N 00F-4439-B0)

Mobile Phase A 0.1% Formic acid in water

Mobile Phase B 0.1% Formic acid in acetonitrile

Gradient

Time (min)	MP A (%)	MP B (%)
0.00	95	5
1.00	95	5
12.00	0	100
3 min Equilabration to 95% A		

Injection Volume 10.00 µL

Column Temperature 50°C

Flow rate 0.75 mL/min

Sample Preparation Protein drop: 100 µL of Drug Substance + 250 µL of cold MeOH + 50 µL of 3 M $MgCl_2$. Spin at 20,000 rpm for 10 min

Standard Preparation Mix 20 µL of 20 mM DL1 (drug-linker compound 1 in DMSO) + 20 µL DMSO + 40 µL MeOH + 20 µL of 200 mM NAC in Diafiltration buffer. Incubate overnight to afford 4 mM DL-NAC. Dilute 4 mM DL-NAC in MeOH to afford a 4 µM DL-NAC standard.

Typical chromatogram showing the NAC standard and the free-drug levels of the final BDS: Fig. 16.

For the ADC1 BDS material shown above, residual free-drug levels below 2.4% (by molar ratio) have been reported.

Example 5: Preparation of an immunoconjugate: a peptide-based conjugate of mAb1 (referred to as ADC2)

5.1 Conjugation process

Antibody Preparation

The antibody mAb1 (as defined herein above) was thawed at 2 - 8°C up to 3 days prior to conjugation and stored at 2 - 8°C until use. The mAb (> 9.5 g) was equilibrated at room temperature on the day of conjugation prior to use. The mAb (9.6 mg/mL) was aliquoted (9.5 g, 989.6 mL) and diluted to 5.59 mg/mL using conjugation buffer (200 mM Histidine, pH 6.5). The mAb solution was added to a 3 L Chemglass jacketed reactor and set to 25 ± 2°C while stirring at 50 rpm.

Reduction of Antibody

Added 8.0 mol eq. (10.5 mL) of 50 mM TCEP solution (50 mM TCEP in conjugation buffer) into the mAb solution vial and the reaction was allowed to proceed at 25 ± 2°C for 3 hours.

Diafiltration

The reduced mAb solution was buffer exchanged against 6 DVs (DV= 1706.9 mL) using a Pellicon 3 (30 kDa) Biomax membrane (1 x 0.11 m2, 300 LMH (550 mL/min), 16 psi TMP, actual loading 86.3 g/m2. Conjugation buffer (200 mM Histidine, pH 6.5) was used to buffer exchange the reduced antibody. After buffer exchanging, the reduced mAb solution was recovered back into the reactor and the membrane flushed with conjugation buffer.

Conjugation

The drug-linker compound 2 (DL2) was weighed and dissolved in DMSO to prepare a 20 mM drug-linker solution. 90% (142.6 mL) of the required DMSO was added to the reactor. Immediately after DMSO addition, 9.5 mol equivalents (31.2 mL) of the 20 mM drug-linker solution was added to the reactor. Then, 10% (15.8 mL) of the remaining required DMSO was

used to rinse the drug-linker vial to ensure total transfer. After final addition, the reaction was allowed to proceed at 25 ± 2°C for 2 hours. Total volume during conjugation reaction was 1894.5 mL

Quench

Added 35 mol eq. (46 mL) of 50 mM NAC to the reactor and the reaction was allowed to proceed at 25 ± 2°C for 45 minutes.

Filtration

The crude conjugate solution was transferred from the reactor and filtered using a Millipak Gamma Gold 60 (MPGL06GH2) to give 1897.3 mL (Filter Load: 308.9 g/m2 [protein], 63.2 L/m2 [solution]) of filtered crude conjugate.

Diafiltration

The filtered crude conjugate solution was buffer exchanged (DV = 1897.3 mL) with a Pellicon 3 (30 kDa) Biomax membrane (1 x 0.11 m2, 300 LMH (550 mL/min), 16 psi TMP, actual loading 84.2 g/m2). The initial 12 DVs were performed using conjugation buffer (200 mM Histidine, pH 6.5) and then switched to standard diafiltration buffer (10 mM Histidine, pH 5.5) for 8 additional DVs. After buffer completing the buffer exchange, the solution was then concentrated to ≥ 25 mg/mL, transferred into a bottle and the membrane flushed with diafiltration buffer. Total pooled volume recovered from UF/DF was 335.7 mL.

Formulation

The concentrated ADC (i.e. ADC2) was diluted to 20.0 mg/mL with 84.7 mL of Diafiltration Buffer (10mM Histidine, pH 5.5). The resulting solution was diluted with 138.6 mL of 4X Formulation Buffer (10 mM Histidine, 12% (w/v) Trehalose Dihydrate, 400 mM NaCl, pH 5.5) for a final target BDS concentration of 15.0 mg/mL.

Filtration

The final formulated ADC was aseptically filtered using a Millipak Gamma Gold 60 (MPGL06GH2) to yield 549.3 mL (Filter Load: 411.4 g/m2 [protein], 27.5 L/m2 [solution]) of ADC2 BDS. The material was packaged into HDPE bottles and stored at ≤ -65°C.

5.2 Methods: Drug substance characterization: ADC2

Size exclusion chromatography (SEC) method

SEC Method Parameters

Wavelength 280 nm

Column Tosoh TSKgel 7.8 mm x 300 mm, 5 µm (P/N 0008541)

Mobile Phase 50 mM Sodium Phosphate Monobasic

0.4 M Sodium Perchlorate

pH 6.3

Injection Volume 1 µL

Column Temperature 25°C

Flow rate 0.5 mL/min

Run Time 30 min

Typical SEC chromatogram showing the purity of the stock mAb and the final BDS: Fig. 17.

For the ADC2 BDS material shown above, 4.2% HMWS and a monomeric purity of 95.8% have been reported.

Reversed-Phase HPLC (RP HPLC) method

RP HPLC Method Parameters

Wavelength 280 nm

Column PLRP-S 1000 Å (50 x 2.1 mm, 8 µm) column, Agilent (P/N PL1912-1802)

Mobile Phase A 0.1% Formic Acid in Water with 0.01% TFA

Mobile Phase B 0.1% Formic Acid in ACN with 0.01% TFA

Gradient

Time (min)	MP A (%)	MP B (%)
0.00	80	20
3.00	80	20
20.00	0	100
22.00	80	20
26.00	0	100
4 min Equilabration to 80% A and 20% B		

Injection Volume 10 µL

Column Temperature 80°C

Flow rate 1.0 mL/min

Run Time 30 min

Sample Preparation Dilute sample to 2 mg/mL and add 40 µL to a micro centrifuge tube. Add 60 µL of the ~8 M Guanidine HCl, ~130 mM Tris, ~1mM EDTA, pH 7.6 buffer. Add 2 µL of 500 mM DTT and vortex to mix. Incubate sample for 30 ± 2 min at 37 ± 2°C.

Typical RP-HPLC chromatogram showing the separation of light and heavy chains: Fig. 18. The chromatogram shows an overlay of the stock mAb and the final BDS.

For the ADC2 BDS material above, a DAR of 7.6 was reported.

Free-drug method

Free-drug method parameters

Wavelength 254 nm

Column Phenomenex Gemini, C18, 2 x 150 mm, 3 µm (P/N 00F-4439-B0)

Mobile Phase A 0.1% Formic acid in water

Mobile Phase B 0.1% Formic acid in acetonitrile

Gradient

Time (min)	MP A (%)	MP B (%)
0.00	95	5
1.00	95	5
12.00	0	100
3 min Equilabration to 95% A		

Injection Volume 10.00 µL

Column Temperature 50°C

Flow rate 0.75 mL/min

Sample Preparation Protein drop: 100 µL of Drug Substance + 250 µL of cold MeOH + 50 µL of 3 M $MgCl_2$. Spin at 20,000 rpm for 10 min

Standard Preparation Mix 20 µL of 20 mM DL2 (drug-linker compound 2 in DMSO) + 20 µL DMSO + 40 µL MeOH + 20 µL of 200 mM NAC in Diafiltration buffer. Incubate overnight to afford 4 mM DL-NAC. Dilute 4 mM DL-NAC in MeOH to afford a 4 µM DL-NAC standard.

Typical chromatogram showing the NAC standard and the free-drug levels of the final BDS: Fig. 19.

For the ADC2 BDS material shown above, residual free-drug levels below 1.9% (by molar ratio) have been reported.

Example 6: An analog of the ADC SAR408701

6.1 The antibody

For the purposes of further comparative experiments, an analog of Sanofi's anti-CEACAM5 ADC SAR408701 was prepared based on a monoclonal antibody having the following sequence:

Heavy chain: SEQ ID NO: 25

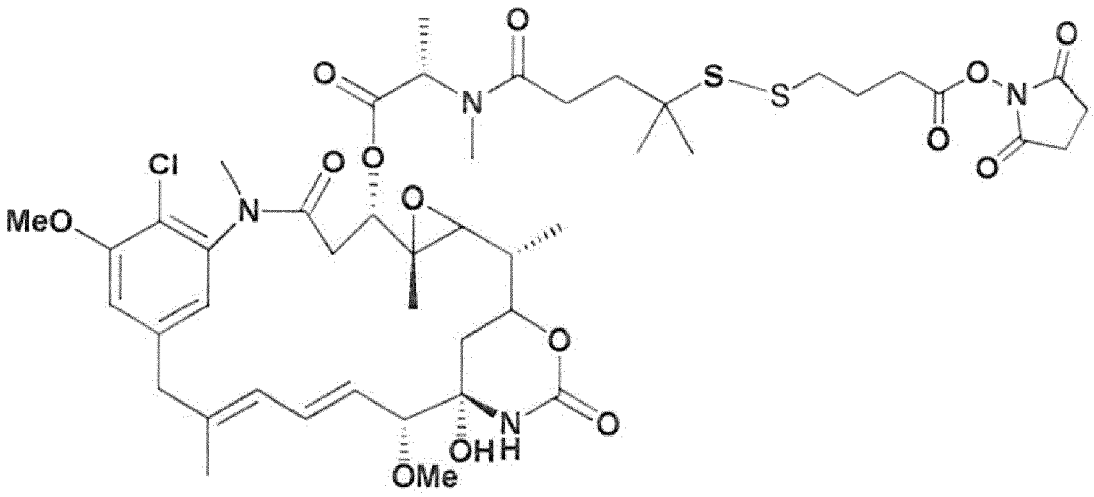
Light chain: SEQ ID NO: 26

6.2 The drug-linker compound

As a drug-linker molecule to be conjugated to the above-mentioned antibody, SPDB-DM4 (obtained from Levena Biopharma) was used:

Product name: SPDB-DM4

Structure:



Expected mass: 994.35

Observed average mass: 995.5 ($Ms+H^+$)

Mass spectral analysis: consistent, exhibited correct MW

HPLC analysis: purity > 95%

Appearance: white powder

6.3 Conjugation

The antibody was thawed at 2 - 8°C up to 3 days prior to conjugation and stored at 2 - 8°C until use. The antibody (175 mg) was equilibrated at room temperature on the day of conjugation prior to use. The antibody (7.9 mg/mL) was diluted to 5 mg/mL using conjugation buffer (PBS pH 7.4) and a 5 mM DMSO solution (8 mol equivalents relative to the antibody) of SPDB-DM4 (Levena Biopharma). The reaction solution was mixed and incubated at 25°C for 4h.

6.4 Preparative size exclusion chromatography, desalting and filtration

The reaction mixture was purified using preparative size-exclusion chromatography. A Superdex 200 pg (50/60) column was connected to an Äkta Avant 25 system (GE Healthcare) and equilibrated with PBS pH 7.4 according to the manufacturer's instructions. Subsequently, the reaction mixture was injected and run through the column with a flowrate of 10 ml/min and PBS pH 7.4 as running buffer. ADC containing fractions were determined via UV light absorption at 280 nm, pooled and concentrated. ADC material was concentrated using 15 ml Amicon Ultra 50 kDa cutoff centrifugal devices (Merck Millipore) according to manufactures instructions. The concentrated ADC material was transferred into formulation buffer (10 mM Histidine, 130 mM Glycine, 5% Sucrose. pH 5.5) using HiPrep 26/10 desalting columns (GE Healthcare) at a flowrate of 10 ml/min on an Äkta Avant 25 system (GE Healthcare) according to the manufactures instructions. The resulting ADC material was filtered using a 0.2 µm filter (Merck Millipore), aliquoted and subsequently shock frozen in liquid nitrogen. The final concentration of the ADC material was 5.82 mg/ml and the material was kept at -80°C until further use. The ADC resulting from this work is also referred to herein as "ADC SAR DM4" or, briefly, as "ADC SAR"; this ADC is an analog of SAR408701.

Example 7: An ADC based on mAb1 and SPDB-DM4

Another ADC was prepared based on the antibody mAb1 (as described herein above) and the drug-linker compound SPDB-DM4, i.e. the same drug-linker compound as in ADC SAR DM4 described above. The ADC resulting from this work is referred to herein as "ADC mAb1 DM4" and was prepared as follows:

7.1 Materials used:

• Antibody: mAb1, 1 mg/mL in 10 mM HEPES, pH 5.8

• Conjugation Buffer: 10 mM HEPES, pH 5.8

• Drug-linker compound: SPDB-DM4, 2 mg/mL in DMF

7.2 Method:

• Conjugation: about 30-fold molar excess of drug-linker molecule was used for conjugation (75 mL antibody + 7.1 mL SPDB-DM4 drug-linker), incubated at room temperature for 5 h with slow rocking

• Purification: ADC was buffer exchanged to 20 mM Histidine, 150 mM NaCl, pH 6.0 to remove free drug

• Formulation Buffer: 20 mM Histidine, 150 mM NaCl, pH 6.0

7.3 Purified ADC Analysis Details:

• Final Yield: 40 mg

• Concentration: 2.2 mg/mL

• DAR: 4.4

Example 8: Characterization of drug release from ADC1 and ADC2

8.1 Materials and Methods

8.1.1 Test Articles

ADC number	**Brief description of ADC**
ADC1	mAb1-glucuronid-exatecan (see also Example 4 above)
ADC2	mAb1-AAN-exatecan (see also Example 5 above)
ADC3	Anti-CEACAM5-CL2A-SN-38 ADC (Creative Biolabs, CAT#: ADC-091LCT, a humanized IgG1 monoclonal anti-CEACAM5 antibody like labetuzumab conjugated to SN38 via a CL2A linker)

8.1.2 Materials

All reagents and buffers were stored according to the instructions of the manufacturers and used before the batch expiration date.

Abbreviated name	Origin and contents
Human Liver Lysosomes	Sekisui Xenotech, H0610.L
Legumain Assay Buffer	50mM MES, 250 mM NaCl
10 x Catabolism Buffer	Sekisui Xenotech
Human serum	Biowest, S4200-100, Lot # S15594 S4200
Mouse serum	Biowest, S2160-100, Lot # S18169 S2160
Cynomolgus serum	Abcam, cat: ab155109, lot: GR316568-1
HEPES	Sigma, cat: H3784-100g lot: SLBR6536V
PIC III	Calbiochem, Protease Inhibitor Cocktail Set III, EDTA-Free, #539134
Methanol	Merck, cat: 1060092500

8.1.3 Instruments

Instrument	Supplier
CO2-Incubator	Heraeus
Thermomixer compact	Eppendorf
HTC PAL Autosampler	CTC Analytics
1200 HPLC	Agilent Technologies
API4000 Triple Quadrupole	Sciex

8.1.4 Procedures

8.1.4.1 Serum sample preparation

2 M HEPES solution: 52.1 g HEPES were dissolved in 75 mL MiliQ-water and 15 mL HCl 25%, adjusted to pH 7.55 and added up to 100 mL. This solution was mixed as 15 %v/v with serum

to obtain a stabilized serum with pH 7.3 - 7.4. Human serum from Biowest (Lot.no. S15594S4200) was thawed. 100 mL serum were mixed with 15mL 2 M HEPES buffer. Mouse serum from Biowest (Lot.no. S18169S2160) was thawed. 100mL serum were mixed with 15mL 2 M HEPES buffer. Cynomolgus serum was thawed and 8.5 mL serum were mixed with 1.5mL 2 M HEPES buffer. The pH was measured (7.37) and serum was sterile filtered. 2mL aliquots were frozen at -20°C.

The prepared serum was thawed at RT. The desired ADC protein concentration was prepared as triplicated with 180µg/mL for subsequent free payload analytics via LC-MS. After adding the ADCs to the serum, the individual batches were mixed and separated into 20 µL aliquots. Additionally, one 96h sample with 20µL for each ADC was pipetted and was used to for total work up analyses to measure recovery. 0h samples were directly frozen at - 80 °C, remaining samples were incubated at 37 °C and 5 % CO2 and reactions were stopped at 2/ 4/ 6/ 24/ 48/ 72, 96 hours incubation via storage at -80°C.

8.1.4.2 Human Liver Lysosome sample preparation

pH was adjusted either to pH 5.0 or pH 4.0 using assay buffer. Lysosomal stability preparations: 80 µL Human Liver Lysosomes were prepared for triplicate measurements as exemplary shown for ADC1 (n=3): 2.76 µL ADC1 + 6 µL Human Liver Lysosomes + 71.2 µL Legumain Assay Buffer. Preparation of MeOH + PIC (1:200): 10µL PIC III + 1990 µL MeOH. The reactions were started upon transferring the Eppendorf tubes to a Thermomix that was preheated to 37°C. Subsequently, 10 µL aliquots were drawn after 0, 1, 2, 4, 24 and 48 hours and mixed with 40µL PIC III (1:200).

8.2 Results

ADC stability for human, mouse and cynomolgus sera (Fig. 20). Conjugated Exatecan concentrations were calculated (initial dose ~10 µM) using free Exatecan (normalized data). Similar profiles were obtained for human, cynomolgus and mouse sera. Only minor warhead release observed, most pronounced in mouse serum for ADC2 (5.9 % free of initial conj. payload at 96h) and ADC1 (1.4%).

ADC3 control stability for mouse serum and buffer (Fig. 21). Conjugated SN38 concentrations were calculated (initial dose 50 µg/mL ADC protein concentration) using free SN38 (not normalized). For both matrices, pronounced SN-38 release observed.

Payload liberation profiles for ADC1 and ADC2 in human liver lysosomes (pH 5.0) (Fig. 22). Conjugated drug concentrations were calculated using e.g. free Exatecan (initial conc. ~10 µM Exatecan), normalized data. Intermediate levels of payload release were observed for ADC1- and ADC2-cleavage mediated payload liberation (both ~40% of initial total conj. Payload).

ADC catabolite profiling confirms free exatecan as lysosomal release product (Fig. 23). To confirm exatecan as major release product, ADC1 catabolite profiling study was performed in human lysosomal extracts. At this, comparison of the TIC-MS and extracted ion chromatograms at various timepoints showed that the expected exatecan catabolite was subsequently released from ADC1 during incubation (0h, 4h, 24h). The retention time 9.33 min, the detected mass *m/z* 436.1671 ($[M+H]^+$, $C_{24}H_{23}O_4N_4$ F) and the MS/MS spectrum of the detected catabolite are consistent with those of exatecan.

Example 9: ADC1 and ADC2 specifically kill cancer cells *in vitro* with high potency

Human cancer cell lines were used to assess the potential of ADC1 and ADC2 to kill cancer cells. ADC1 and ADC2 showed sub-nanomolar *in vitro* potency against different CEACAM5-positive and minor effect on CEACAM5-negative cell lines (Table 2 below). As shown in exemplary dose-response curves (Fig. 24A/B), ADC1 and ADC2 were very potent against CEACAM5-positive cell lines SK-CO-1 and SNU-16. In contrast, effects of ADC1 and ADC2 on antigen-negative MDA-MB-231 were limited to the highest concentrations tested (Fig. 24C).

Rituximab isotype control ADCs utilizing the respective linker and payload as for ADC1 and ADC2 showed much lower effects on SK-CO-1 cell line (Fig. 25).

In conclusion, ADC1 and ADC2 specifically kill CEACAM5 expressing human cancer cell lines *in vitro* with high potency.

	MKN-45 IC50 [M]; N≥3	**SNU-16 IC50 [M]; N≥3**	**SK-CO-1 IC50 [M]; N≥4**	**LS174T IC50 [M]; N≥3**	**MDA-MB-468 IC50 [M]; N≥2**	**MDA-MB-231 IC50 [M]; N≥3**
ADC1	6.2E-10 (-78%)	3.2E-10 (-91%)	8.5E-11 (-97%)	3.8E-10 (-72%)	>1E-08 (-85%)	>1E-08 (-67%)
ADC2	4.7E-10 (-90%)	2.8E-10 (-96%)	6.9E-11 (-98%)	2.8E-10 (-81%)	6.7E-09 (-97%)	1.5E-08 (-84%)
PAYLOAD	8.9E-10 (-96%)	4.4E-10 (-99%)	2.5E-10 (-99%)	5.3E-10 (-88%)	2.1E-10 (-99%)	9.3E-10 (-92%)
CEACAM5	Positive	Positive	Positive	Positive	Negative	Negative

Table 2. Potency of ADC1, ADC2 and free payload against multiple human cell lines. Maximal effects compared to untreated controls at the highest tested compound concentration are indicated in brackets. For each cell line, CEACAM5 expression is indicated.

Method – Viability Assay:

Cytotoxicity effects of the ADC on the cancer cell lines were measured by cell viability assays. Cells were seeded in a volume of 90 µL in 96-well plates the day before treatment. Test compounds (ADCs or free payloads) were formulated at 10-fold the starting concentration in cell culture medium. Test compounds were serial diluted (1:4) and 10 µL of each dilution was added to the cells in triplicates. Plates were cultured at 37 °C in a CO2 incubator for six days. For cell viability measurement, Cell Titer-Glo® reagent (Promega™ Corp, Madison, WI) was added to each well, and plates processed according to the manufacturer's instructions. Luminescence signals were measured using a Varioskan plate reader (Thermo Fisher). Luminescence readings were converted to % viability relative to untreated cells. Data was fitted with non-linear regression analysis, using log (inhibitor) vs. response, variable slope, 4-parameter fit equation using GraphPad Prism. Data is shown as % relative cell viability vs. molar compound concentration, error bars indicating standard deviation (SD) of triplicates. Geometric mean values of IC50s derived from multiple experiments were calculated.

Using the same method as above, ADC1 and ADC2 were also compared to ADC SAR DM4 in terms of their cytotoxic effects on antigen-positive SK-CO-1 and antigen-negative MDA-MB-231 cell line. ADC1 and ADC2 showed 2.9- and 2.7-fold higher potencies than ADC SAR DM4 against SK-CO-1 cancer cells, respectively (Fig. 26A). Non-specific effects against antigen-negative MDA-MB-231 were slightly higher for ADC SAR DM4 compared to ADC1 and ADC2 (Fig. 26B). ADC SAR DM4 and ADC mAb1 DM4 showed comparable potencies against SK-CO-1, with a slight tendency for higher potency of ADC mAb1 DM4 (Fig. 26A).

Example 10: ADC1 and ADC2 mediate potent bystander effect against antigen-negative cells in co-culture with antigen-positive cells

The potential of ADC1 and ADC2 to mediate a bystander effect against antigen-negative cells in close proximity to antigen-positive cells was evaluated in bystander assays. ADC1 and ADC2 showed a potent bystander effect against CEACAM5-negative MDA-MB-231 cells in the presence of CEACAM5-positive SK-CO-1 (Fig. 27A). The co-culture experiments were performed at an ADC concentration of 1 nM which, for ADC1, causes maximal inhibition of CEACAM5-positive SK-CO-1 cell viability (Fig. 26A) but no effect on CEACAM5-negative MDA-MB-231 cells (Fig. 26B). In line with these findings, no non-specific effect of ADC1 or ADC2 was observed in the bystander assay setup for MDA-MB-231 only controls (Fig. 27B).

In conclusion, ADC1 and ADC2 mediate a potent bystander effect against antigen-negative cancer cells in co-culture with antigen-positive cells. These findings indicate the potential to effectively target tumors with heterogeneous target expression.

In view of utilizing the same drug-linker and Fv region, it is expected that ADC1-M, ADC2-M, ADC3-M, and ADC6-M and ADC7-M mediate similar potent bystander effects like ADC1 and ADC2.

Compared to ADC SAR, ADC1 and ADC2 mediated a much more potent bystander effect on antigen-negative cells in co-culture with antigen-positive cells (Fig. 28A, Fig. 28B). ADC mAb1 DM4 utilizing the same antibody as in ADC1 and ADC2 (i.e. mAb1) with the drug-linker molecule utilized in ADC SAR DM4 (i.e. SPDB-DM4) also showed a more pronounced bystander effect than ADC SAR DM4 (Fig. 28A, Fig. 28B). This indicated that mAb1 contributes to the higher bystander effect observed for ADC1 and ADC2 in comparison with ADC SAR utilizing a different antibody. It is therefore expected that also ADC1-M, ADC2-M, ADC3-M, ADC6-M and ADC7-M mediate more potent bystander effects than ADC SAR.

For all ADCs tested, the extent of bystander effect increased with increasing the number of antigen-positive cells added to a constant number of antigen-negative cells (compare Fig. 28A and Fig. 28B). Without wishing to be bound by theory this may be a result of more ADC being processed by the higher number of antigen-positive cells to release more free payload, which is responsible for the bystander effect on antigen-negative cells.

No non-specific effects of tested ADCs were observed on MDA-MB-231 cells alone (Fig. 28C).

Method – Bystander Assay

Cytotoxicity effects of ADCs on antigen-negative cancer cell lines in co-culture with antigen-positive cancer cell lines were measured by bystander assays. One thousand CEACAM5-negative MDA-MB-231 cells were seeded in co-culture experiments with 750 or 3000 CEACAM5-positive SK-CO-1 cells per well. As a control, 1000 MDA-MB-231 cells only were seeded in parallel. Cells were seeded in a total volume of 90 µL in 96-well plates the day before treatment. Test compounds were formulated at 10-fold the final concentration of 1E-9 M in cell culture medium and 10 µL was added to the cells in duplicates. Plates were cultured at 37 °C in a CO2 incubator for six days.

Prior to immunofluorescence staining, medium was removed and cells were treated with 100% methanol (-20°C) for 30 minutes. After methanol removal and one PBS wash step, cells were treated with 2.5% paraformaldehyde (PFA) with 0.2% Triton X-100 in PBS for 15 minutes at room temperature. After solution removal and one PBS wash step, cells were treated with 1% BSA / 0.1% Tween / 0.1% sodium azide in PBS for at least one hour at room temperature. Antigen-positive and antigen-negative cells were discriminated by immunofluorescence

staining with 10 µg/mL human anti-CEACAM5 (mAb1) primary antibody and 1:2000 dilution of donkey anti-human IgG fluorescently (phycoerythrin) labeled secondary antibody (Jackson ImmunoResearch #709-116-149). Cells were identified by nuclei staining using 1 µg/mL Hoechst 33342 (Life technologies, cat# H3570) dye. Staining was carried out in 1% BSA / 0.1% sodium azide PBS solutions for 30 minutes at room temperature. Secondary antibody staining was combined with Hoechst dye staining. Between and after staining steps, cells were washed thrice with PBS.

Plates were imaged with the confocal quantitative image cytometer CQ1 (Yokogawa® Electric Corporation, Tokyo, Japan). Analysis was adapted from the CQ1 software (Yokogawa) template "Nucleus and pseudo-Cell body" and FCS export files were analyzed using FlowJo (BD). Based on staining or absence of staining with fluorescently labeled antibody around the nucleus, antigen-positive and antigen-negative cells were distinguished and quantified. Bar graphs show the number of identified antigen-positive and antigen-negative cells per treatment condition.

Example 11: Efficacy of ADC1 and ADC2 in a colorectal cancer (CRC) patient-derived xenograft (PDX) mouse model

Anti-tumor efficacy in vivo has been evaluated in the human patient-derived CRC xenograft model COPF217 (Shanghai LideBiotech CO., LTD). COPF217 tumor fragments were transplanted subcutaneously into the right flank of six to eight weeks old immunodeficient female mice (NU-Foxn1nu, Charles River). When tumors reached a mean volume of 165 mm^3, 6 mice/group were treated once intravenously with vehicle (saline solution) or with ADC1 or ADC2 (each at a dose of 10mg/kg; day 0). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using the formula L×(W^2)/2.

The single treatment with ADC1 or ADC2 at a dose of 10 mg/kg led to a significant anti-tumor effect. The two substances show a comparable effect leading to tumor stasis (Fig. 29). Treatment with ADC1 or ADC2 had no significant impact on body weight (data not shown).

Further experiments with other CRC PDX models:

In additional experiments corresponding to the one above described for COPF217, a single treatment with ADC1 also resulted in tumor stasis or tumor regression in 12 other CRC PDX models with high CEACAM5 expression.

Example 12: Efficacy of ADC1 in a non-small cell lung cancer (NSCLC) PDX mouse model

Anti-tumor efficacy *in vivo* has been evaluated in the human patient-derived NSCLC xenograft model LUPF160151 (Shanghai LideBiotech CO., LTD). LUPF160151 tumor fragments were transplanted subcutaneously into the right flank of six to eight weeks old immunodeficient

female mice (NU-Foxn1nu, Charles River). When tumors reached a mean volume of 180 mm³, 5 mice/group were treated once intravenously with vehicle (saline solution) or with ADC1 (6 mg/kg; day 0). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using the formula L×(W^2)/2.

The single treatment with ADC1 at a dose of 6 mg/kg led to a significant anti-tumor effect (Fig. 30) with no impact on body weight (data not shown). The model LUPF160151 showed a heterogeneous CEACAM5 expression with CEACAM5 negative tumor cells adjacent to CEACAM5 positive tumor cells. The good efficacy of ADC1 in this model thus indicates a potent bystander effect of the ADC.

Example 13: Efficacy of ADC1 in gastric cancer PDX mouse model

Anti-tumor efficacy *in vivo* was evaluated in the human patient-derived gastric cancer xenograft model GAX066 (Shanghai ChemPartner Co., Ltd). Tumor fragments were transplanted subcutaneously into the right flank of immunodeficient female mice (Nu/Nu mice, Beijing Vital River Lab Animal Technology Co. Ltd, 18-22g). When tumors reached a mean volume of 220 mm³, 6 mice/group were treated once intravenously with vehicle (saline solution) or with ADC1 (3 or 10 mg/kg; Day 0). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using the formula L×(W^2)/2.

The single treatment with 3 or 10 mg/kg ADC1 caused a significant anti-tumor effect (Fig. 31) with no impact on body weight (data not shown). In five of six tumors, treatment with 10 mg/kg resulted in complete tumor regression.

Example 14: Efficacy of ADC1 compared to ADC3 in a pancreatic cell line derived tumor model

Efficacy of ADC1 in comparison to ADC3 has been evaluated in the human pancreatic cell line derived xenograft model HPAF-II (ATCC, CRL-1997). $5x10^6$ HPAF-II cells were injected subcutaneously into the right flank of six to eight weeks old immunodeficient female mice (Hsd:Athymic Nude-Foxn1nu, Envigo). When tumors reached a mean volume of 150 mm³, 10 mice/group were treated once intravenously with vehicle (saline solution) or ADC1 (1 mg/kg or 6mg/kg; day 0) or with ADC3 (1 mg/kg or 6mg/kg; day 0). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using L×(W^2)/2.

The single treatment with ADC1 at a dose of 6 mg/kg led to a significant anti-tumor effect. The effect is dose-dependent, as the single treatment with 1 mg/kg only led to a mild but significant, transient anti-tumor effect. In contrast, the single treatment with same doses of ADC3 showed no significant anti-tumor effect at either dose (Fig. 32). All treatments had no significant effect on body weight (data not shown). It is expected that ADC1-M, ADC2-M, ADC3-M, ADC6-M and ADC7-M compare similarly favorably to ADC3 in this regard as does ADC1.

Example 15: Efficacy of ADC1 compared to ADC SAR DM4 in two CRC PDX mouse models

Anti-tumor efficacy *in vivo* was evaluated in the human patient-derived CRC xenograft models COPF230 and REPF210 (Shanghai LideBiotech CO., LTD). Tumor fragments were transplanted subcutaneously into the right flank of six to eight weeks old immunodeficient female mice (NU-Foxn1nu, Charles River). When tumors reached a mean volume of about 170 mm^3, 6 mice/group were treated once (Day 0) intravenously with vehicle (saline solution), ADC1 (6 mg/kg) or ADC SAR DM4 (6 mg/kg). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using the formula L×(W^2)/2.

Single treatment with ADC1 led to a significant anti-tumor effect in both PDX models COPF230 (Fig. 33) and REPF210 (Fig. 34). In contrast, a single treatment with the same dose of ADC SAR DM4 showed no anti-tumor effect in either of the CRC PDX models (Fig. 33, Fig 34). No significant effect on body weight was observed in any of the treatment groups (data not shown). It is expected that ADC1-M, ADC2-M, ADC3-M, ADC6-M and ADC7-M compare similarly favorably to ADC SAR DM4 in this regard as does ADC1.

Example 16: Efficacy of ADC1 compared to ADC SAR DM4 in a gastric PDX mouse model (GAPF313)

Anti-tumor efficacy *in vivo* was evaluated in the human patient-derived gastric xenograft model GAPF313 (Shanghai LideBiotech CO., LTD). Tumor fragments were transplanted subcutaneously into the right flank of six to eight weeks old immunodeficient female mice (NU-Foxn1nu, Charles River). When tumors reached a mean volume of about 180 mm^3, 6 mice/group were treated 3 times every second week starting from day 0 intravenously with vehicle (saline solution), ADC1 (4 mg/kg or 7mg/kg Q2Wx3) or ADC SAR DM4 (4.7 mg/kg Q2Wx3). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using the formula L×(W^2)/2.

Interim data analysis of the ongoing experiment demonstrates a clear anti-tumor efficacy of ADC1 in this model and no effect of ADC SAR DM4 (Fig. 35). All treatments were well tolerated (data not shown). It is expected that ADC1-M, ADC2-M, ADC3-M, ADC6-M and ADC7-M compare similarly favorably to ADC SAR DM4 in this regard as does ADC1.

Example 17: Safety profile of ADC1 – a pilot toxicity study in cynomolgus monkeys

To investigate the safety profile, ADC1 was administered by 30-min i.v. infusion to cynomolgus monkeys, three times with a 3-week interval (on day 1, 22 and 43), at dosages of 0, 3, 10, and 30 mg/kg, and animals were sacrificed on day 50 for gross and histopathological examination. As a result, it was found that ADC1 has a comparatively favorable safety profile in that ADC1 lacks toxicity in certain organs which are affected by toxic side effects of known ADCs.

Example 18: Expression and purification of modified antibodies and of Transglutaminase

In the following, the expression and purification of modified antibodies will be described. These modified antibodies can be used for the production of a respective antibody-drug-conjugate. The features of the modified antibodies mAb1-M, mAb2-M, mAb3-M, mAb4-M, mAb5-M, mAb6-M and mAb7-M are outlined in the following Table 4:

Antibody/Antibody--drug conjugate name	**Antibody features**	**Antibody-drug conjugate features, incl. preferred drug-to-antibody ratio, linker type and drug**	**Amino acid sequence for heavy chain (HC) and light chain (LC) of the mAb antibody**
mAb1-M, ADC1-M	anti-CEACAM5 mAb LALA mutation YTE mutation	interchain cysteine conjugation DAR=8 maleimido-b-glucuronide-exatecan (DL1)	HC = SEQ ID NO: 34 LC = SEQ ID NO: 14
mAb2-M, ADC2-M	anti-CEACAM5 mAb LALA mutation YTE mutation MTG Tag	MTG coupling DAR=4 NH2-GGG-b-glucuronide-exatecan (DL1-M)	HC = SEQ ID NO: 34 LC = SEQ ID NO: 36
mAb3-M, ADC3-M	anti-CEACAM5 mAb LALA mutation YTE mutation K222R mutation (MTG optimization) MTG Tag	MTG coupling DAR=4 NH2-GGG-b-glucuronide-exatecan (DL1-M)	HC = SEQ ID NO: 35 LC = SEQ ID NO: 36
mAb4-M, ADC4-M	anti-CD20 Rituximab based isotype control	interchain cysteine conjugation DAR=8 maleimido-b-glucuronide-exatecan (DL1)	HC = SEQ ID NO: 64 LC = SEQ ID NO: 65 (Rituximab mAb)

mAb5-M, ADC5-M	anti-CD20 Rituximab based isotype control LALA mutation MTG Tag	MTG coupling DAR=4 NH2-GGG-b-glucuronide-exatecan (DL1-M)	HC = SEQ ID NO: 40 LC = SEQ ID NO: 41
mAb6-M, ADC6-M	anti-CEACAM5 mAb LALA mutation	interchain cysteine conjugation DAR=8 maleimido b-glucuronide-exatecan (DL1)	HC = SEQ ID NO: 51 LC = SEQ ID NO: 14
mAb7-M, ADC7-M	anti-CEACAM5 mAb LALA mutation MTG Tag	MTG coupling DAR=4 NH2-GGG-b-glucuronide-exatecan (DL1-M)	HC = SEQ ID NO: 51 LC = SEQ ID NO: 36

Table 4

DNA sequences encoding for antibodies mAb1-M, mAb2-M, mAb3-M, mAb5-M, mAb6-M and mAb7-M were synthesized and cloned onto pTT5 plasmids for recombinant expression at GeneArt (Life Technologies). Produced plasmids were used for transient transfection and recombinant protein expression in shaking flasks using the ExpiCHO expression system (GibcoTM, Thermo Fisher Scientific Inc.). Seven days post transfection, supernatants were harvested and expressed antibodies purified using a standard stepwise process including protein A affinity chromatography (HiTrap MabSelect SuRe columns, Cytiva) and size-exclusion chromatography (HiLoad Superdex 200 pg columns, Cytiva).

mAb4-M/Rituximab (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was purchased in pharmacy grade quality.

Expression and purification of transglutaminase

A DNA sequence encoding for transglutaminase enzyme mTG (Seq ID 46) was synthesized and cloned onto a pET30a plasmid for recombinant expression at GeneArt (Life Technologies). An Escherichia coli BL21 (DE3) strain transformed with the generated plasmid was cultivated in shaking flasks in lysogenic broth medium supplemented with, 5 g/l glucose, 10 ml/100 ml 10x phosphate buffered saline and 30 mg/l kanamycin overnight at 28 °C and 130 rpm (50 mm throw). This culture was used to inoculate a fermenter containing 9.5 l liter growth medium (50 g/l yeast extract, 10 g/l peptone, 0.5 g/l $MgSO_4$ x 7 H_2O and 2 ml 50% Desmophen (antifoam by Rhein Chemie Rheinau) to an optical density of 0.00002. The fermenter was run at 28 °C

with 800 U/min revolutions, 5 Nl/min aeration and pH 7.0-7.4 over night (16 h). At OD 5 the culture was induced with 0.1 mM IPTG until an OD of ~30 was reached (5-6 hours). In case of foam formation or a drop in oxygen concentration below 2 mg/ml, more Desmophen was added, or the revolutions increased to 1000 rpm, respectively. The cell mass was harvested by continuous flow-through centrifugation.

50 g of cell pellet were resuspended in 250 ml 50 mM Na-acetate,1 mM DTT, 5 mM $MgCl_2$, 25 U/ml Benzonase (Millipore) pH 5.5 and disrupted using a French Press. The supernatant was clarified by centrifugation and filtration (0.8/0.2 µm pore size), pH adjusted to 5.5 and loaded onto a Fractogel® SO3- (M) 85 ml column (Millipore) followed elution with a linear gradient of 20 CV from 0 – 1 M NaCl. Fractions with purified protein of expected size were identified by SDS-PAGE, pooled and dialyzed overnight at 4 °C against 50 mM Tris/HCl, 300 mM NaCl, pH 8.0. Transglutaminase was then proteolytic processed at a final concentration of 2 mg/ml using 0.5 U/ml Dispase®I (Sigma Aldrich) and 2 mM $CaCl_2$ for 60 min at 37 °C followed by addition of 5 mM EDTA. The reaction mix was dialyzed overnight at 4°C against 50 mM sodium phosphate buffer pH 6.0, loaded onto a Fractogel® SO3- column (Millipore) and eluted with a linear gradient of 20 CV from 0 – 1 M NaCl. Fractions with efficiently cleaved and purified protein were identified by SDS-PAGE, pooled, concentrated and purified using a HiLoad Superdex 75pg size exclusion column (Cytiva) with 24 HEPES pH 7, 100 mM NaCl as a running buffer. Transglutaminase containing fractions were pooled, concentrated to >20 mg/ml, flash frozen in liquid nitrogen and stored at -80°C. Enzyme activity was determined using ZediXclusive Microbial Transglutaminase assay (Zedira).

Example 19: ADC preparation, conjugation and characterization of ADC1-M, ADC4-M and ADC6-M

19.1. Antibody Preparation and conjugation

Monoclonal antibodies (mAb) formulated in 50 mM Histidine, 100 mM NaCl, pH 6.5 were stored at -80°C. Prior conjugation, mAbs were thawed at RT and protein concentration was adjusted to 5 mg/ml via dilution with formulation buffer. Subsequently, mAbs were reduced adding 10-12 molar equivalents excess (relative to the mAb) of TCEP and incubated for 2-3h at 37°C. 16-24 molar equivalents (relative to the mAb) of DL1 (in a 10 mM stock solution in DMSO) were added and incubated for 60 min at 25°C. The reaction mix was quenched adding 25 molar equivalents (relative to the mAb) of N-acetyl-cysteine (from a 25 mM DMSO stock solution) and incubated for 30 min at 25°C. ADCs were separated from DL1 and possible high molecular weight species (HMWS) via size exclusion chromatography (SEC). Prior to SEC purification, samples were centrifuged at 4000 x g for 2 min to remove possible precipitates. SEC was carried out using a HiLoad Superdex 200 26/60 Increase column in combination with an Äkta LC system (Cytiva) at a flow rate of 2.5 ml/min and 50 mM Histidine, 100 mM NaCl,

pH 6.5 as running buffer. Fractionated samples containing the ADC material were pooled and concentrated to 6 mg/ml using Amicon Ultra 15 50K centrifugal (Millipore) followed by a final buffer exchange into 10 mM Histidine, 40 mM NaCl, 6% Trehalose, 0.05% TWEEN, pH 5.5 using HiTrap Desalting columns in combination with an Äkta LC system (Cytiva). Final bulk drug substance (BDS) was filtered through a 0.22 µm sterile filter unit (Millipore) and shock frozen in liquid nitrogen till further use.

19.2. Quality attributes

ADC1-M

Concentration[mg/ml]	6.0
Drug-to-Antibody Ratio	8.16
Purity SEC [%]	98.9
Free drug content [%]	0.08
Endotoxin content [EU/mg]	1.84

ADC6-M

Concentration[mg/ml]	7.09
Drug-to-Antibody Ratio	8.14
Purity SEC [%]	100
Free drug content [%]	0.01
Endotoxin content [EU/mg]	0.39

ADC4-M

Concentration[mg/ml]	5.82
Drug-to-Antibody Ratio	7.87
Purity SEC [%]	97.8
Free drug content [%]	0.03
Endotoxin content [EU/mg]	0.13

9.3 Drug substance characterization

Size exclusion chromatography (SEC) method

Monomer content and purity were assessed by size exclusion chromatography on a Waters Bio Resolve SEC mAb Column.

Wavelength	214 nm
Column	Waters Bio Resolve SEC mAb Column, 200Å, 2.5 µm, 4.6 x 150mm
Mobile Phase	50 mM Sodium Phosphate Monobasic
	0.4 M Sodium Perchlorate, pH 6.3
Injection Volume	5 µL
Column Temperature	25°C
Flow rate	0.35 mL/min

Typical SEC chromatograms showing the purity of the input mAb and the final BDS are shown in Figure 36.

Reversed-Phase HPLC (RP HPLC) method

RP HPLC Method Parameters

Wavelength	214 nm
Column	PLRP-S 1000 Å (50 x 2.1 mm, 8 µm) column, Agilent
Mobile Phase A	Lichrosolv H2O + 0.1 % TFA
Mobile Phase B	100% AcN + 0.1 % TFA
Gradient	30-45 % B in 7.5 min
Injection Volume	10 µL
Column Temperature	65°C
Flow rate	1.0 mL/min
Sample Preparation	40 µl sample (2.2 mg/ml) were mixed with 4 µl 0.5 M TCEP and incubated for 5 min at RT. Afterwards, 40 µl of 0.5 M Iodoacetamide were added and incubated for 15 min at 37°C. 10 µl of this reaction was injected.

Typical RP-HPLC chromatograms illustrating DAR determination of the final BDS are shown in Figure 37.

Free-drug method

Wavelength	254 nm
Column	Phenomenex Gemini, C18, 2 x 150 mm, 3 µm (P/N 00F-4439-B0)
Mobile Phase A	0.1% Formic acid in water
Mobile Phase B	0.1% Formic acid in acetonitrile
Gradient	

Time (min)	MP A (%)	MP B (%)
0.00	95	5
1.00	95	5
12.00	0	100
3 min Equilabration to 95% A		

Injection Volume	10.00 µL
Column Temperature	50°C
Flow rate	0.75 mL/min
Sample Preparation	Protein drop: 100 µL of Drug Substance + 250 µL of cold MeOH + 50 µL of 3 M MgCl2. Spin at 20,000 rpm for 10 min
Standard Preparation	Mix 20 µL of 20 mM DL (MSC2702209A in DMSO) + 20 µL DMSO + 40 µL MeOH + 20 µL of 200 mM NAC in Diafiltration buffer. Incubate overnight to afford 4 mM DL-NAC. Dilute 4 mM DL-NAC in MeOH to afford a 4 µM DL-NAC standard.

Endotoxin characterization

Endotoxin was determined by kinetic chromogenic LAL assay using an Endosafe PTS endotoxin system (Charles River). Buffers and antibodies were diluted 10-fold in LAL reagent water. The ADCs were diluted 10-fold in LAL reagent water. All samples were analyzed on 0.01 – 1 EU/mL cartridges. The EU/mL value was converted to EU/mg by dividing by the ADC [P] mg/mL.

Example 20: ADC preparation, conjugation and characterization of ADC7-M, ADC2-M, ADC5-M and ADC3-M

20.1. Antibody Preparation and conjugation

Monoclonal antibodies (mAb) were stored at -80°C. Prior conjugation, mAbs were thawed at RT and buffer was exchanged to 24 mM HEPES, pH 7.0 using HiTrap Desalting columns in combination with an Äkta liquid chromatography (LC) system (Cytiva). To couple the drug linker (DL) to the antibody, a microbial transglutaminase (mTG) was used. The reaction setup was as follows: 5 mg/ml mAb, 5 molar equivalents of DL1-M per conjugation site, 20 U/ml mTG, 7 % DMSO, 24 mM HEPES, pH 7.0. The reaction was carried out at 37°C for 18 h. ADCs were separated from DL and mTG via size exclusion chromatography (SEC). Prior to SEC purification, NaCl concentration of the samples was adjusted to 100 mM using a 5 M NaCl stock solution. SEC was carried out using a HiLoad Superdex 200 26/60 Increase column in combination with an Äkta LC system (Cytiva) at a flow rate of 2.5 ml/min and 10 mM Histidine, 100 mM NaCl, pH 5.5 as running buffer. Fractionated samples containing the ADC material were pooled and concentrated to 8 mg/ml using Amicon Ultra 15 50K centrifugal (Millipore) followed by a final buffer exchange into 10 mM Histidine, 40 mM NaCl, 6% Trehalose, 0.05%

TWEEN, pH 5.5 using HiTrap Desalting columns in combination with an Äkta LC system (Cytiva). Final bulk drug substance (BDS) was filtered through a 0.22 µm sterile filter unit (Millipore) and shock frozen in liquid nitrogen till further use.

20.2 Quality attributes

ADC7-M

Concentration[mg/ml]	7.11
Drug-to-Antibody Ratio	3.87
Purity SEC [%]	99.9
Free drug content [%]	0.12
Endotoxin content [EU/mg]	0.34

ADC2-M

Concentration[mg/ml]	6.9
Drug-to-Antibody Ratio	3.86
Purity SEC [%]	99.7
Free drug content [%]	0.03
Endotoxin content [EU/mg]	0.19

ADC3-M

Concentration[mg/ml]	7.98
Drug-to-Antibody Ratio	4.2
Purity SEC [%]	100
Free drug content [%]	0.13
Endotoxin content [EU/mg]	0.14

ADC5-M

Concentration[mg/ml]	5.92
Drug-to-Antibody Ratio	3.88
Purity SEC [%]	99.8
Free drug content [%]	0.15
Endotoxin content [EU/mg]	>0.5

20.3. Methods: Drug substance characterization

Size exclusion chromatography (SEC) method

Monomer content and purity were assessed by size exclusion chromatography on a TOSOH TSKgel column. Injection volumes ranged from 1 – 5 µl (up to 10 µg protein).

Wavelength 214 nm

Column Tosoh TSKgel 7.8 mm x 300 mm, 5 µm

Mobile Phase 50 mM Sodium Phosphate Monobasic, 0.4 M Sodium Perchlorate, pH 6.3

Injection Volume 1-5 µL

Column Temperature 25°C

Flow rate 0.5 mL/min

Typical SEC chromatograms showing the purity of the input mAb and the final BDS are shown in Figure 38.

Reversed-Phase HPLC (RP HPLC) method

RP HPLC Method Parameters

Wavelength 214 nm

Column PLRP-S 1000 Å (50 x 2.1 mm, 8 µm) column, Agilent

Mobile Phase A Lichrosolv H2O + 0.1 % TFA

Mobile Phase B 100% AcN + 0.1 % TFA

Gradient 30-45 % B in 7.5 min

Injection Volume 10 µL

Column Temperature 65°C

Flow rate 1.0 mL/min

Sample Preparation 40 µl sample (2.2 mg/ml) were mixed with 4 µl 0.5 M TCEP and incubated for 5 min at RT. Afterwards, 40 µl of 0.5 M Iodoacetamide were added and incubated for 15 min at 37°C. 10 µl of this reaction was injected.

Typical RP-HPLC chromatograms illustrating DAR determination of the final BDS are shown in Figure 39.

Free-drug method

Wavelength 254 nm

Column Phenomenex Gemini, C18, 2 x 150 mm, 3 µm (P/N 00F-4439-B0)

Mobile Phase A 0.1% Formic acid in water

Mobile Phase B 0.1% Formic acid in acetonitrile

Gradient

Time (min)	MP A (%)	MP B (%)
0.00	95	5
1.00	95	5
12.00	0	100
3 min Equilabration to 95% A		

Injection Volume 10.00 µL

Column Temperature 50°C

Flow rate 0.75 mL/min

Sample Preparation Protein drop: 100 µL of Drug Substance + 250 µL of cold MeOH + 50 µL of 3 M MgCl2. Spin at 20,000 rpm for 10 min

Standard Preparation Mix 20 µL of 20 mM DL (MSC2702209A in DMSO) + 20 µL DMSO + 40 µL MeOH + 20 µL of 200 mM NAC in Diafiltration buffer. Incubate overnight to afford 4 mM DL-NAC. Dilute 4 mM DL-NAC in MeOH to afford a 4 µM DL-NAC standard.

Endotoxin characterization

Endotoxin was determined by kinetic chromogenic LAL assay using an Endosafe PTS endotoxin system (Charles River). Buffers and antibodies were diluted 10-fold in LAL reagent water. The ADCs were diluted 10-fold in LAL reagent water. All samples were analyzed on

0.01 – 1 EU/mL cartridges. The EU/mL value was converted to EU/mg by dividing by the ADC [P] mg/mL.

Example 21: ADC1-M and ADC2-M specifically kill cancer cells *in vitro* with high potency

Human cancer cell lines were used to assess the potential of ADC1-M and ADC2-M to kill cancer cells. ADC1-M and ADC2-M showed sub-nanomolar and sub-nanomolar to single digit nanomolar *in vitro* potency against different CEACAM5-positive cell lines, respectively **(Table 3)**. In contrast, effects of ADC1-M and ADC2-M were minor on the CEACAM5-negative cell line MDA-MB-231 **(Table 3)**. As shown in exemplary dose-response curves, ADC1-M and ADC2-M were very potent against CEACAM5-positive cell lines SK-CO-1, SNU-16, MKN-45 and LS174T **(Fig. 40a-d & Fig. 41a-d)**. In contrast, ADC1-M and ADC2-M had only minor effects on antigen-negative MDA-MB-231 cell viability **(Fig. 40e & Fig. 41e)**.

Isotype control ADCs utilizing the same linker payloads as ADC1-M and ADC2-M showed much lower effects on the tested CEACAM5-positive cell lines **(Fig. 40 & Fig. 41)**.

In conclusion, ADC1-M and ADC2-M specifically kill CEACAM5 expressing human cancer cell lines *in vitro* with high potency.

	SK-CO-1 IC50 [M]; n≥2	SNU-16 IC50 [M]; n≥2	MKN-45 IC50 [M]; n≥2	LS174T IC50 [M]; n≥2	MDA-MB-231 IC50 [M]; n≥3
ADC1-M	7.6E-11 (-95%)	3.6E-10 (-97%)	5.2E-10 (-96%)	6.0E-10 (-86%)	>1E-08 (-71%)
ADC2-M	1.1E-10 (-92%)	5.1E-9 (-78%)	1.2E-9 (-80%)	8.5E-10 (-67%)	n/a (-16%)
Payload	2.2E-10 (-97%)	2.9E-10 (-99%)	6.6E-10 (-100%)	3.0E-10 (-91%)	6.0E-10 (-91%)
CEACAM5	Positive	Positive	Positive	Positive	Negative

Table 3. Potency of ADC1-M, ADC2-M and free payload against multiple human cell lines. Maximal effects compared to untreated controls at the highest tested compound concentration are indicated in brackets. Cut-off for potency reporting -50% effect, otherwise “n/a” stated. For each cell line, CEACAM5 expression is indicated.

Method – Viability Assay:

Cytotoxicity effects of the ADC on the cancer cell lines were measured by cell viability assays. Cells were seeded in a volume of 90 µL in 96-well plates the day before treatment. Test compounds (ADCs or free payloads) were formulated at 10-fold the starting concentration in cell culture medium. Test compounds were serial diluted (1:4) and 10 µL of each dilution was added to the cells in triplicates. Plates were cultured at 37 °C in a CO2 incubator for six days. For cell viability measurement, Cell Titer-Glo® reagent (Promega™ Corp, Madison, WI) was added to each well, and plates processed according to the manufacturer's instructions. Luminescence signals were measured using a Varioskan plate reader (Thermo Fisher). Luminescence readings were converted to % viability relative to untreated cells. Data was fitted with non-linear regression analysis, using log (inhibitor) vs. response, variable slope, 4-parameter fit equation using Genedata Screener or GraphPad Prism. Data is shown as % relative cell viability vs. molar compound concentration, error bars indicating standard deviation (SD) of duplicates or triplicates. Geometric mean values of IC50s derived from multiple experiments were calculated.

ADC1-M and ADC2-M were also compared to ADC SAR DM4 in terms of their cytotoxic effects on antigen-positive SK-CO-1 and antigen-negative MDA-MB-231 cell lines. ADC1-M and ADC2-M showed similar potency as ADC SAR DM4 against SK-CO-1 cancer cells (Fig. 40a and Fig. 41a compared to Fig. 26a). Non-specific effects against antigen-negative MDA-MB-231 were higher for ADC SAR DM4 compared to ADC1-M and ADC2-M (Fig. 40e and Fig. 41e compared to Fig. 26b) and the difference was more pronounced for ADC2-M than ADC1-M.

ADC1-M and ADC2-M were also generated utilizing an antibody backbone lacking YTE mutation. Both these ADCs (ADC6-M and ADC7-M) showed comparable results like respective ADCs with YTE mutation (ADC1-M and ADC2-M).

Example 23: Pharmacokinetic studies of ADCs

Pharmacokinetic studies in human FcRn transgenic (276 hemizygous model) mice were performed, following single intravenous administration of 3 mg/kg of ADC1, ADC1-M, ADC6-M, ADC7-M, ADC2-M. Samples were taken at 1, 24, 48, 72, 144, 168, 240, 336 and 504 h after dose, from 6 animals per treatment group for ADC1-M, ADC6-M, ADC7-M and ADC2-M, and at 0.08 (about 5 minutes), 4, 24, 48, 72, 168, 240, 336 and 504 h after dose for ADC1 from 9 animals. Data were pooled for PK analysis, see Fig. 42 for plasma concentrations per treatment. Back extrapolation of plasma concentration at time 0 (C0) resulted in slightly lower than theoretical maximal concentration for ADC1-M, ADC7-M, and ADC2-M and close to the theoretical maximum for ADC6-M and ADC1 (assuming dilution in 40 mL/kg plasma volume). The plasma concentration decreased with a biphasic profile: steep plasma concentration decrease was observed up to 24 h followed by a slower decrease up to the last sampling time

(504 h) in all molecule groups except for animals treated with ADC6-M that was quantifiable up to 336 h.

The longest terminal half-life (t1/2) value was calculated for ADC2-M (190 h) a Lala-YTE-mutant with DAR=4, followed by ADC7-M (122 h) a Lala-mutant with DAR=4, ADC1-M (64.5 h) a Lala-YTE-mutant and DAR=8 and ADC6-M (33.8 h) a Lala-mutant with DAR=8 and finally ADC1 (29.6 h) an IgG1.4 with DAR=8. The % of extrapolated AUCinf was lower than 20% allowing reliable calculation of AUC0-inf and derived parameters (Cl, Vz and Vss). The highest AUC0-inf and the lowest Cl values were calculated for ADC2-M (Lala-YTE-mutant, DAR=4) followed by ADC7-M, ADC6-M, ADC1-M, and ADC1 in transgenic mice. TheAUC0-inf and Cl values ranged from 1360000 to 10200000 h*ng/mL and from 0.293 to 1.16 mL/h/kg respectively. No relevant differences in the volume of distribution (Vss) were observed with Vss ranging from 49.8 to 113 mL/kg.

In conclusion, after single iv administration of 3 mg/kg in transgenic mice the best PK profile was shown by ADC2-M with the highest plasma exposure, lowest Cl and longest t1/2. PK profiles improved by moving from IgG1.4 to a Lala-mutant, further by including a YTE-mutant the strongest effect was observed by reducing DAR from 8 to 4.

Table 5: PK parameters for Ceacam5 back-up molecules after 3 mg/kg i.v. administration. The antibody-drug-conjugate (ADC) and drug-to-antibody ratio (DAR) is indicated as well.

Dose (mg/kg)	ADC	Anti-body back-bone	DAR	Strain	t1/2 (h)	C0 (ng/mL)	Tlast (h)	Clast (ng/mL)	AUClast (h*ng/mL)	AUCinf (h*ng/mL)	%_Ext (%)	Vz (mL/kg)	Cl (mL/h/kg)	Vss (mL/kg)
3	ADC 1	IgG1.4	8	HuFcRn	29.6	76800	240	74.4	1360000	1360000	0.2	93.9	2.20	65.8
3	ADC 1-M	Lala-YTE-mutant	8	HuFcRn	64.5	49900	504	1190	2580000	2590000	0.4	108	1.16	113
3	ADC 2-M	Lala-YTE-mutant	4	HuFcRn	190	56100	504	6810	8360000	10200000	18.4	80.2	0.293	87.7
3	ADC 6-M	Lala-Mutant	8	HuFcRn	33.8	84100	336	52.0	2850000	2850000	0.1	51.3	1.05	49.8
3	ADC 7-M	Lala-Mutant	4	HuFcRn	122	55800	504	2400	5720000	6140000	6.9	86.0	0.488	93.4

Example 24: Efficacy of ADC1-M and ADC2-M in a pancreatic cell line derived tumor model

Efficacy of ADC1-M and ADC2-M has been evaluated in the human pancreatic, cell line derived xenograft model BxPC3 (ATCC, CRL-1687). 5x106 BxPC3 cells were injected subcutaneously into the right flank of six to eight weeks old immunodeficient female mice (Hsd:Athymic Nude-Foxn1nu, Envigo). When tumors reached a mean volume of 85 mm^3, 10 mice/group were treated once intravenously with vehicle (saline solution) or ADC1-M (5mg/kg; day 0) or with ADC2-M (5 mg/kg or 10mg/kg; day 0). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using L×(W^2)/2.

The single treatment with ADC1-M at a dose of 5 mg/kg and with ADC2-M at a dose of 5 mg/kg or 10mg/kg led to a significant anti-tumor effect. Both ADCs have comparable efficacy in this model (Fig. 43). The treatments had no significant effect on body weight (data not shown).

Example 25: Preparation of ADC8, an analog of the ADC labetuzumab govitecan

25.1 The antibody

For the purposes of further comparative experiments, a further analog of the ADC labetuzumab govitecan was prepared based on a monoclonal antibody having the following sequence:

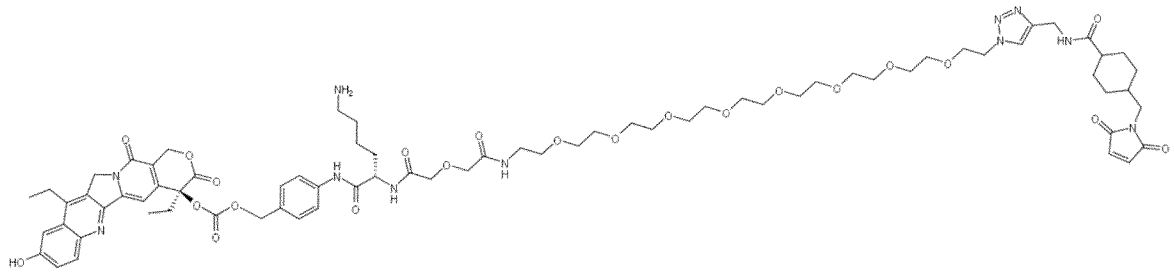
Heavy chain: DIQLTQSPSS LSASVGDRVT ITCKASQDVG TSVAWYQQKP GKAPKLLIYW TSTRHTGVPS RFSGSGSGTD FTFTISSLQP EDIATYYCQQ YSLYRSFGQG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GECEVQLVES GGGVVQPGRS LRLSCSASGF DFTTYWMSWV RQAPGKGLEW IGEIHPDSST INYAPSLKDR FTISRDNAKN TLFLQMDSLR PEDTGVYFCA SLYFGFPWFA YWGQGTPVTV SSASTKGPSV FPLAPSSKST SGGTAALGCL VKDYFPEPVT VSWNSGALTS GVHTFPAVLQ SSGLYSLSSV VTVPSSSLGT QTYICNVNHK PSNTKVDKRV EPKSCDKTHT CPPCPAPELL GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG

QPENNYKTTP PVLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP GK (SEQ ID NO: 62)

Light chain: DIQLTQSPSS LSASVGDRVT ITCKASQDVG TSVAWYQQKP GKAPKLLIYW TSTRHTGVPS RFSGSGSGTD FTFTISSLQP EDIATYYCQQ YSLYRSFGQG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC (SEQ ID NO: 63)

25.2 The drug-linker compound

As a drug-linker molecule to be conjugated to the above-mentioned antibody, a molecule of the following structure was used:



This drug-linker molecule was purchased from SyntaBio LLC, 10239 Flanders Ct, San Diego, CA 92121. Lot No. S041070422.

25.3 Conjugation

The monoclonal antibody (mAb) was thawed at 2 - 8°C up to 3 days prior to conjugation and stored at 2 - 8°C in PBS pH 6.8 until further use. On the day of conjugation, the pH of the mAb solution was adjusted by addition of 0.5 M Tris, 0.025 M EDTA, pH 8.5 to a final concentration of 5% (v/v). After pH adjustment, the mAb was reduced using 10 molar equivalents of TCEP and an incubation at 20°C for 120 min. Subsequently, the mAb solution was diluted 1:1 with 20 mM Histidine, 80 mM NaCl, pH 5.5, the DMSO concentration was adjusted to 10% (v/v) and 16 molar equivalents of the above-mentioned drug-linker were added to start the reaction. The reaction was incubated at 20°C for 60 min and was finally quenched by addition of 100 mM NAC (n-acetyl-cysteine). Afterwards, the conjugated mAb (i.e. the ADC) was processed via preparative size exclusion chromatography.

25.4 Preparative size exclusion chromatography, desalting and filtration

The reaction mixture was purified using preparative size-exclusion chromatography. A GE HiLoad 26/60 Superdex S200 column was connected to an Äkta Avant 25 system (GE Healthcare) and equilibrated with 20 mM Histidine, 80 mM NaCl, pH 5.5 according to the manufacturers' instructions. Subsequently, the reaction mixture was injected and run through the column with a flowrate of 5 ml/min using 20 mM Histidine, 80 mM NaCl, pH 5.5 as running buffer. ADC-containing fractions were determined via UV light absorption at 280 nm, pooled and concentrated. ADC material was concentrated using Vivaspin VS2022 devices (Sartorius UK Ltd.) according to manufacturer's instructions. The concentrated ADC material was transferred into formulation buffer (10mM Histidine 100 mM NaCl, 3% trehalose, 0.05% (w/v) PS20, pH 5.5) using HiPrep 26/10 desalting columns (GE Healthcare) at a flowrate of 10 ml/min on an Äkta Avant 25 system (GE Healthcare) according to the manufacturer's

instructions. The final ADC material was filtered using a 0.2 µm filter (0.2 µm PES filters, Merck Millipore), aliquoted and subsequently shock frozen in liquid nitrogen. Final concentration of the ADC material (drug substance) was 7.7 mg/ml. The material was kept at -80°C until further use. The ADC resulting from this work is referred to herein as "ADC8"; this ADC is an analog of labetuzumab govitecan.

25.5 Further characterization of ADC8 drug substance

The ADC8 drug substance obtained above was further analyzed by (a) size exclusion chromatography (SEC), showing a monomeric purity of 99.3%, (b) reversed-phase HPLC (RP HPLC), showing a DAR of 7.7, and (c) an RP HPLC-based free-drug method, showing residual free-drug levels below 0.02% (by molar ratio).

Example 26: ADC1-M, ADC2-M, ADC6-M and ADC7-M kill cancer cells with higher specificity than ADC SAR DM4 and ADC8

Using the same method as described in Example 21, human cancer cell lines were used to compare effects of ADC1-M, ADC2-M, ADC6-M and ADC7-M, as well as ADC SAR DM4 and ADC8, on cancer cells with CEACAM5 expression relative to effects on cancer cells lacking CEACAM5 expression. A fold reduction in IC50, defined as a SPECIFICITY FACTOR, was calculated by dividing the IC50 against CEACAM5-negative MDA-MB-231 cells by the IC50 against each CEACAM5-positive cell line (see Table 6). The larger the value of the SPECIFICITY FACTOR is, the more specific is the tested ADC. ADC1-M, ADC2-M, ADC6-M and ADC7-M showed much lower IC50s in the CEACAM5-positive SK-CO-1, SNU-16, and MKN-45 cells than in the CEACAM5-negative MDA-MB-231 cells, which resulted in SPECIFICITY FACTORS in the range of 116 to 874. SPECIFICITY FACTORS for ADC2-M and ADC7-M are likely underestimated due to the lack of effect on MDA-MB-231 cells in the tested concentration range (as shown for ADC2-M in Table 3 and Figure 41e). For this reason, for ADC2-M and ADC7-M only, the IC50 was set to the highest tested concentration of 100 nM to calculate SPECIFICITY FACTORS. In contrast, ADC SAR DM4 and ADC8 showed lower SPECIFICITY FACTORS in the range of 26 to 73 and 1.8 to 4.8, respectively. In conclusion, a more selective killing of CEACAM5-positive cancer cells by ADC1-M, ADC2-M, ADC6-M and ADC7-M compared with ADC SAR DM4 and ADC8 in vitro was shown.

	SK-CO-1 n≥2	SNU-16 n≥2	MKN-45 n≥2	MDA-MB-231 n≥2
ADC1-M	874.4	241.9	201.2	1
ADC2-M	>631.9	>207.2	>166.6	1
ADC6-M	710.2	194.9	157.7	1
ADC7-M	>595.8	>178.9	>116.2	1
ADC SAR DM4	46.5	73.2	26.0	1
ADC8	4.8	1.8	2.5	1
CEACAM5	Positive	Positive	Positive	Negative

Table 6. Relative potency increases of ADCs in CEACAM5-positive cell lines compared to CEACAM5-negative MDA-MB-231 cell line (SPECIFICITY FACTOR). IC50 in MDA-MB-231 was divided by IC50 in each of the three CEACAM5-positive cell lines to calculate the respective SPECIFICITY FACTOR for each ADC (the theoretical factor of 1 for MDA-MB-231 being reported only for illustration). Calculations were based on IC50 geometric means of 2 to 4 individual experiments.

Example 27: ADC1-M, ADC2-M, ADC6-M, ADC7-M mediate a more potent bystander effect than ADC SAR DM4 against antigen-negative cells in co-culture with antigen-positive cells

Using the same method as in Example 10, the potential of ADC1-M, ADC2-M, ADC6-M, ADC7-M and ADC SAR DM4 to mediate a bystander effect against antigen-negative cells in close proximity to antigen-positive cells was evaluated in bystander assays. ADC1-M, ADC2-M, ADC6-M and ADC7-M showed a potent bystander effect against CEACAM5-negative MDA-MB-231 cells in the presence of CEACAM5-positive SK-CO-1 (Fig. 44). The co-culture experiments were performed at an ADC concentration of 1 nM which, for ADC1, causes maximal inhibition of CEACAM5-positive SK-CO-1 cell viability (Fig. 26A) but no effect on CEACAM5-negative MDA-MB-231 cells alone (Fig. 26B). In line with these findings, no non-specific effect of ADC1-M, ADC2-M, ADC6-M, ADC7-M and ADC SAR DM4 was observed in the bystander assay setup for MDA-MB-231 only controls.

In conclusion, ADC1-M, ADC2-M, ADC6-M, ADC7-M mediate a potent bystander effect against antigen-negative cancer cells in co-culture with antigen-positive cells. These findings indicate the potential to effectively target tumors with heterogeneous target expression.

Compared to ADC SAR DM4, ADC1-M, ADC2-M, ADC6-M and ADC7-M mediated a much more potent bystander effect on antigen-negative cells in co-culture with antigen-positive cells (Fig. 44).

For all ADCs tested, the extent of bystander effect increased with increasing the number of antigen-positive cells added to a constant number of antigen-negative cells. Without wishing to be bound by theory, this may be a result of more ADC being processed by the higher number of antigen-positive cells to release more free payload, which is responsible for the bystander effect on antigen-negative cells.

No non-specific effects of tested ADCs were observed on MDA-MB-231 cells alone for all tested ADCs at 1E-9M testing concentration in bystander assays.

Example 28: Efficacy of ADC1-M and ADC3-M compared to ADC8

Efficacy of ADC1-M and ADC3-M in comparison to ADC8 was evaluated in the human pancreatic adenocarcinoma, cell line-derived xenograft model HPAF-II (ATCC, CRL-1997). Six to eight weeks old immunodeficient female mice (Hsd:Athymic Nude-Foxn1nu, Envigo) were injected subcutaneously in the right flank with $5x10^6$ HPAF-II cells. When tumors reached a mean volume of 150 mm^3, 10 mice/group were treated once intravenously with ADC1-M (1 mg/kg or 6mg/kg) or with ADC3-M (1 mg/kg or 6mg/kg) or with ADC8 (1 mg/kg or 6mg/kg). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using L×W^2/2.

The single treatment with ADC1-M or ADC3-M at a dose of 1 or 6 mg/kg led to a significant anti-tumor effect in comparison to the vehicle control. The effect is dose-dependent, as the single treatment with 1mg/kg only led to a minor and temporary anti-tumor effect, while 6 mg/kg showed a much stronger anti-tumor effect. In contrast, the single treatment with ADC8 showed no significant anti-tumor effect at either dose (Fig. 45). All treatments had no significant effect on body weight (data not shown).

Example 29: Efficacy of ADC1-M and ADC3-M compared to ADC SAR DM4 in a CRC PDX mouse model

Anti-tumor efficacy of ADC1-M and ADC3-M in comparison to ADC SAR DM4 was in the human CRC xenograft model COPF230. The study was performed at Lide Biotech (Shanghai). Six to eight weeks old immunodeficient female mice (Crl: NU-Foxn1nu) were transplanted subcutaneously in the right flank with COPF230 tumor fragments. When tumors reached a mean volume of 165 mm^3 6 mice/group were treated once intravenously with vehicle (saline

solution) or with ADC1-M, ADC3-M or ADC SAR DM4 (each 6 mg/kg). Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using L×W^2/2.

The single treatment with 6 mg/kg ADC1-M or ADC3-M led to a comparable and significant anti-tumor effect in comparison to vehicle control group. In contrast, single treatment with 6 mg/kg ADC SAR DM4 demonstrated only minor anti-tumor effect (Fig 46). All treatments had no significant impact on body weight (data not shown).

Example 30: Efficacy of ADC1-M andADC3-M compared to ADC SAR DM4 in a GC PDX mouse model

Anti-tumor efficacy of ADC1-M and ADC3-M in comparison to ADC SAR DM4 has been evaluated in the human GC xenograft model GAPF313. The study was performed at Lide Biotech (Shanghai). Six to eight weeks old immunodeficient female mice (Crl: NU-Foxn1nu) were transplanted subcutaneously in the right flank with GAPF313 tumor fragments. When tumors reached a mean volume of 176 mm^3 6 mice/group were treated intravenously every other week (3 cycles) with vehicle (saline solution) or with 4 mg/kg ADC1-M or ADC3-M or 4.7 mg/kg ADC SAR DM4. Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using L×W^2/2.

The multiple treatment with ADC1-M or ADC3-M led to a comparable and significant anti-tumor effect in comparison to the vehicle control. ADC SAR DM4 is significantly less effective in comparison to ADC1-M and ADC3-M (Fig 47). All treatments had no significant impact on body weight (data not shown).

CLAIMS

1. An isolated antibody which binds to human CEACAM5 protein;

and wherein the isolated antibody comprises

(i) at least one light chain constant region (CL) that comprises a sequence selected from the group consisting of GGTLQSPP, LLQGA, GGLLQGPP, TLQSG, TLQSPP and TLQSA and preferably comprising this sequence at the C-terminus of said light chain constant region;

and/or

(ii) at least one heavy chain constant region (CH) comprising one or more of the following amino acid substitutions:

(a) L234A and L235A (LALA mutation);

(b) L234A and L235A and P329G (LALA-PG mutation);

(c) L235A and G237A (LAGA mutation);

(d) M252Y and S254T and T256E (YTE mutation);

(e) K222R;

and wherein Eu numbering is used for said amino acid substitutions;

and wherein said isolated antibody comprises a CDR1-H consisting of the amino acid sequence of SEQ ID NO: 3, a CDR2-H consisting of the amino acid sequence of SEQ ID NO: 4, a CDR3-H consisting of the amino acid sequence of SEQ ID NO: 5, a CDR1-L consisting of the amino acid sequence of SEQ ID NO: 6, a CDR2-L consisting of the amino acid sequence of SEQ ID NO: 7, and a CDR3-L consisting of the amino acid sequence of SEQ ID NO: 8.

2. The isolated antibody of claim 1, wherein said isolated antibody comprises framework regions FR1, FR2, FR3, FR4, FR5, FR6, FR7 and FR8 having the structure FR1 - CDR1-H – FR2 – CDR2-H – FR3 – CDR3-H – FR4 and FR5 - CDR1-L – FR6 – CDR2-L – FR7 – CDR3-L – FR8; wherein FR1 consists of SEQ ID NO: 54, FR2 consists of SEQ ID NO: 55, FR3 consists of SEQ ID NO: 56, FR4 consists of SEQ ID NO: 57, FR5 consists of SEQ ID NO: 58, FR6 consists of SEQ ID NO: 59, FR7 consists of SEQ ID NO: 60 and FR8 consists of SEQ ID NO: 61.

3. An isolated antibody which binds to human CEACAM5 protein;

and wherein the isolated antibody comprises

(i) at least one light chain constant region (CL) that comprises a sequence selected from the group consisting of GGTLQSPP, TLQSPP, TLQSA and TLQSG, and preferably comprising this sequence at the C-terminus of said light chain constant region;

and/or

(ii) at least one heavy chain constant region (CH) comprising one or more of the following amino acid substitutions:

(a) L234A and L235A (LALA mutation);

(b) L234A and L235A and P329G (LALA-PG mutation);

(c) L235A and G237A (LAGA mutation);

(d) M252Y and S254T and T256E (YTE mutation);

(e) K222R;

and wherein Eu numbering is used for said amino acid substitutions;

and wherein preferably said isolated antibody comprises a CDR1-H consisting of the amino acid sequence of SEQ ID NO: 3, a CDR2-H consisting of the amino acid sequence of SEQ ID NO: 4, a CDR3-H consisting of the amino acid sequence of SEQ ID NO: 5, a CDR1-L consisting of the amino acid sequence of SEQ ID NO: 6, a CDR2-L consisting of the amino acid sequence of SEQ ID NO: 7, and a CDR3-L consisting of the amino acid sequence of SEQ ID NO: 8;

and wherein said isolated antibody comprises framework regions FR1, FR2, FR3, FR4, FR5, FR6, FR7 and FR8 having the structure FR1 - CDR1-H – FR2 – CDR2-H – FR3 – CDR3-H – FR4 and FR5 - CDR1-L – FR6 – CDR2-L – FR7 – CDR3-L – FR8; wherein FR1 consists of SEQ ID NO: 54, FR2 consists of SEQ ID NO: 55, FR3 consists of SEQ ID NO: 56, FR4 consists of SEQ ID NO: 57, FR5 consists of SEQ ID NO: 58, FR6 consists of SEQ ID NO: 59, FR7 consists of SEQ ID NO: 60 and FR8 consists of SEQ ID NO: 61.

4. The isolated antibody of any one of claims 1-3, wherein both heavy chain constant regions (CH) comprise one or more of said amino acid substitutions (a) through (e) and/or wherein both light chain constant regions comprise said sequence GGTLQSPP.

5. The isolated antibody of any one of claims 1-3, wherein at least one heavy chain constant region (CH) comprises the amino acid sequence of SEQ ID NO: 31 or SEQ ID NO: 32 and/or wherein at least one of said light chain constant regions (CL) comprises the amino acid sequence of SEQ ID NO: 33.

6. The isolated antibody of any one of claims 1-3, wherein said heavy chain constant regions (CH) and light chain constant regions (CL) have any of the following sequence combinations:

(1) both CH comprise a sequence of SEQ ID NO: 31 and both CL comprise a sequence of SEQ ID NO: 12; or

(2) both CH comprise a sequence of SEQ ID NO: 31 and both CL comprise a sequence of SEQ ID NO: 33; or

(3) both CH comprise a sequence of SEQ ID NO: 32 and both CL comprise a sequence of SEQ ID NO: 33; or

(4) both CH comprise a sequence of SEQ ID NO: 50 and both CL comprise a sequence of SEQ ID NO: 12; or

(5) both CH comprise a sequence of SEQ ID NO: 50 and both CL comprise a sequence of SEQ ID NO: 33; or

(6) one CH comprise a sequence of SEQ ID NO: 31 and one CL comprise a sequence of SEQ ID NO: 12; or

(7) one CH comprise a sequence of SEQ ID NO: 31 and one CL comprise a sequence of SEQ ID NO: 33; or

(8) one CH comprise a sequence of SEQ ID NO: 32 and one CL comprise a sequence of SEQ ID NO: 33; or

(9) one CH comprise a sequence of SEQ ID NO: 50 and one CL comprise a sequence of SEQ ID NO: 12; or

10) one CH comprise a sequence of SEQ ID NO: 50 and one CL comprise a sequence of SEQ ID NO: 33.

7. Antibody according to any one of claims 1 to 6, which comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence of SEQ ID NO: 9 and a light chain variable region (VL) comprising an amino acid sequence that is at least 85 % identical to the amino acid sequence of SEQ ID NO: 10.

8. Antibody according to claim 7, which comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO: 10.

9. Antibody according to any one of claims 1 to 8, which comprises

(i) a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 34 and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 14; or

(ii) a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 34 and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 36; or

(iii) a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 35 and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 36; or

(iv) a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 51 and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 14; or

(v) a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 51 and a light chain (LC) comprising the amino acid sequence of SEQ ID NO: 36.

10. An isolated antibody which competes for binding to A2-B2 domain of human CEACAM5 protein with an antibody comprising a heavy chain variable region (VH) of the amino acid sequence of SEQ ID NO: 9 and a light chain variable region (VL) of the amino acid sequence of SEQ ID NO: 10 and wherein said heavy chain constant regions (CH) and light chain constant regions (CL) are as defined in any one of claims 1-9.

11. Antibody according to claim 10, wherein the antibody also competes for binding to A2-B2 domain of *Macaca fascicularis* CEACAM5 protein with an antibody comprising a heavy chain variable region (VH) of the amino acid sequence of SEQ ID NO: 9 and a light chain variable region (VL) of the amino acid sequence of SEQ ID NO: 10 and wherein said heavy chain constant regions (CH) and light chain constant regions (CL) are as defined in any one of claims 1-9.

12. Antibody according to any one of claims 1 to 11, wherein the antibody does not significantly cross-react with human CEACAM1, human CEACAM6, human CEACAM7, human CEACAM8 and *Macaca fascicularis* CEACAM6.

13. Antibody according to any one of claims 1 to 12, wherein the antibody is an antibody fragment.

14. Antibody according to claim 13, wherein the antibody is an antibody fragment selected from the group consisting of Fv, Fab, F(ab')2, Fab', dsFv, (dsFv)2, scFv, sc(Fv)2, and diabodies.

15. Antibody according to any one of claims 1 to 14, which is a bispecific or a multispecific antibody.

16. An isolated antibody which binds to human CEACAM5 protein and which

consists of two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 34 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 14; or

which consists of two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 34 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 36; or

which consists of two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 35 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 36; or

which consists of two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 51 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 14; or

which consists of two identical heavy chains (HC) comprising the amino acid sequence of SEQ ID NO: 51 and two identical light chains (LC) comprising the amino acid sequence of SEQ ID NO: 36.

17. An isolated nucleic acid comprising a nucleic acid sequence encoding an antibody according to any one of claims 1 to 16.

18. A host cell which has been transformed with a nucleic acid according to claim 17.

19. An immunoconjugate comprising an antibody according to any one of claims 1 to 16 covalently linked via a linker to at least one growth inhibitory agent.

20. Immunoconjugate according to claim 19, wherein the growth inhibitory agent is a cytotoxic drug or a radioactive moiety.

21. Immunoconjugate according to claim 19 or 20, wherein the growth inhibitory agent is selected from a group consisting of chemotherapeutic agents, enzymes, antibiotics, toxins such as small molecule toxins or enzymatically active toxins, toxoids, vincas, taxanes, maytansinoids or maytansinoid analogs, tomaymycin or pyrrolobenzodiazepine derivatives, cryptophycin derivatives, leptomycin derivatives, auristatin or dolastatin analogs, prodrugs, topoisomerase I inhibitors, topoisomerase II inhibitors, DNA alkylating agents, anti-tubulin agents, CC-1065 and CC-1065 analogs.

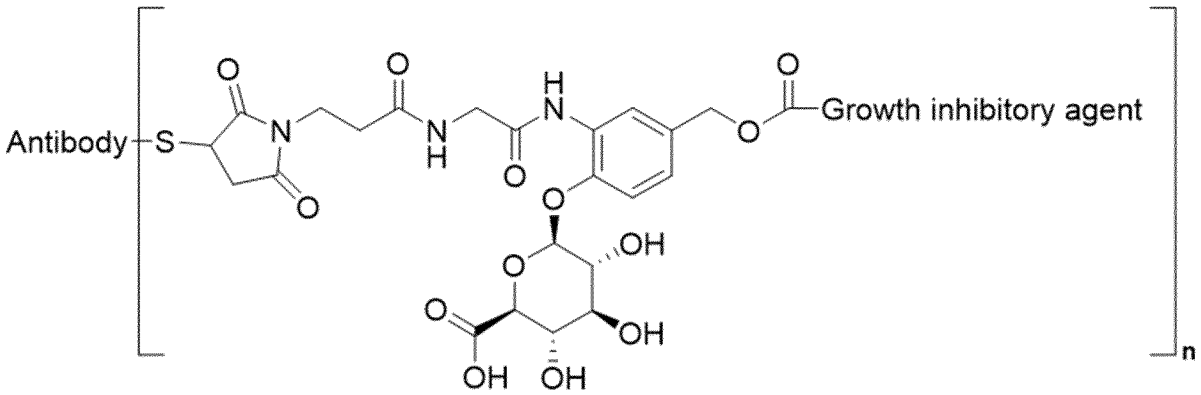
22. Immunoconjugate according to any one of claims 19 to 21, wherein the growth inhibitory agent is exatecan.

23. Immunoconjugate according to any one of claims 19 to 22, wherein linker is a cleavable linker.

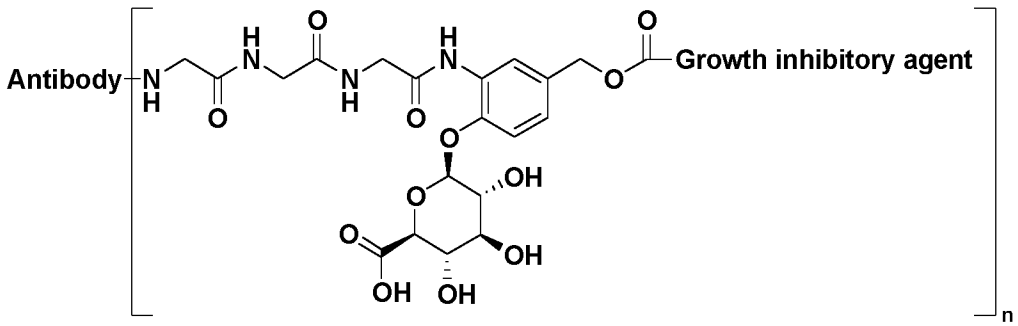
24. Immunoconjugate according to any one of claims 19 to 23, wherein the linker is a linker cleavable in an endosome of a mammalian cell.

25. Immunoconjugate according to any one of claims 19 to 24, wherein the linker is a linker cleavable by the human enzyme glucuronidase.

26. Immunoconjugate according to any one of claims 19 to 25, wherein the immunoconjugate has the following **formula (II) or formula (IIA)**:



(II),

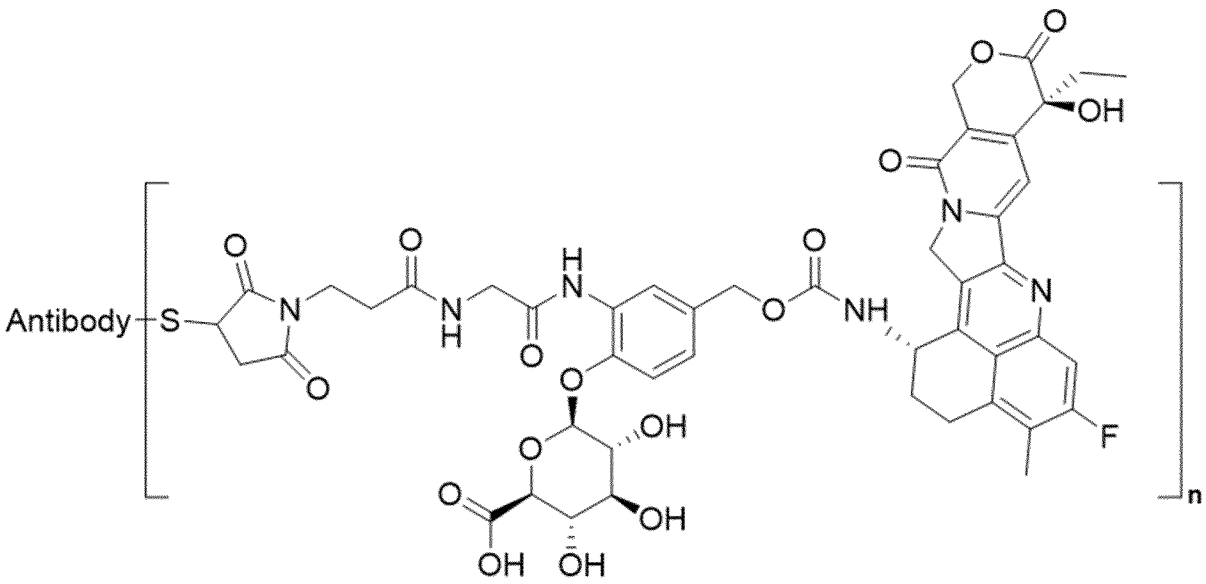


(IIA),

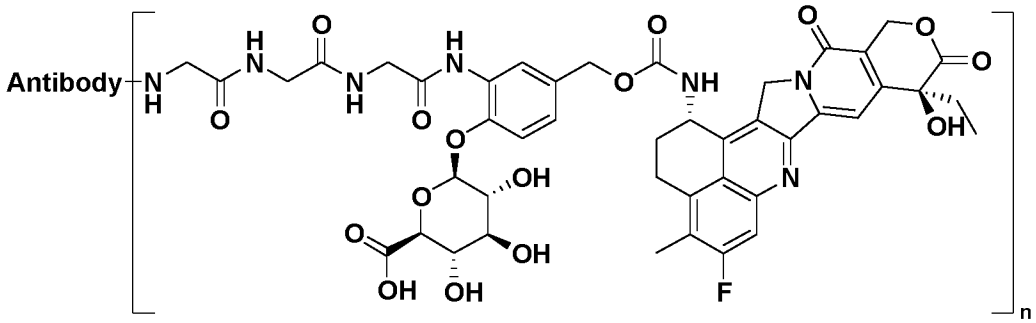
wherein S is a sulfur atom of the antibody, and wherein n is a number of [(linker)–(growth inhibitory agent)] moieties covalently linked to the antibody.

27. Immunoconjugate according to claim 26, wherein n is between 1 and 10 and preferably 4.

28. Immunoconjugate according to any one of claims 19 to 26, wherein the immunoconjugate has the following **formula (IV) or formula (IVA)**:



(IV),



(IVA), wherein S is a sulfur atom of the antibody, and wherein n is a number of [(linker)–(exatecan)] moieties covalently linked to the antibody.

29. Immunoconjugate according to claim 28, wherein the immunoconjugate has formula (IVA) and n is between 1 and 10 and preferably 4.

30. Immunoconjugate according to any one of claims 19 to 29, wherein the S is a sulfur atom of a cysteine of the antibody.

31. Immunoconjugate according to claim 30, wherein the cysteine of the antibody is one of the cysteines capable of forming an interchain disulfide bond.

32. Immunoconjugate according to any one of claims 26 to 31, wherein n is between 7 and 8 for formulas (II)and (IV); and wherein n is between 3 and 5 for formulas (IIA) and (IVA).

33. Immunoconjugate according to any one of claims 26 to 32, wherein n is between 7.5 and 8.0 for formulas (II)and (IV); and wherein n is between 3.5 and 4.5 for formulas (IIA) and (IVA) and preferably n is 4 for formulas (IIA) and (IVA).

34. A pharmaceutical composition comprising an antibody according to any one of claims 1 to 16 or an immunoconjugate according to any one of claims 19 to 33, further comprising a pharmaceutically acceptable carrier, diluent and/or excipient.

35. An antibody according to any one of claims 1 to 16 or an immunoconjugate according to any one of claims 19 to 33 or a pharmaceutical composition according to claim 34 for use as a medicament.

36. An antibody according to any one of claims 1 to 16 or an immunoconjugate according to any one of claims 19 to 33 or a pharmaceutical composition according to claim 34 for use in the treatment of cancer.

37. Antibody, immunoconjugate or pharmaceutical composition for the use according to claim 36, wherein the cancer is a CEACAM5 expressing cancer.

38. Antibody, immunoconjugate or pharmaceutical composition for the use according to claim 36 or 37, wherein the cancer is a colorectal cancer, gastric cancer, lung cancer, pancreatic cancer, esophageal cancer or prostate cancer.

39. Method of treating cancer, comprising administering to a subject an antibody according to any one of claims 1 to 16 or an immunoconjugate according to any one of claims 19 to 33 or a pharmaceutical composition according to claim 34.

40. Method according to claim 39, wherein the cancer is a CEACAM5 expressing cancer.

41. Method according to claim 39 or 40, wherein the cancer is a colorectal cancer, gastric cancer, lung cancer, pancreatic cancer, esophageal cancer or prostate cancer.

42. Method for detecting CEACAM5 expression *ex vivo* in a biological sample from a subject using an antibody according to any one of claims 1 to 16.

43. Method according to claim 42, wherein the antibody is labelled with a detectable molecule.

44. Use of an antibody according to any one of claims 1 to 16 for detecting CEACAM5 expression *ex vivo* in a biological sample from a subject.

45. Use according to claim 44, wherein the antibody is labelled with a detectable molecule.

DRAWINGS:

Fig. 1:

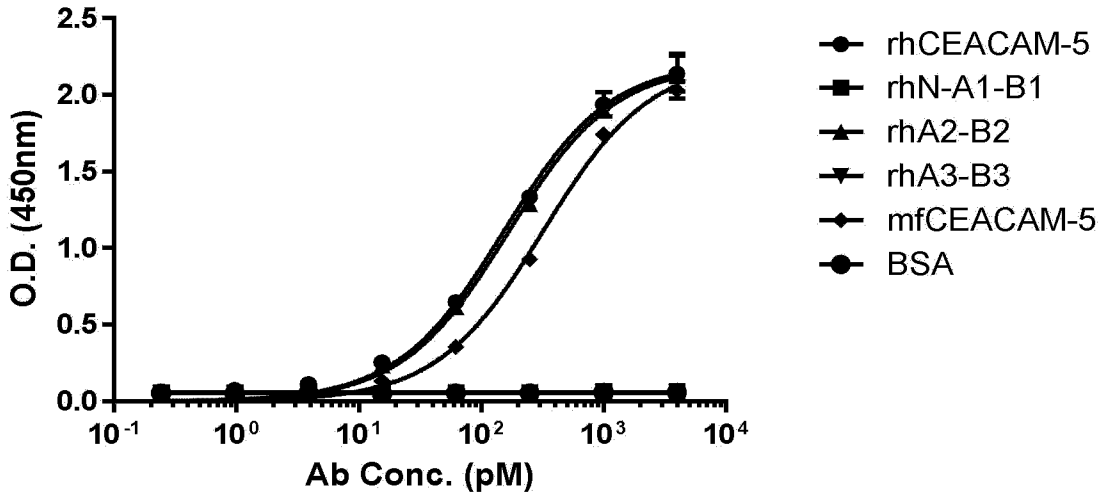


Fig. 2:

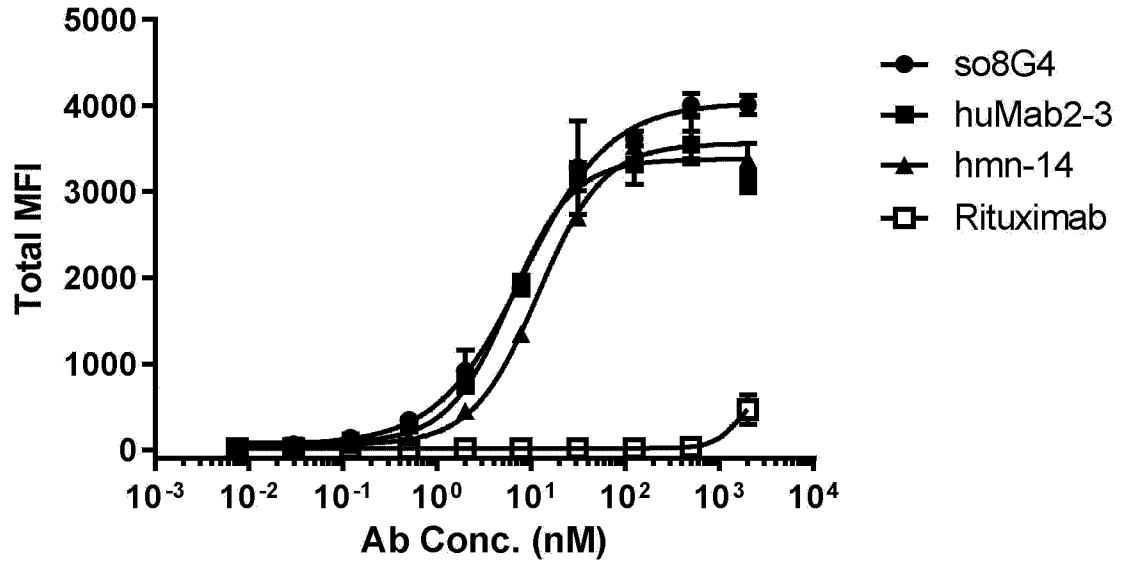


Fig. 3:

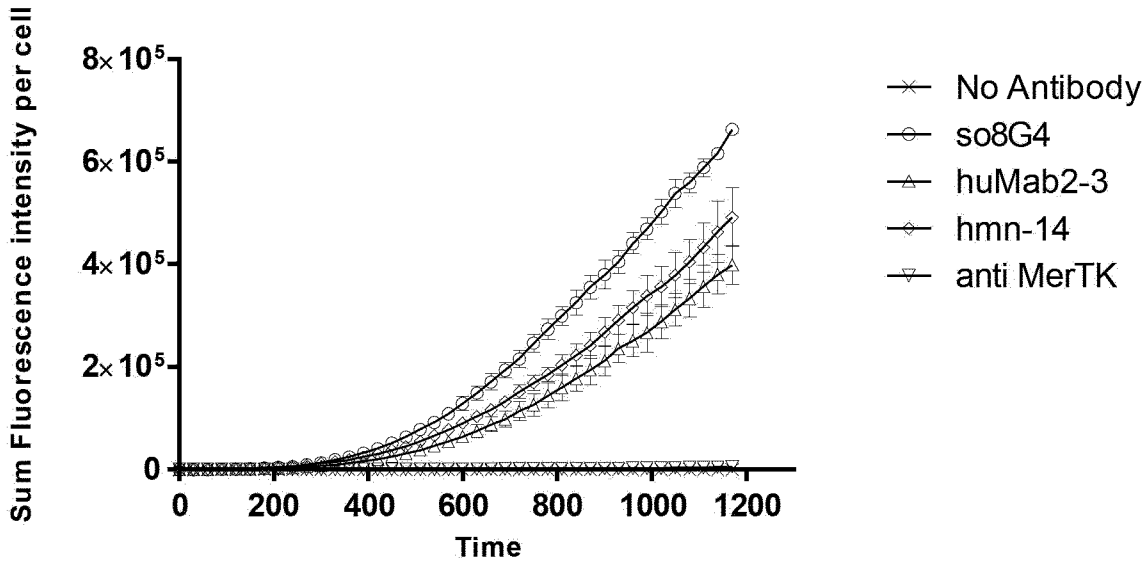

Sum Fluorescence intensity per cell
8×10^5
6×10^5
4×10^5
2×10^5
0
0
200
400
600
800
1000
1200
Time
No Antibody
so8G4
huMab2-3
hmn-14
anti MerTK

Fig. 4:

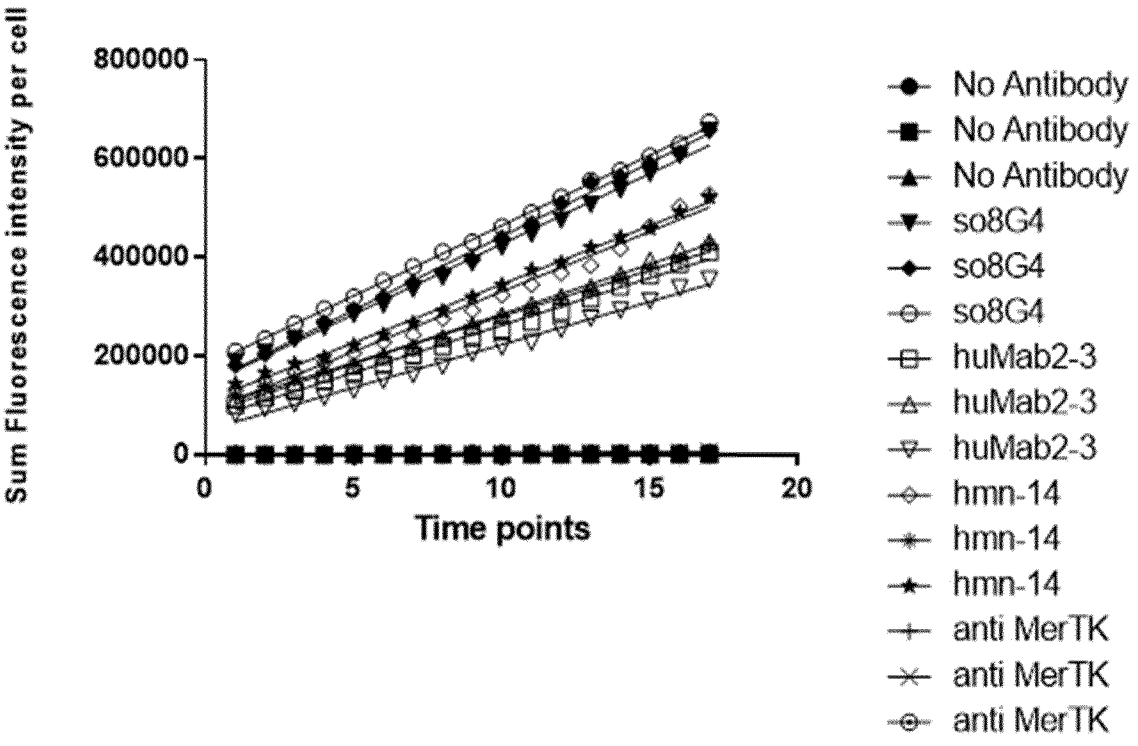

Sum Fluorescence intensity per cell
800000
600000
400000
200000
0
0
5
10
15
20
Time points
No Antibody
No Antibody
No Antibody
so8G4
so8G4
so8G4
huMab2-3
huMab2-3
huMab2-3
hmn-14
hmn-14
hmn-14
anti MerTK
anti MerTK
anti MerTK

Fig. 5:

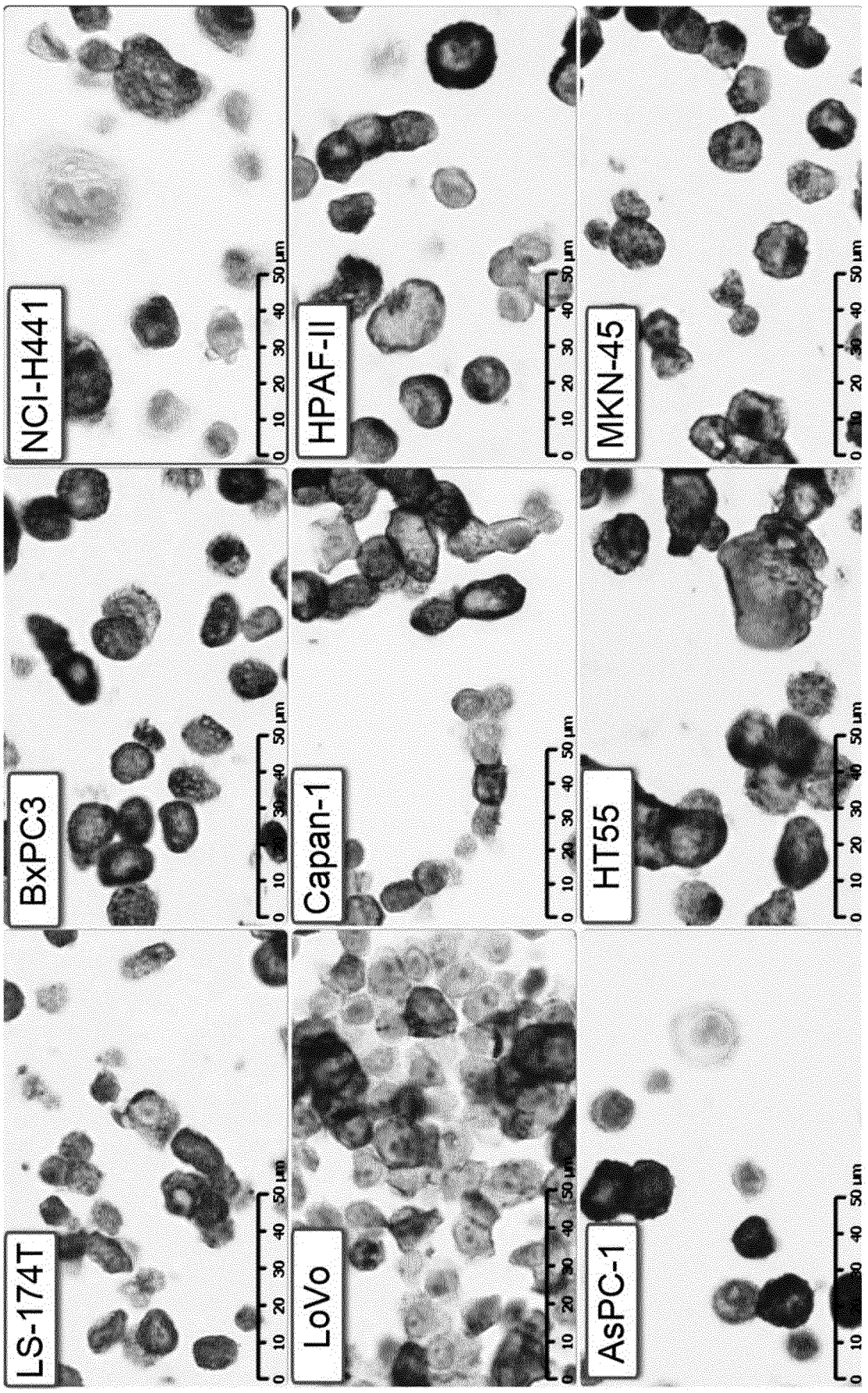

NCI-H441
BxPC3
HPAF-II
Capan-1
MKN-45
HT55
LS-174T
LoVo
AsPC-1
0 10 20 30 40 50 µm

Fig. 6:

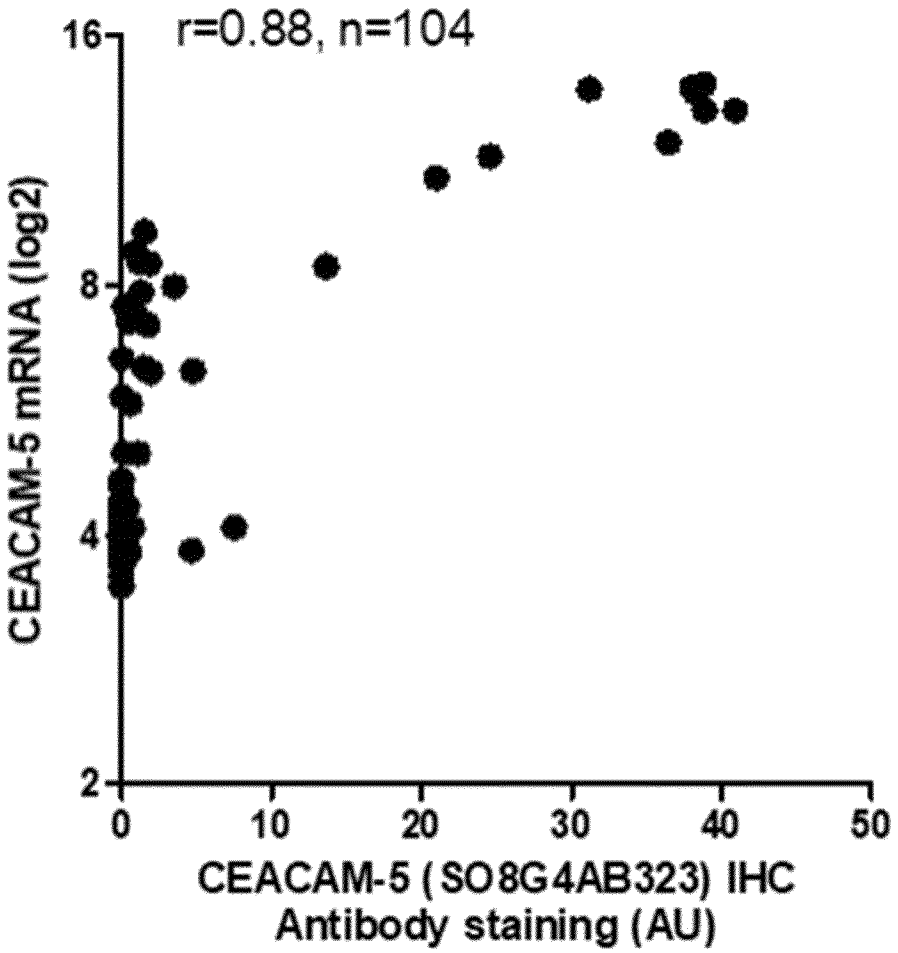

r=0.88, n=104
16
8
4
2
CEACAM-5 mRNA (log2)
0
10
20
30
40
50
CEACAM-5 (SO8G4AB323) IHC
Antibody staining (AU)

Fig. 7:

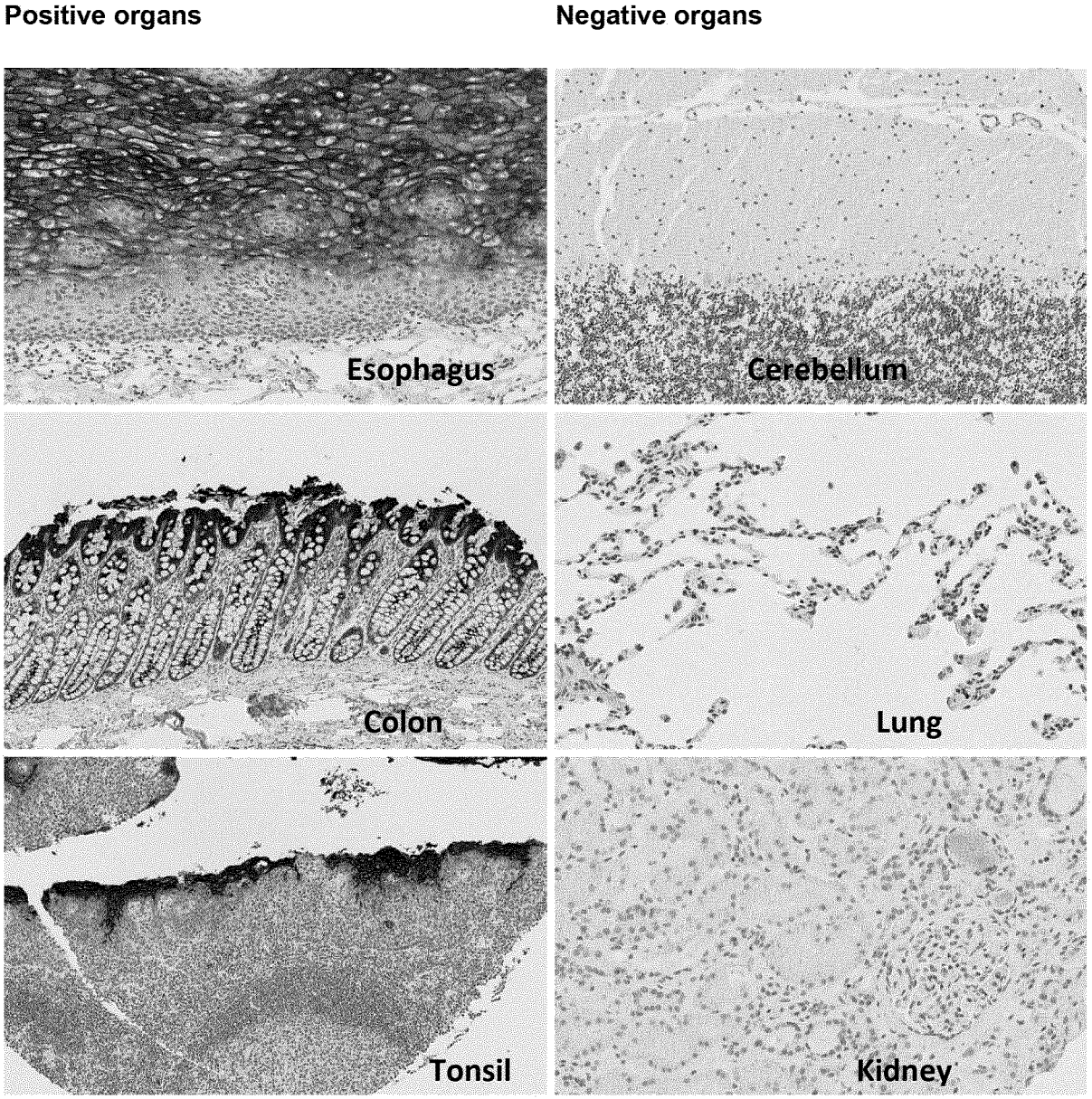

Positive organs
Negative organs
Esophagus
Cerebellum
Colon
Lung
Tonsil
Kidney

Fig. 8

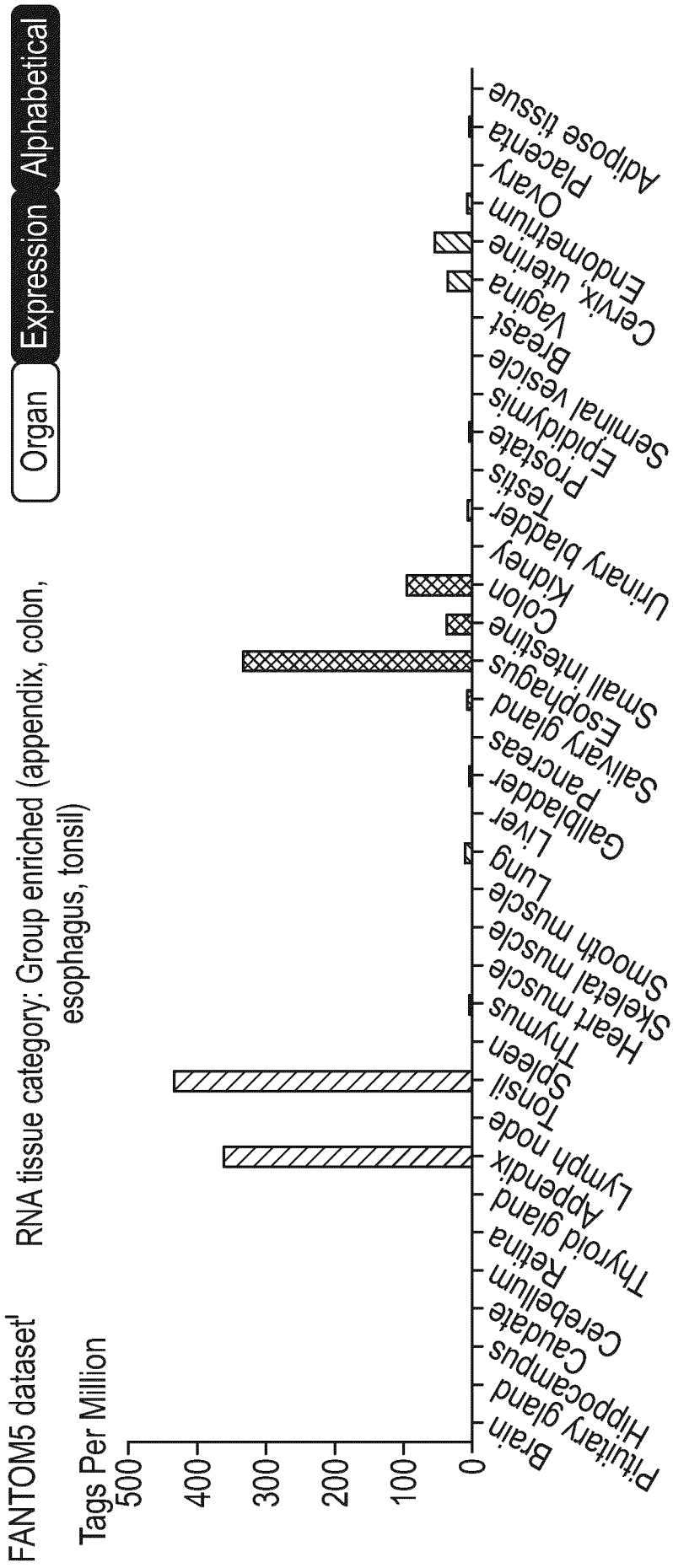

Organ
Expression
Alphabetical
RNA tissue category: Group enriched (appendix, colon, esophagus, tonsil)
FANTOM5 dataset
Tags Per Million
500
400
300
200
100
0
Brain
Pituitary gland
Hippocampus
Caudate
Cerebellum
Retina
Thyroid gland
Appendix
Lymph node
Tonsil
Spleen
Thymus
Heart muscle
Skeletal muscle
Smooth muscle
Lung
Liver
Gallbladder
Pancreas
Salivary gland
Esophagus
Small intestine
Colon
Kidney
Urinary bladder
Testis
Prostate
Epididymis
Seminal vesicle
Breast
Vagina
Cervix, uterine
Endometrium
Ovary
Placenta
Adipose tissue

Fig. 9:

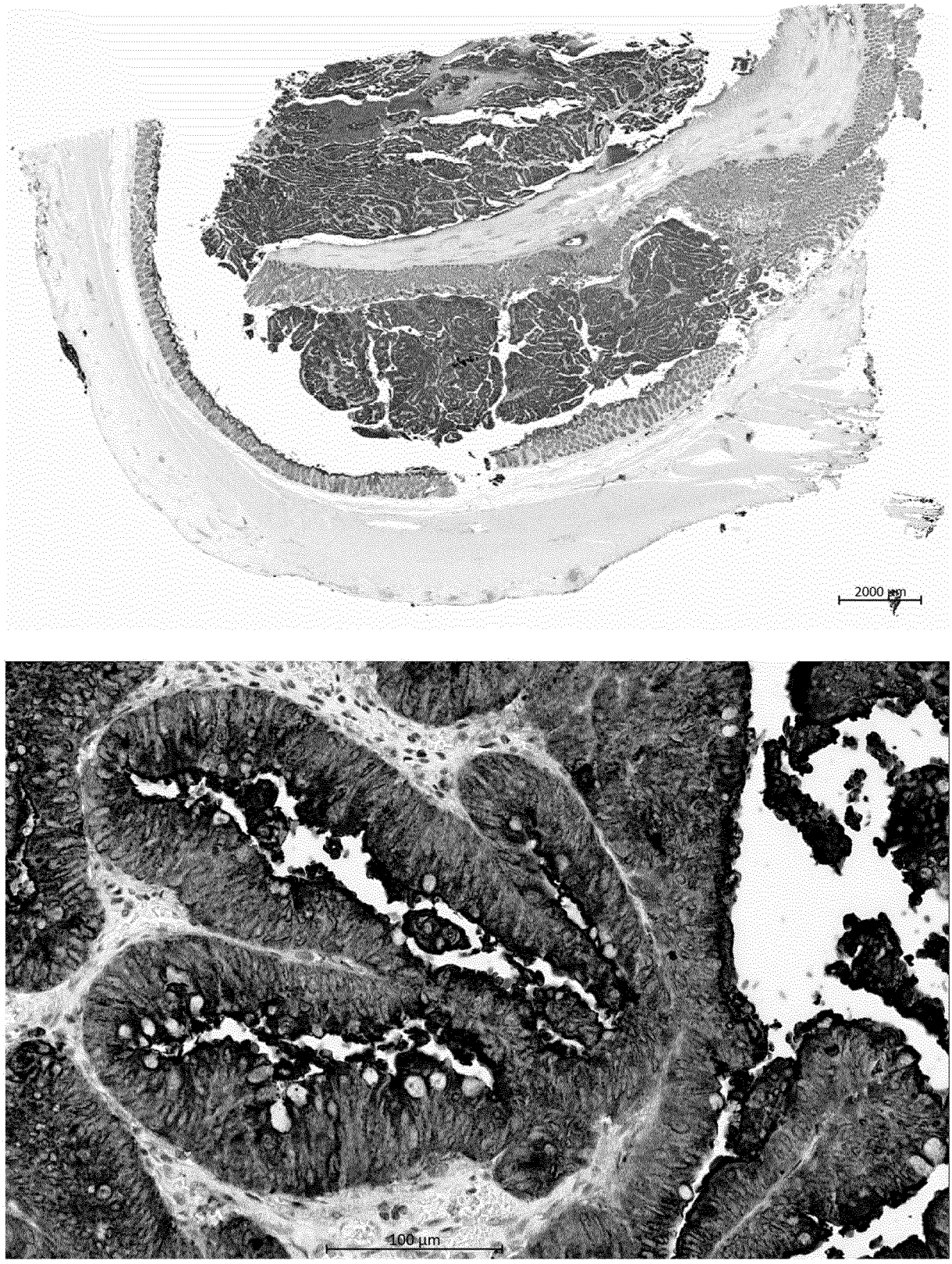


Fig. 10:

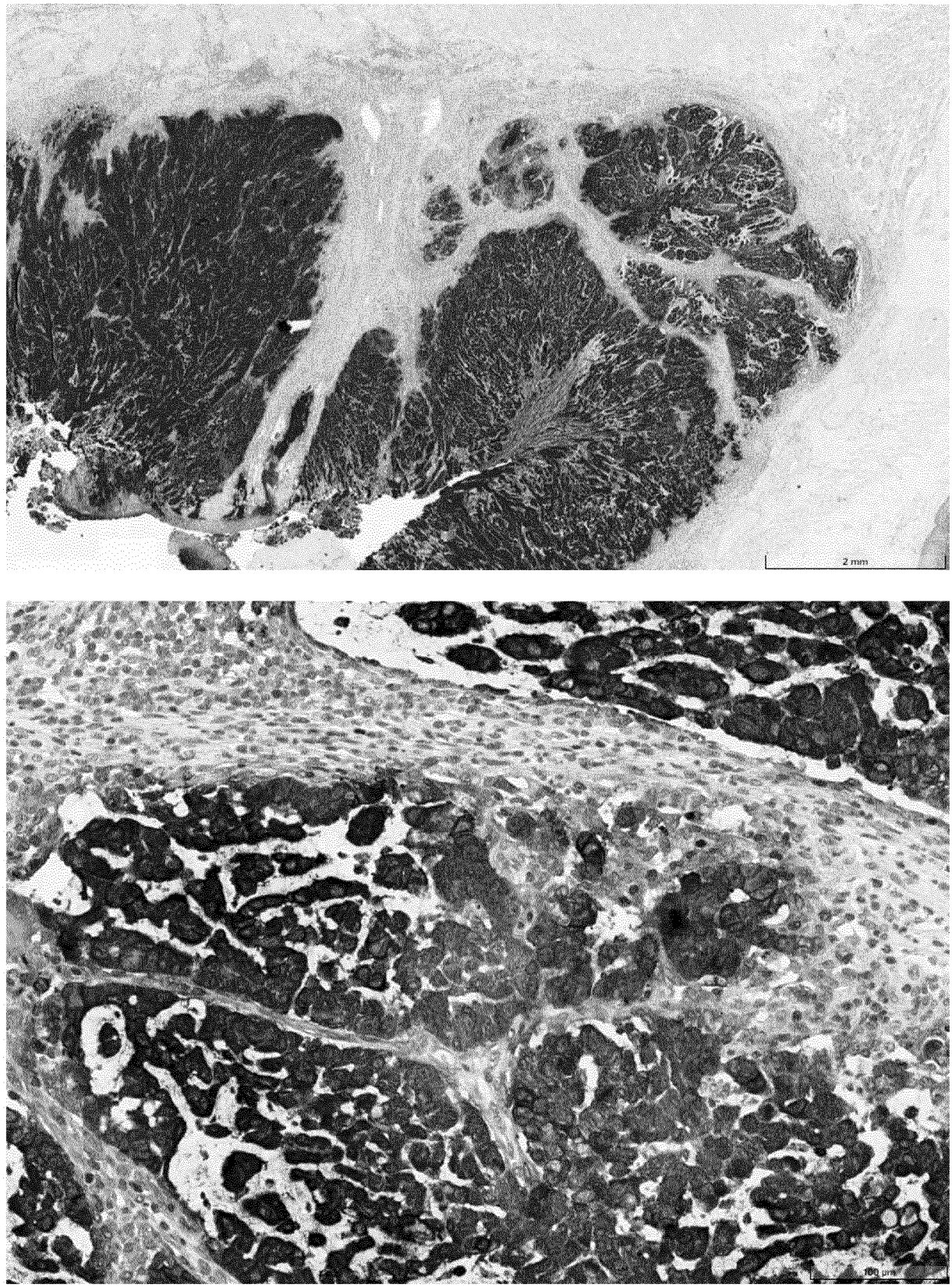


Fig. 11:

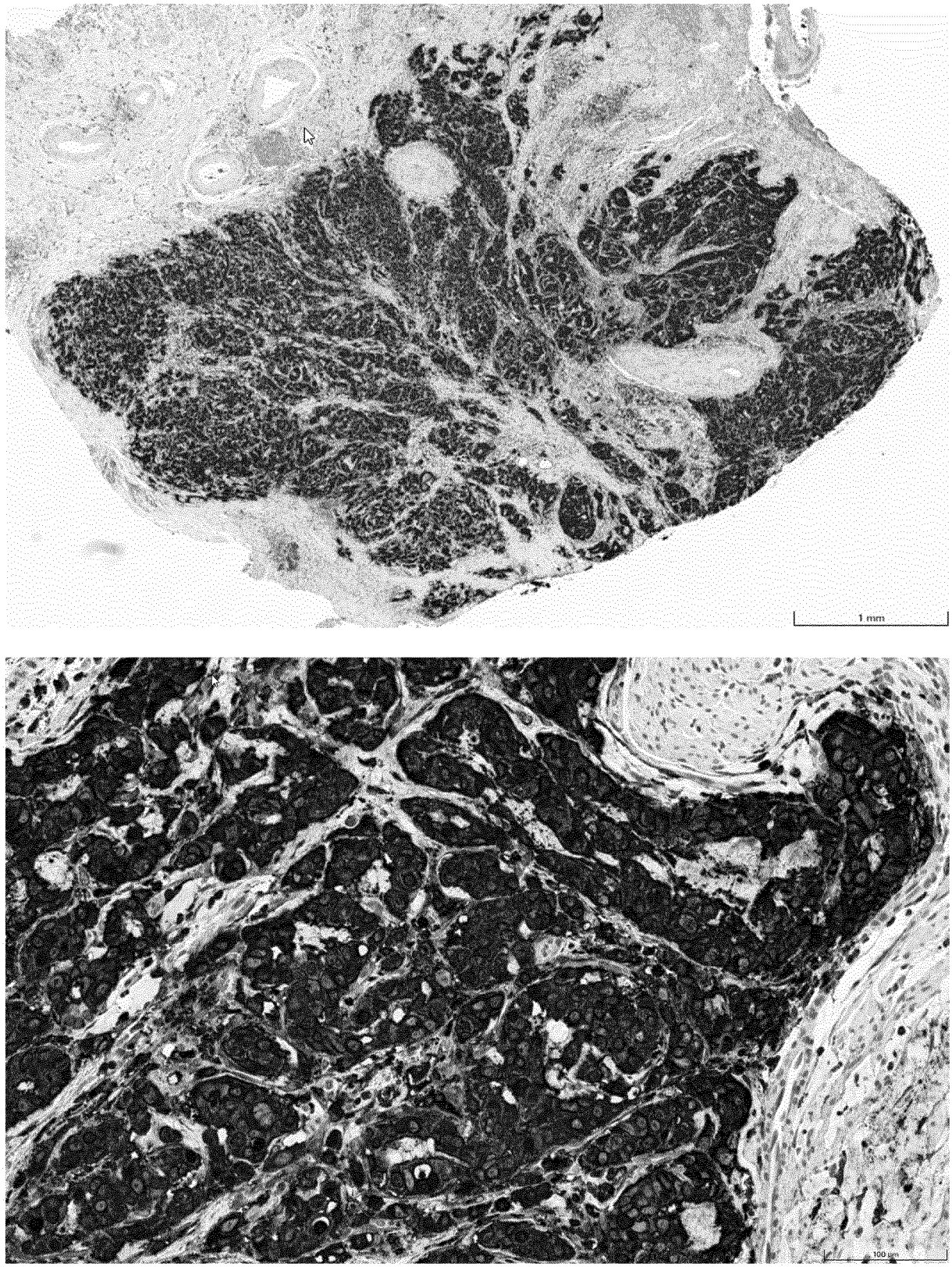
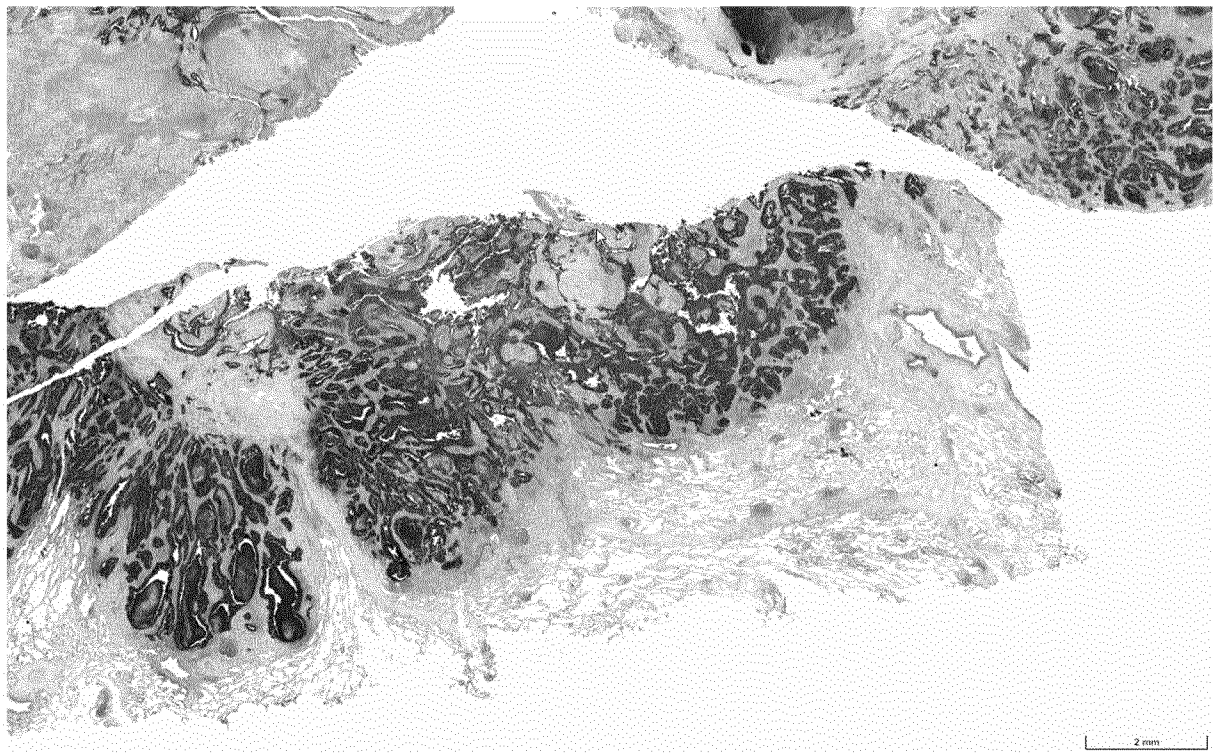


Fig. 12:



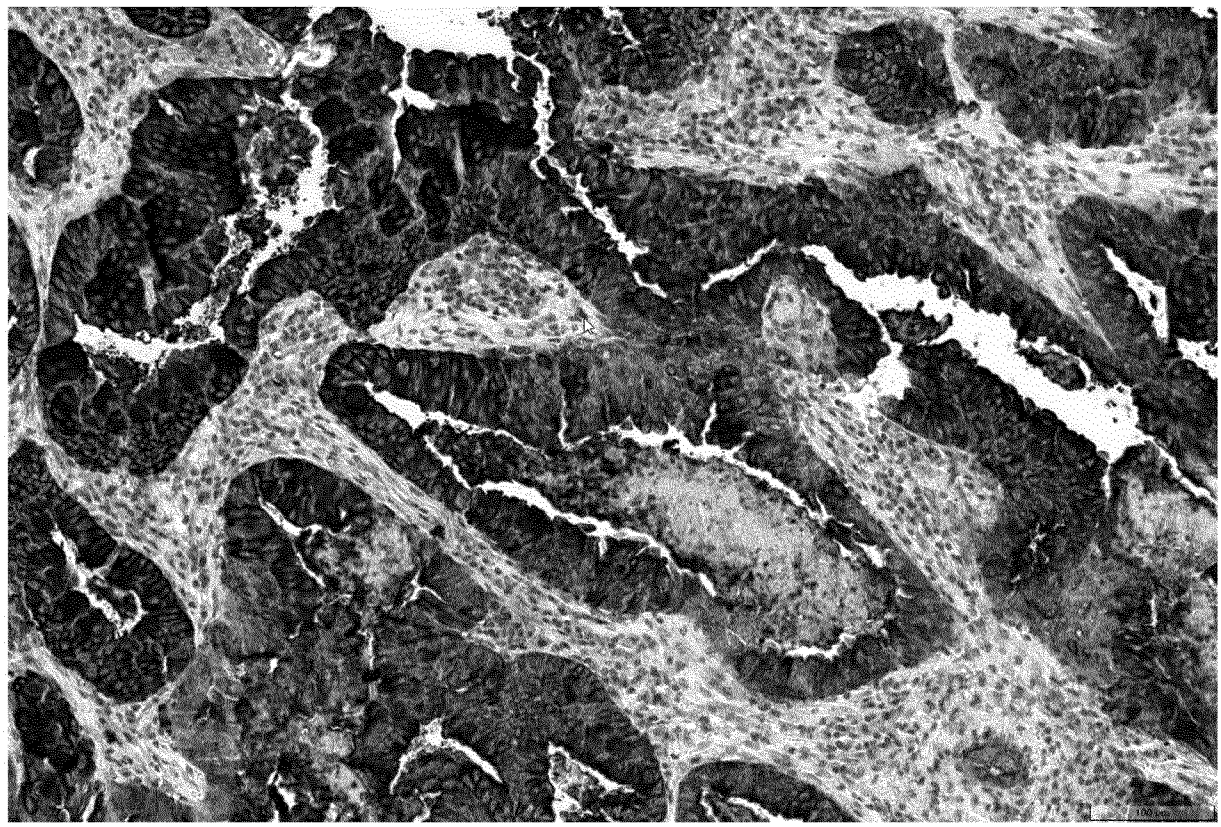


Fig. 13A:

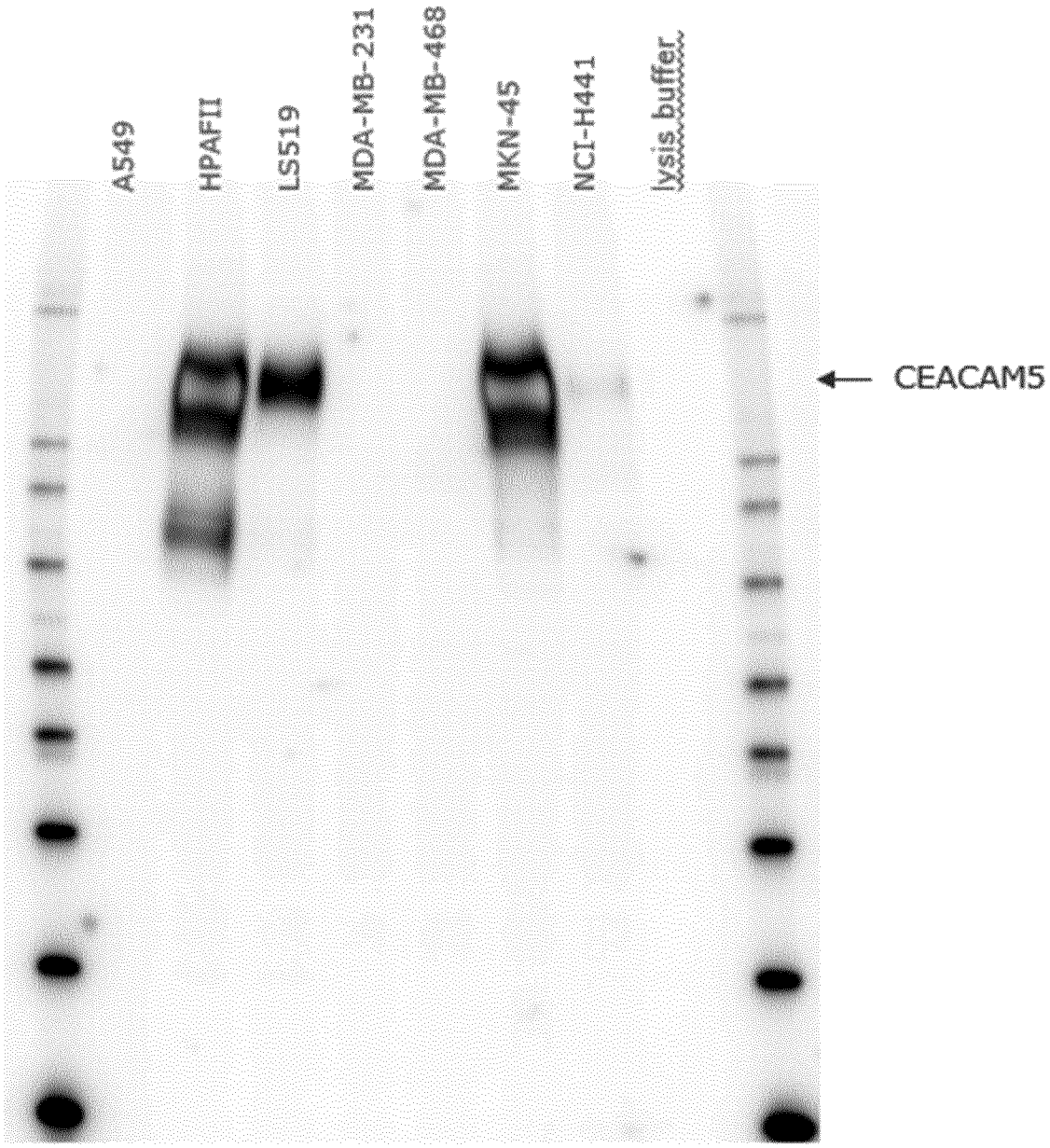
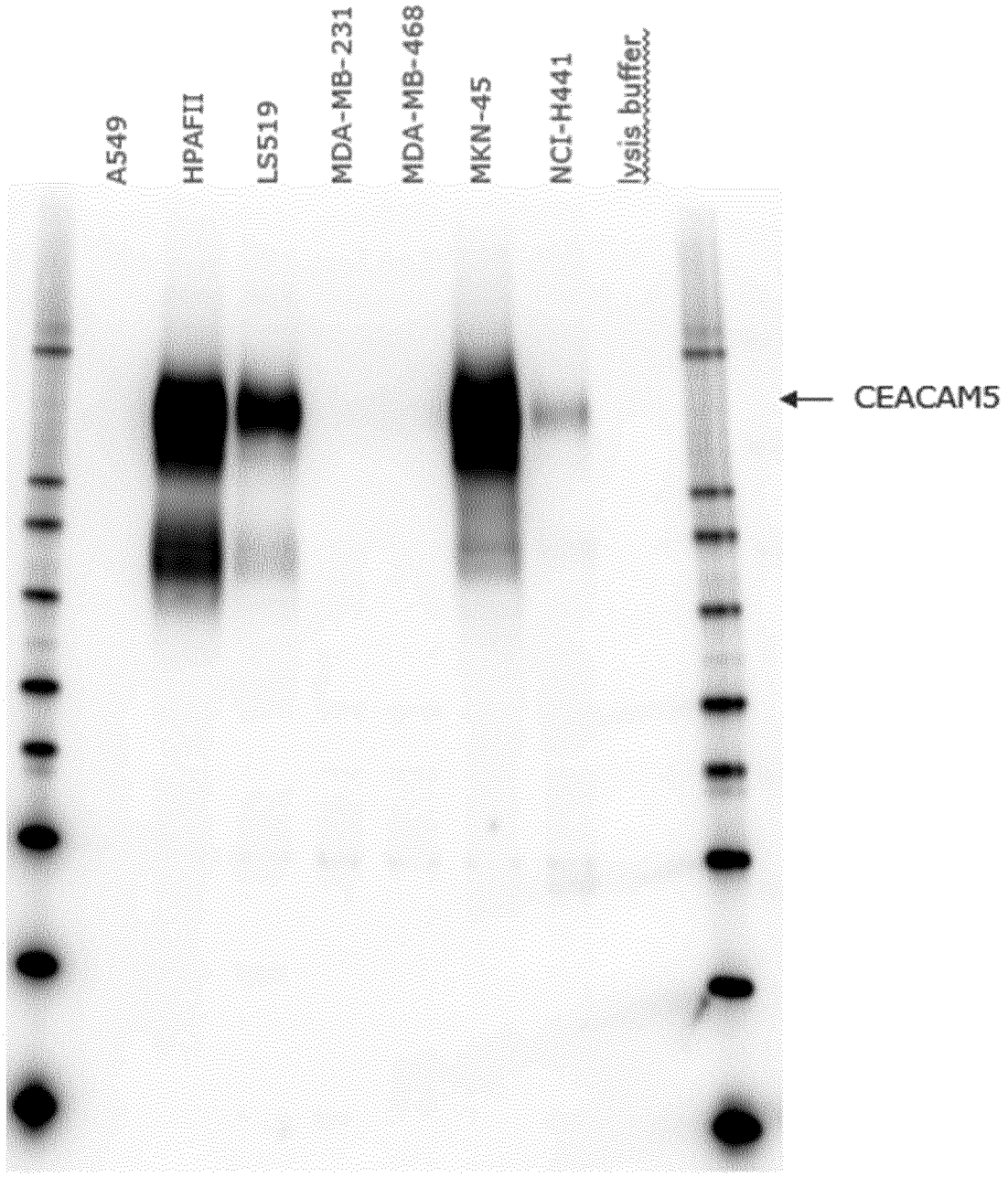

A549
HPAFII
LS519
MDA-MB-231
MDA-MB-468
MKN-45
NCI-H441
lysis buffer
CEACAM5

Fig. 13B: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NCI-H441
lysis buffer
CEACAM5

Fig. 14:

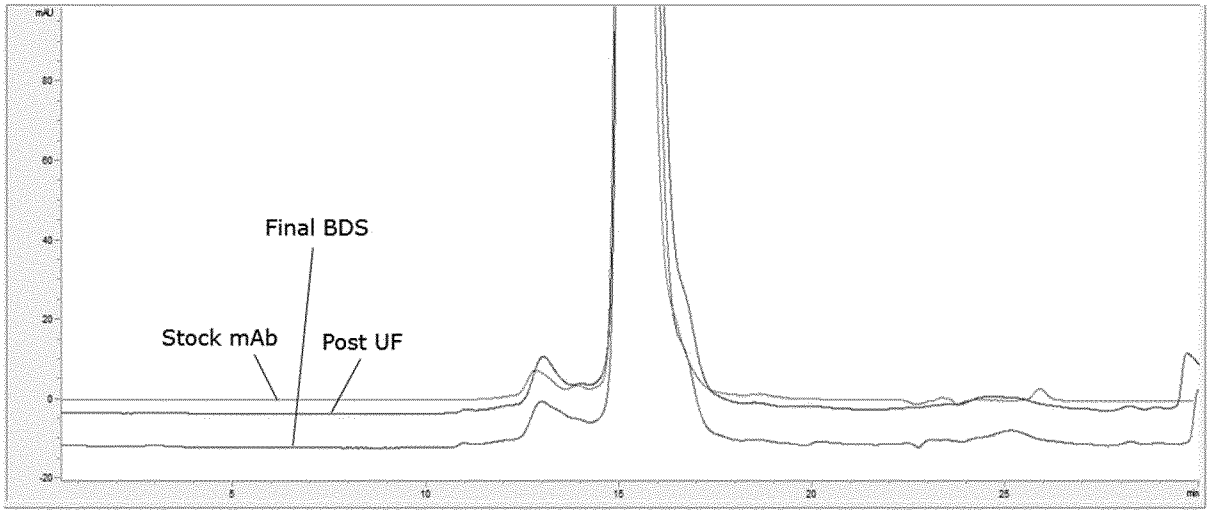


Fig. 15:

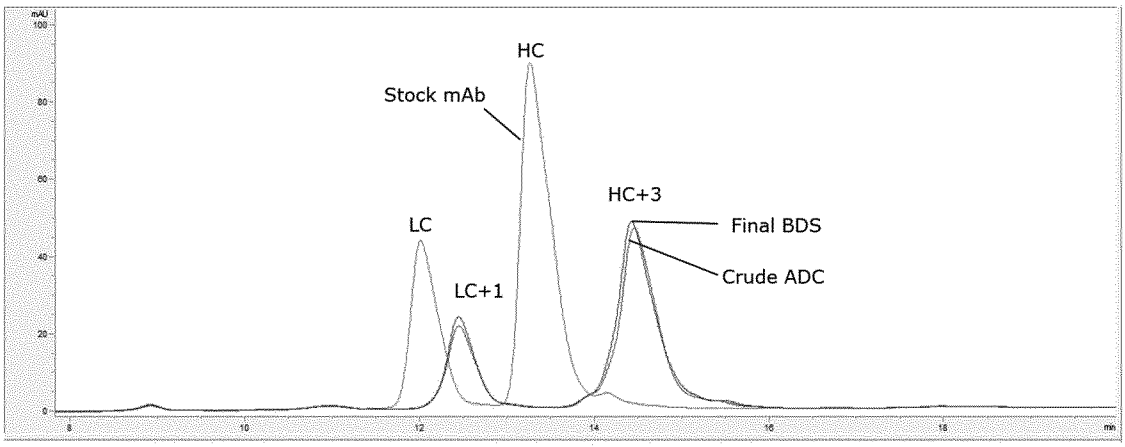


Fig. 16:

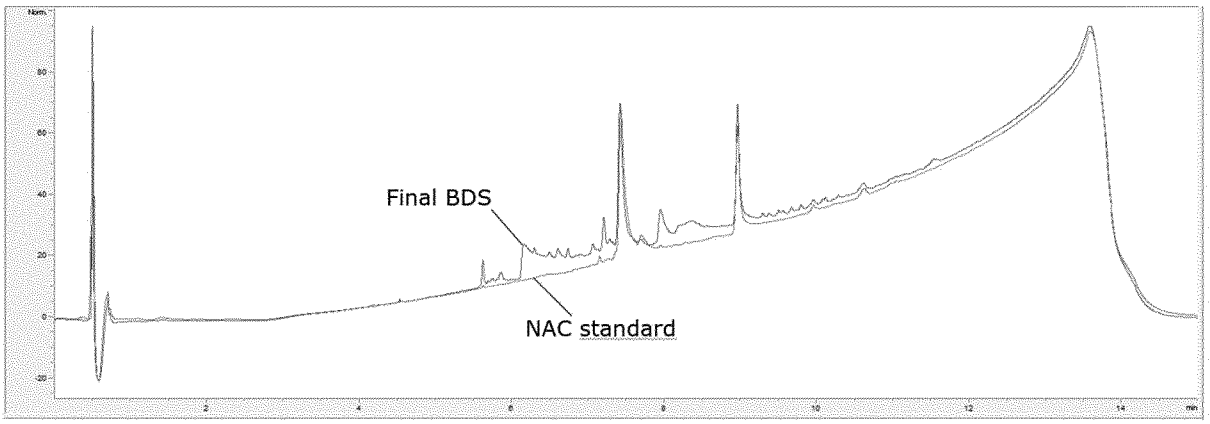


Fig. 17:

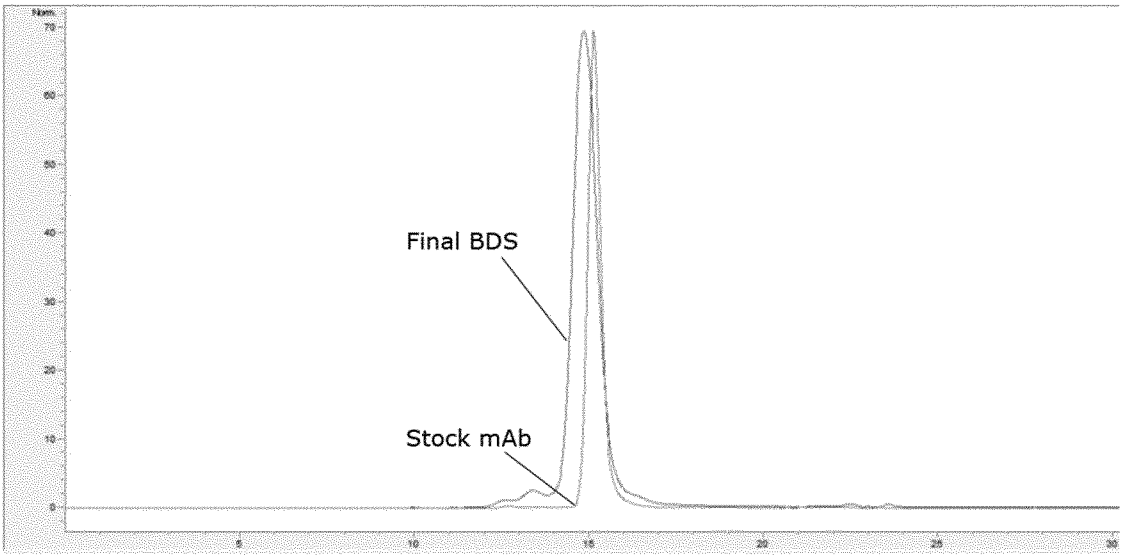


Fig. 18:

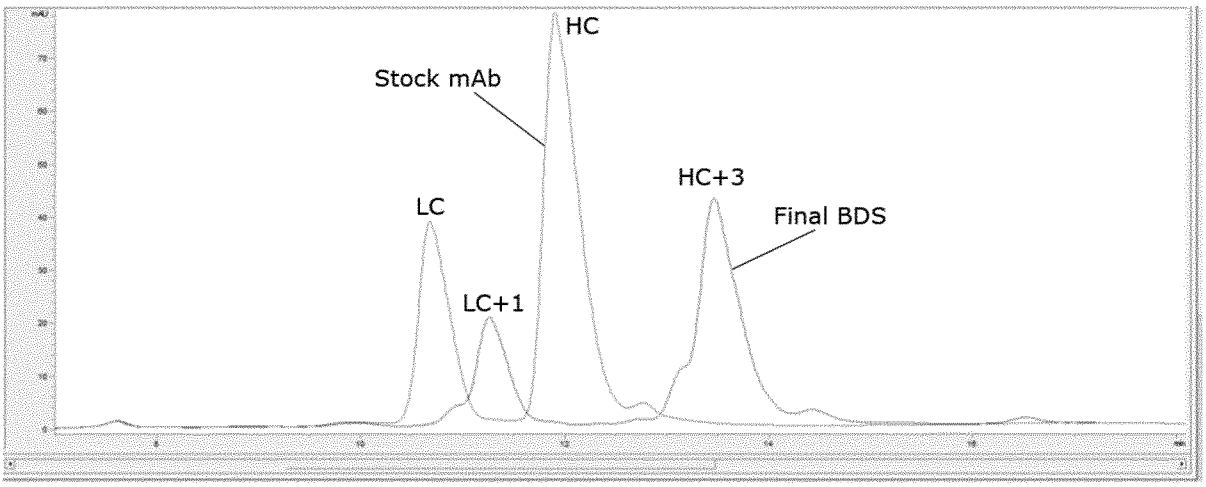


Fig. 19:

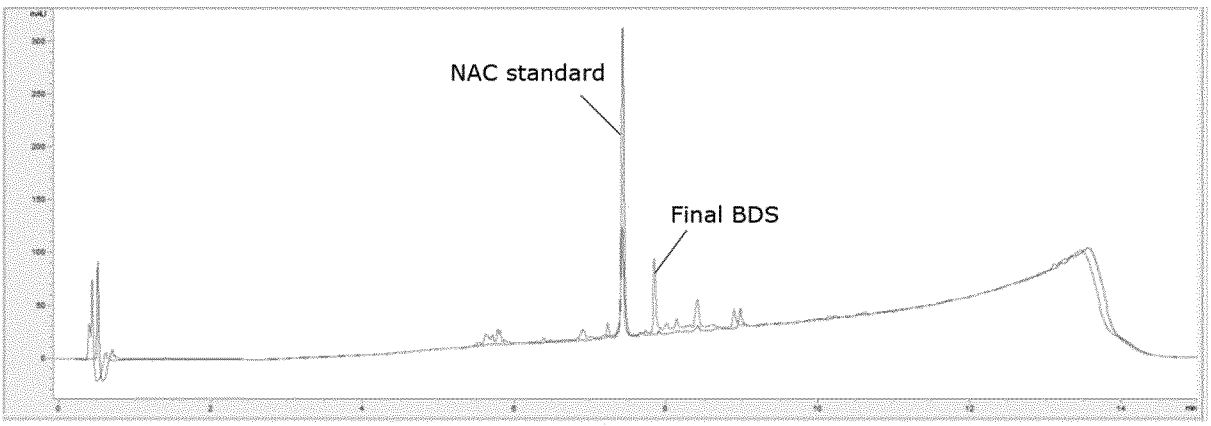


Fig. 20:

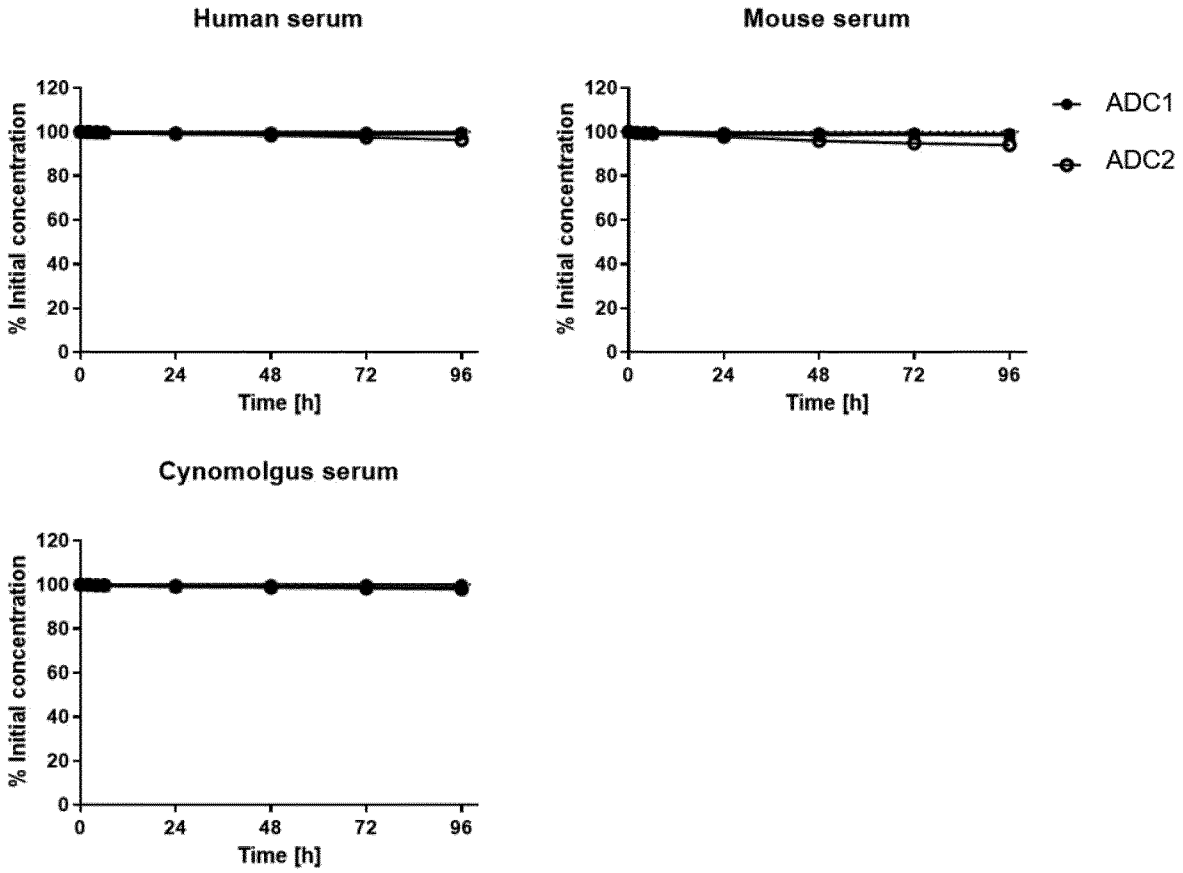

Human serum
Mouse serum
Cynomolgus serum
% Initial concentration
Time [h]
0
24
48
72
96
20
40
60
80
100
120
ADC1
ADC2

Fig. 21:

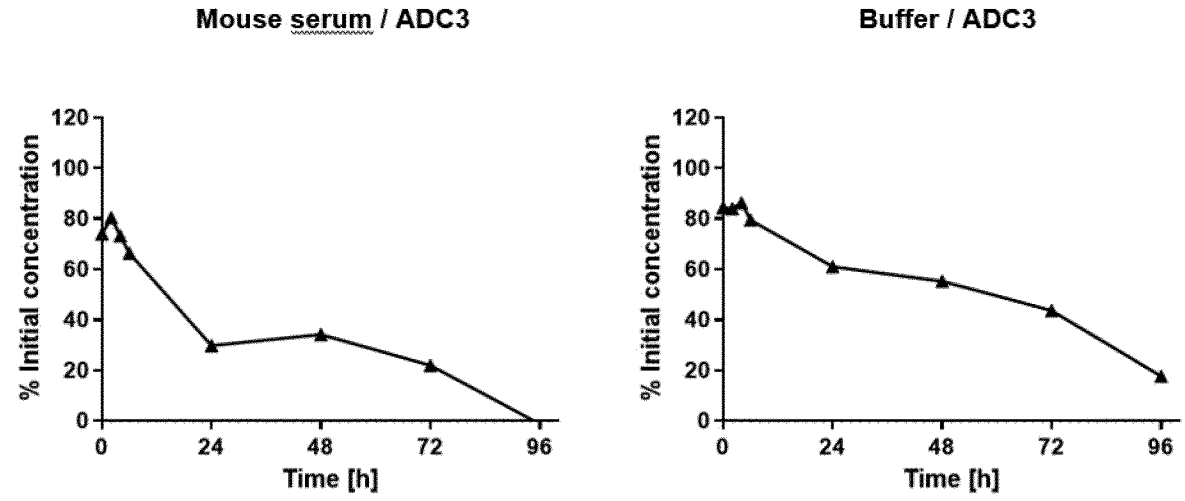

Mouse serum / ADC3
Buffer / ADC3
% Initial concentration
Time [h]
0
24
48
72
96
20
40
60
80
100
120

Fig. 22:

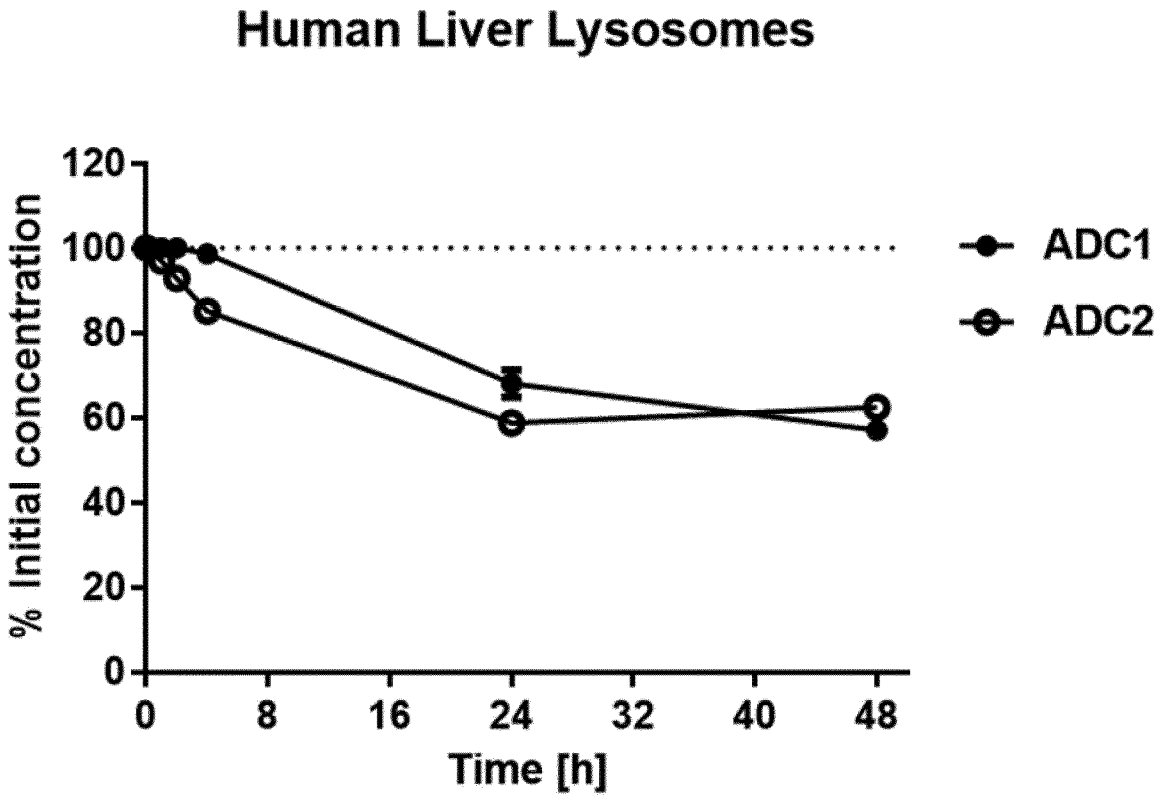

Human Liver Lysosomes
% Initial concentration
120
100
80
60
40
20
0
0
8
16
24
32
40
48
Time [h]
ADC1
ADC2

Fig. 23:

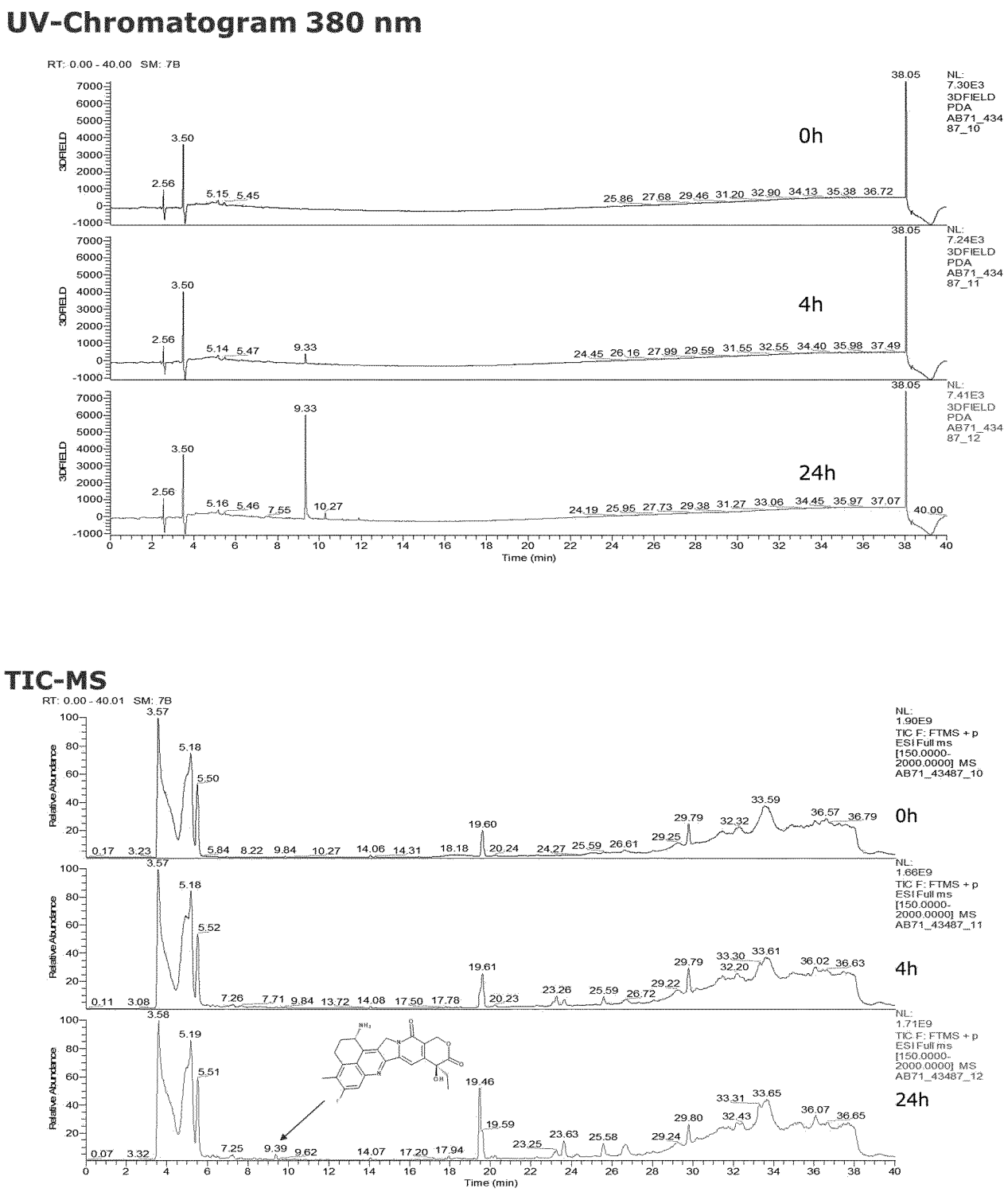

UV-Chromatogram 380 nm
RT: 0.00 - 40.00 SM: 7B
3DFIELD
0h
4h
24h
NL: 7.30E3 3DFIELD PDA AB71_43487_10
NL: 7.24E3 3DFIELD PDA AB71_43487_11
NL: 7.41E3 3DFIELD PDA AB71_43487_12
Time (min)
TIC-MS
RT: 0.00 - 40.01 SM: 7B
Relative Abundance
NL: 1.90E9 TIC F: FTMS + p ESI Full ms [150.0000-2000.0000] MS AB71_43487_10
NL: 1.66E9 TIC F: FTMS + p ESI Full ms [150.0000-2000.0000] MS AB71_43487_11
NL: 1.71E9 TIC F: FTMS + p ESI Full ms [150.0000-2000.0000] MS AB71_43487_12
Time (min)

Fig. 24A:

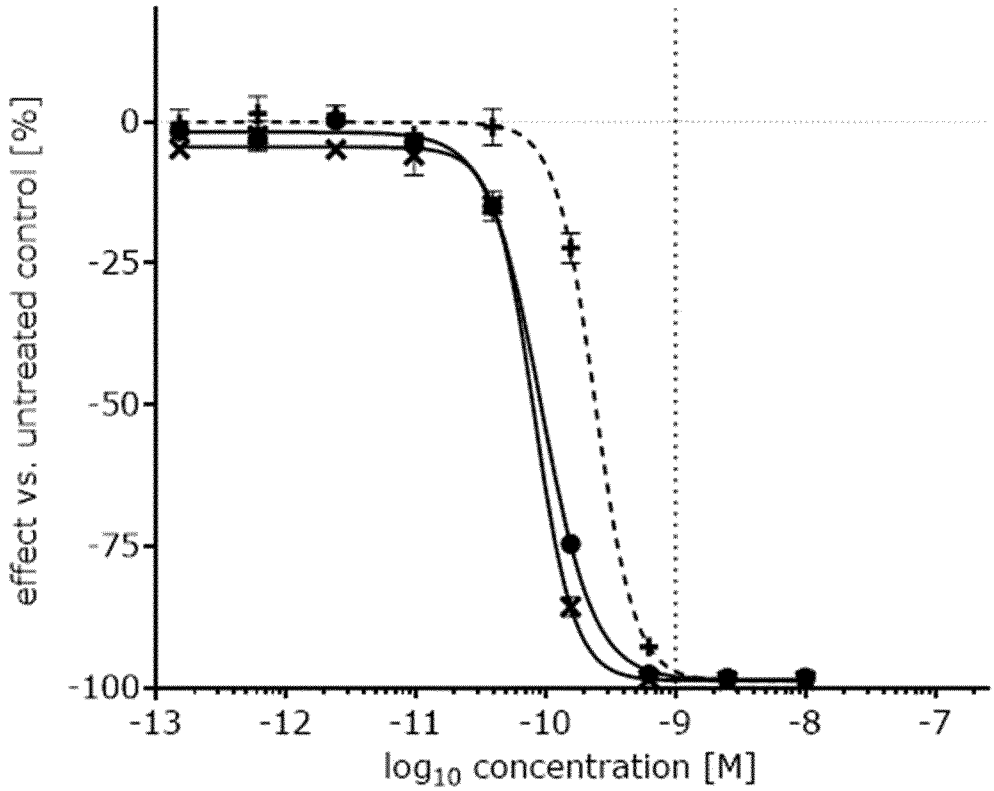


Fig. 24B:

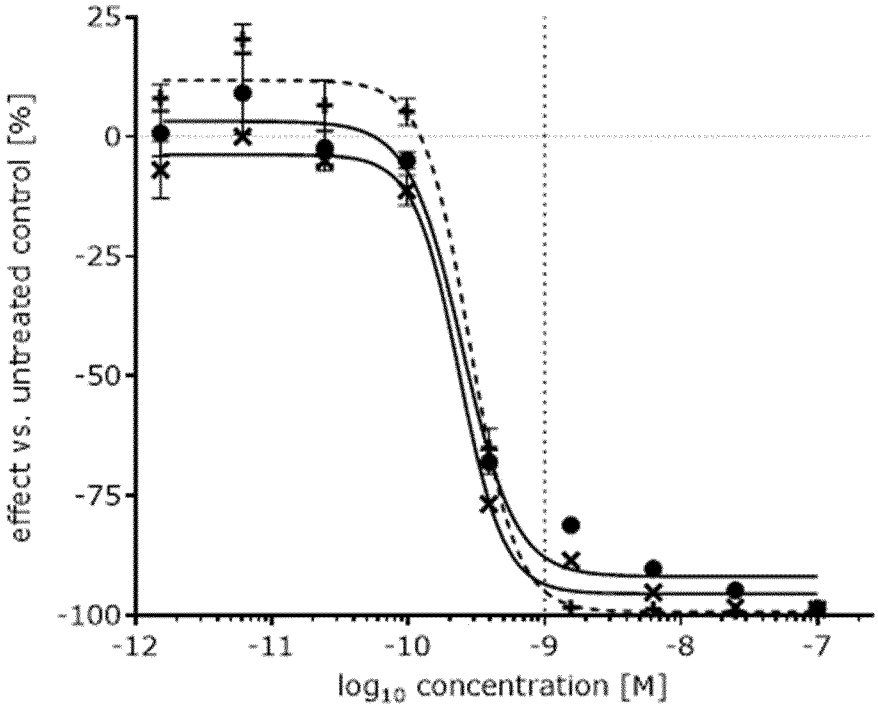


Fig. 24C:

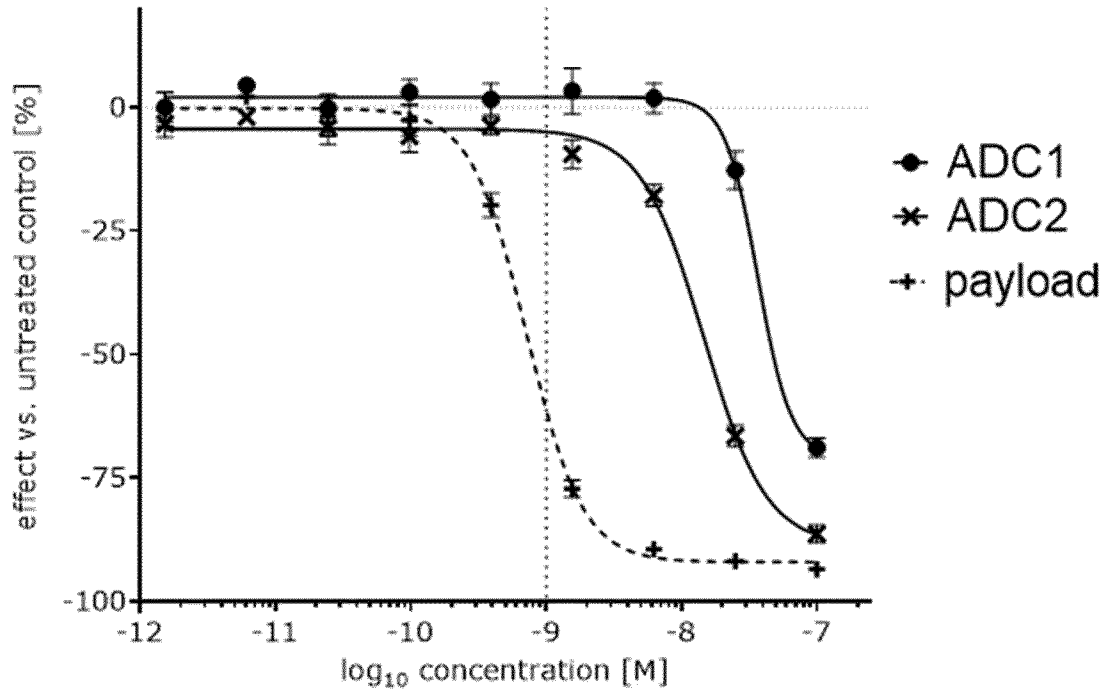


Fig. 25:

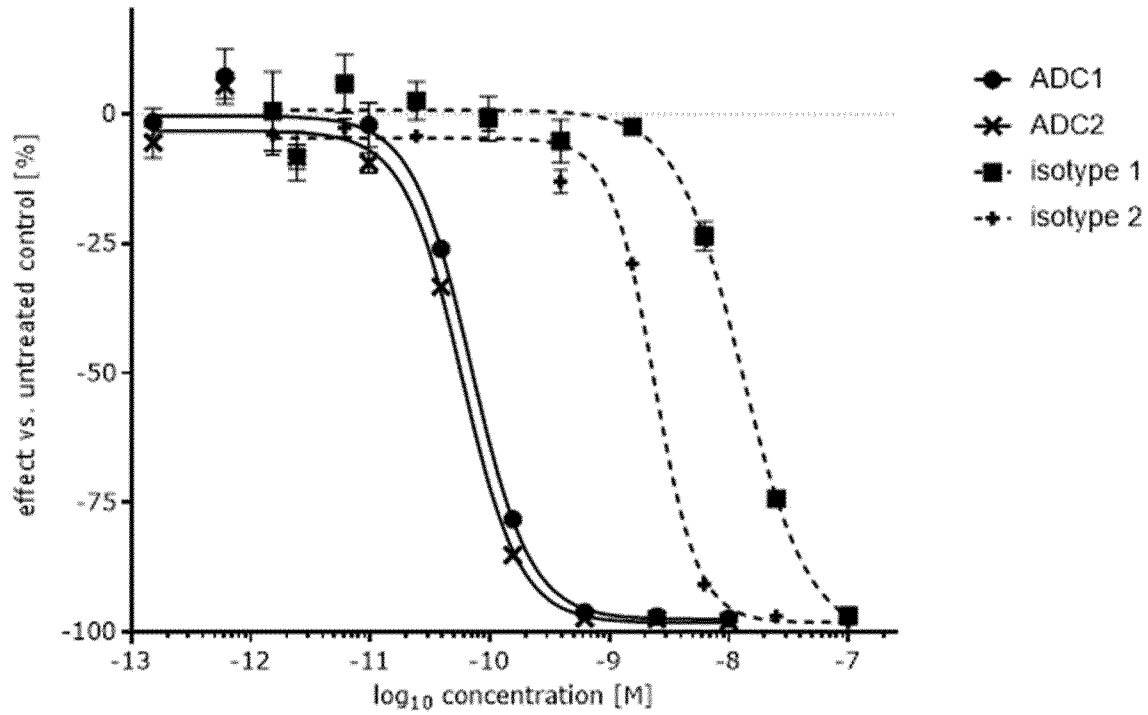


Fig. 26A:

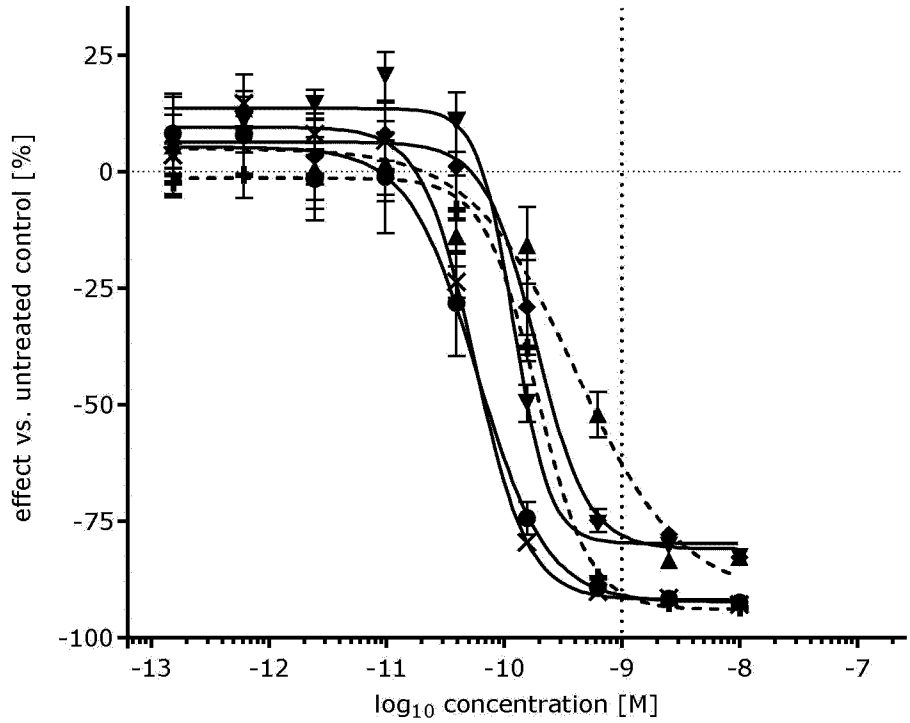


Fig. 26B:

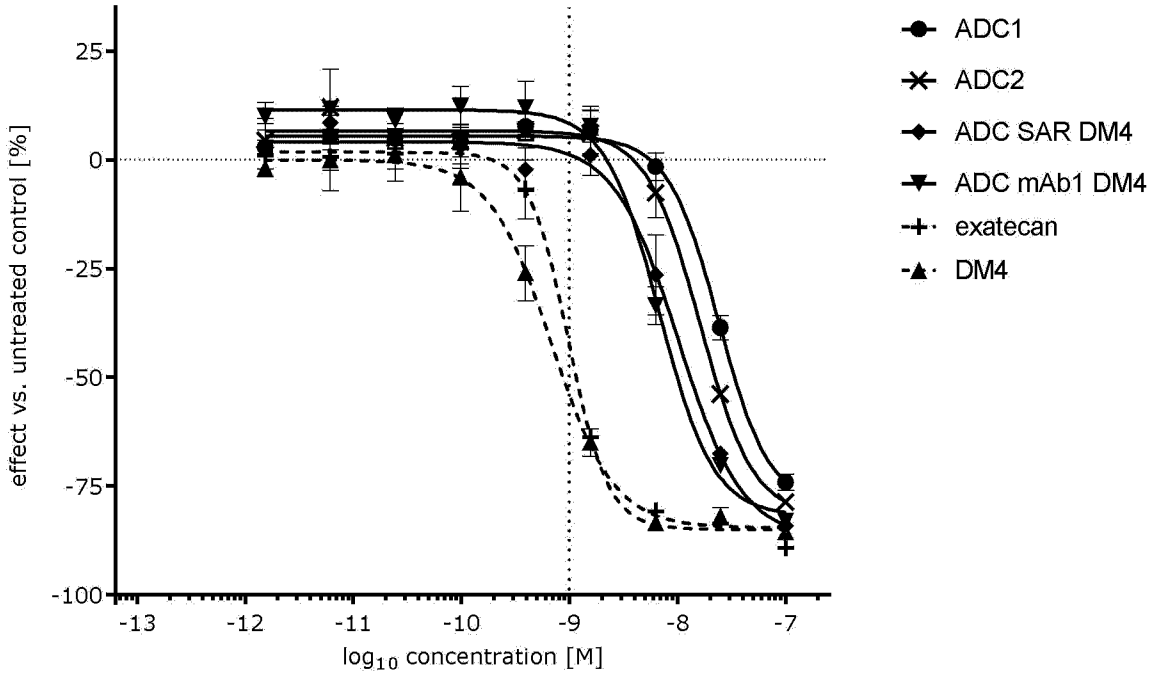


Fig. 27A:

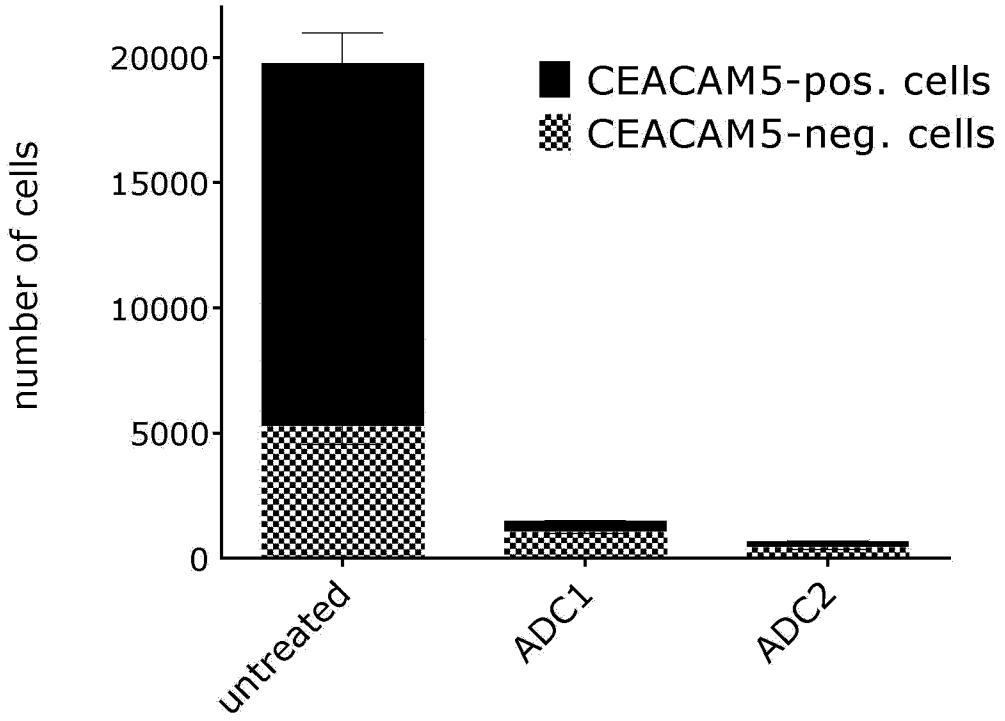


Fig. 27B:

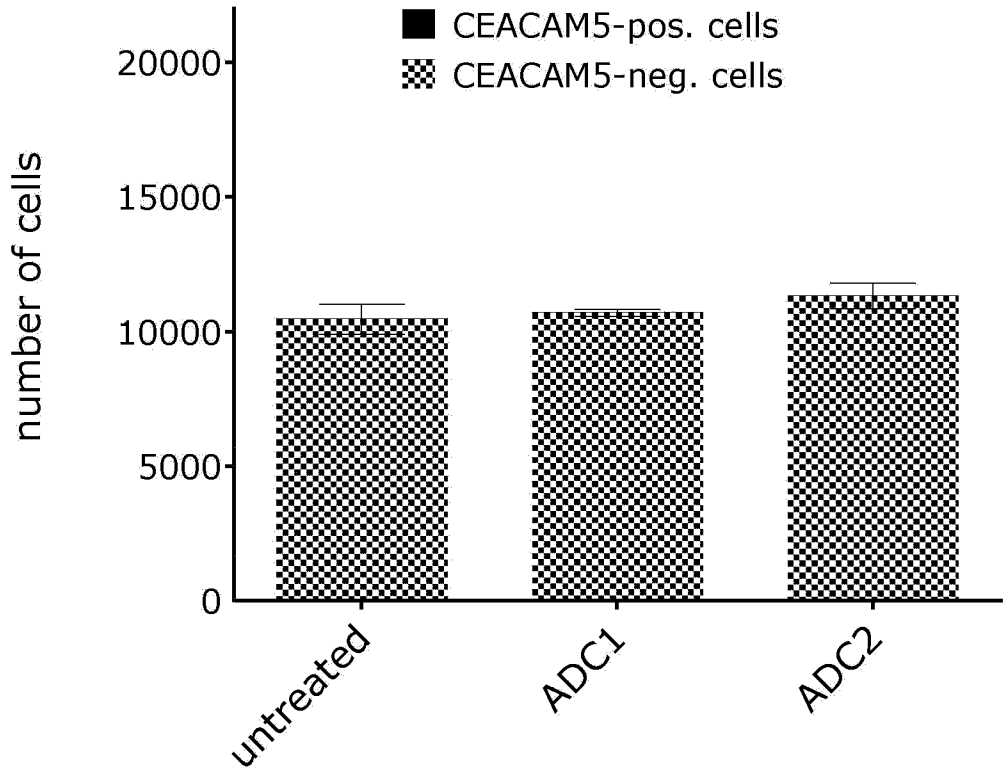


Fig. 28A:

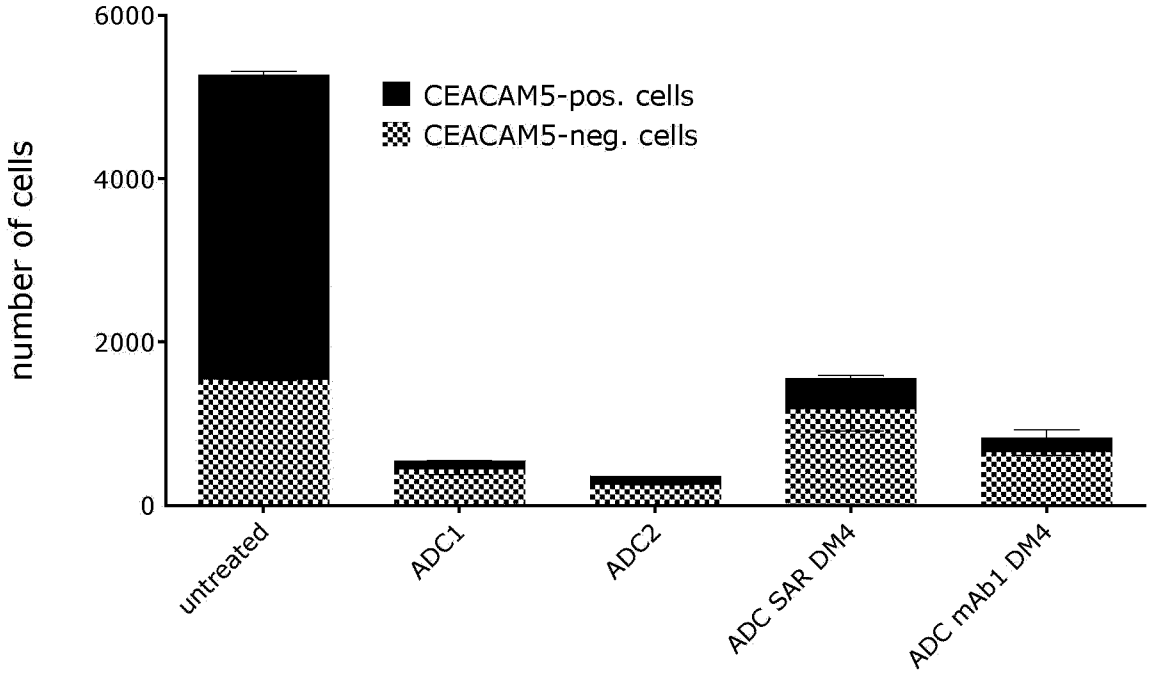


Fig. 28B:

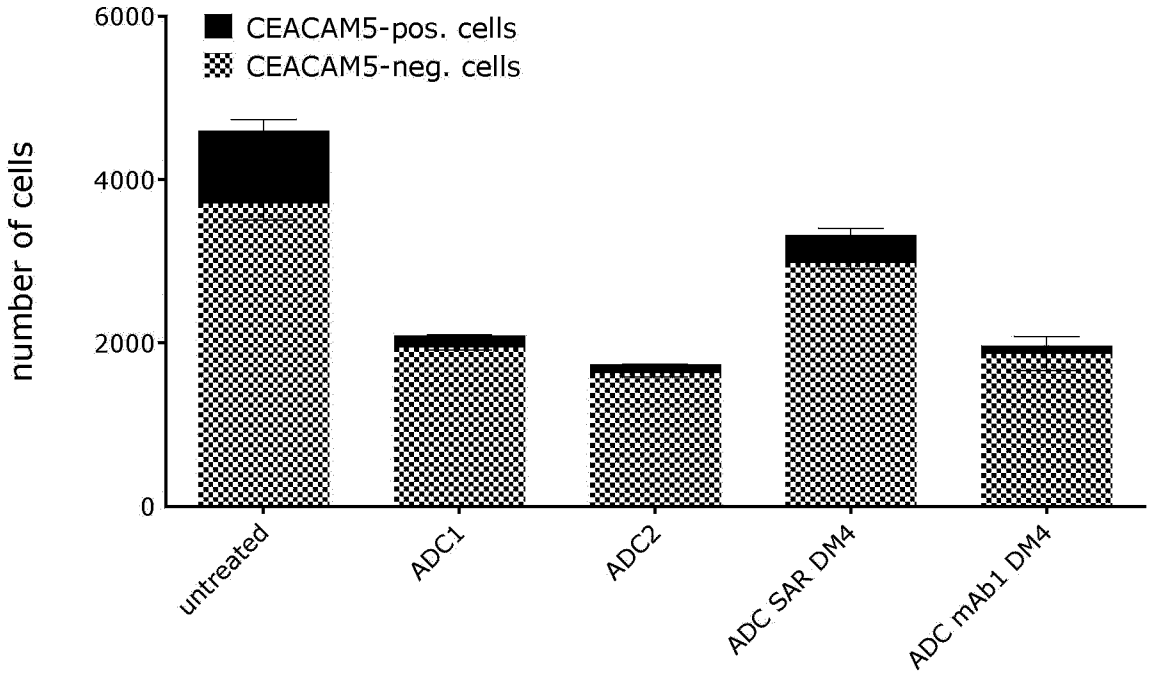


Fig. 28C:

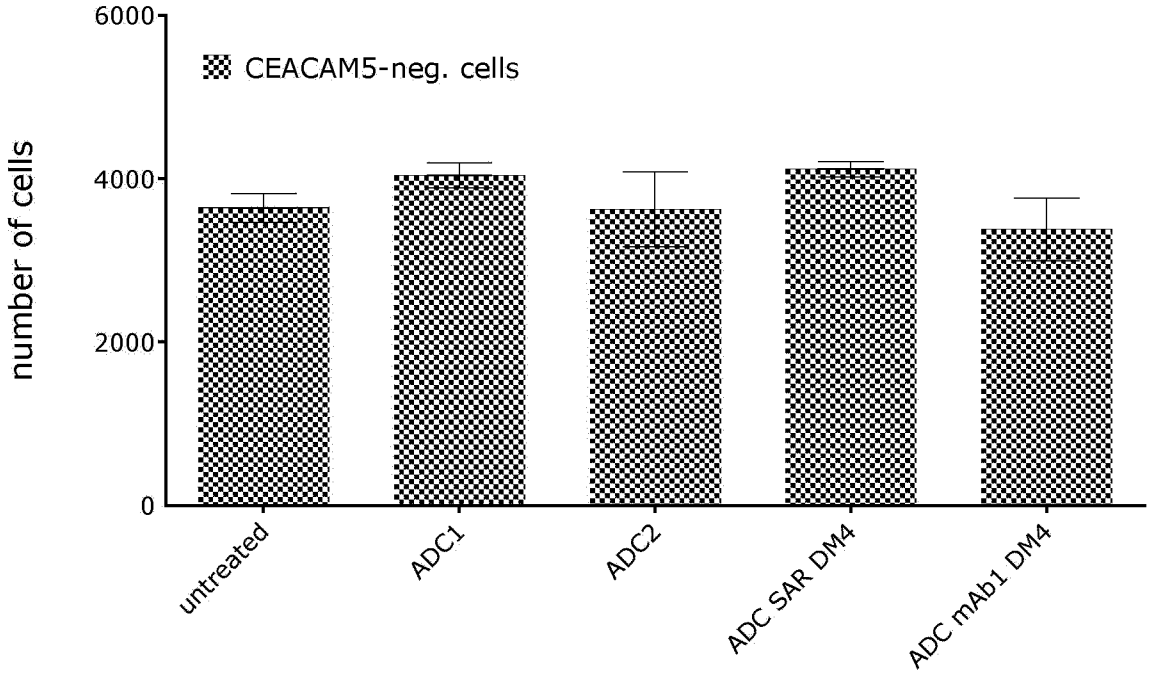


Fig. 29:

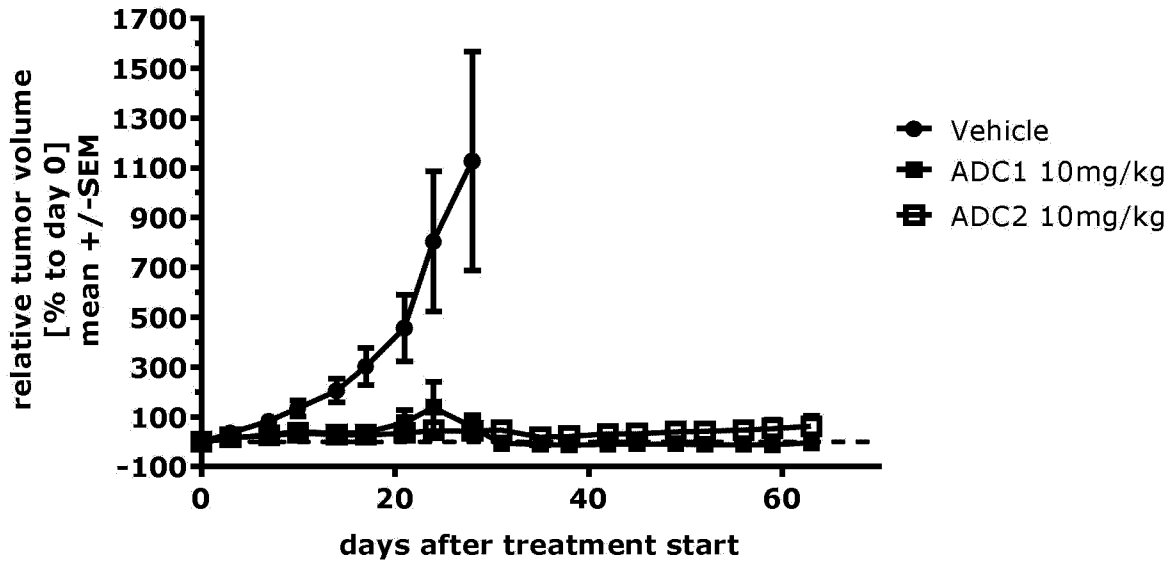


Fig. 30:

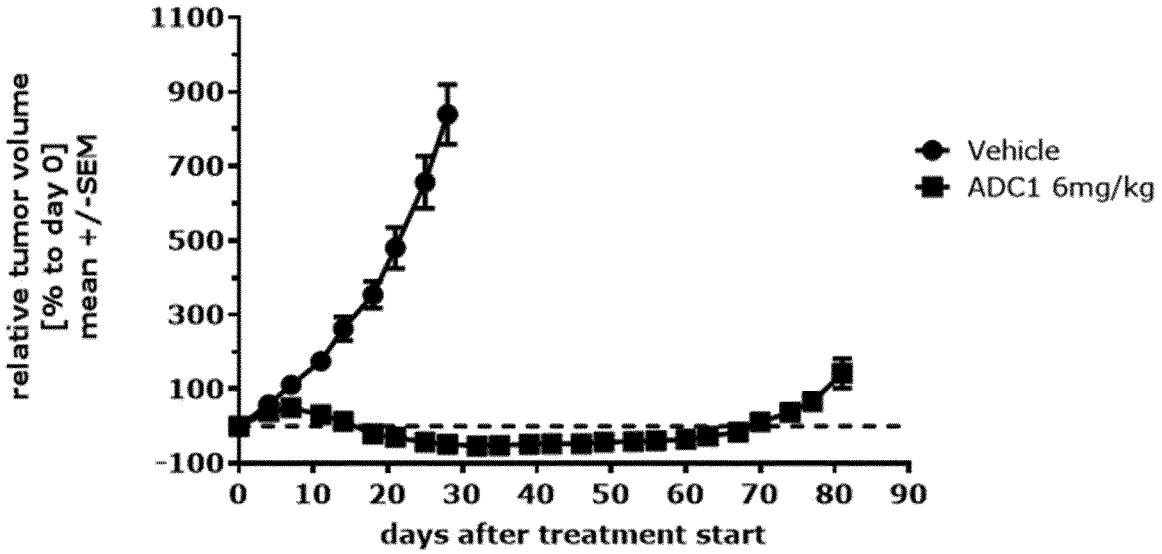

relative tumor volume
[% to day 0]
mean +/-SEM
1100
900
700
500
300
100
-100
0
10
20
30
40
50
60
70
80
90
days after treatment start
Vehicle
ADC1 6mg/kg

Fig. 31:

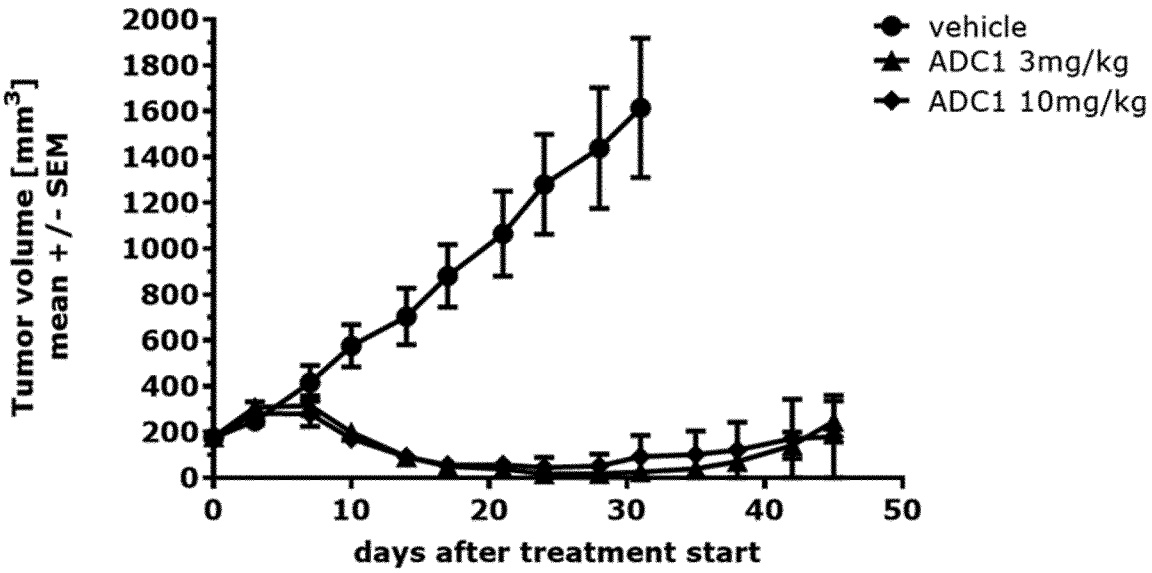

Tumor volume [mm3]
mean +/- SEM
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
days after treatment start
vehicle
ADC1 3mg/kg
ADC1 10mg/kg

Fig. 32:

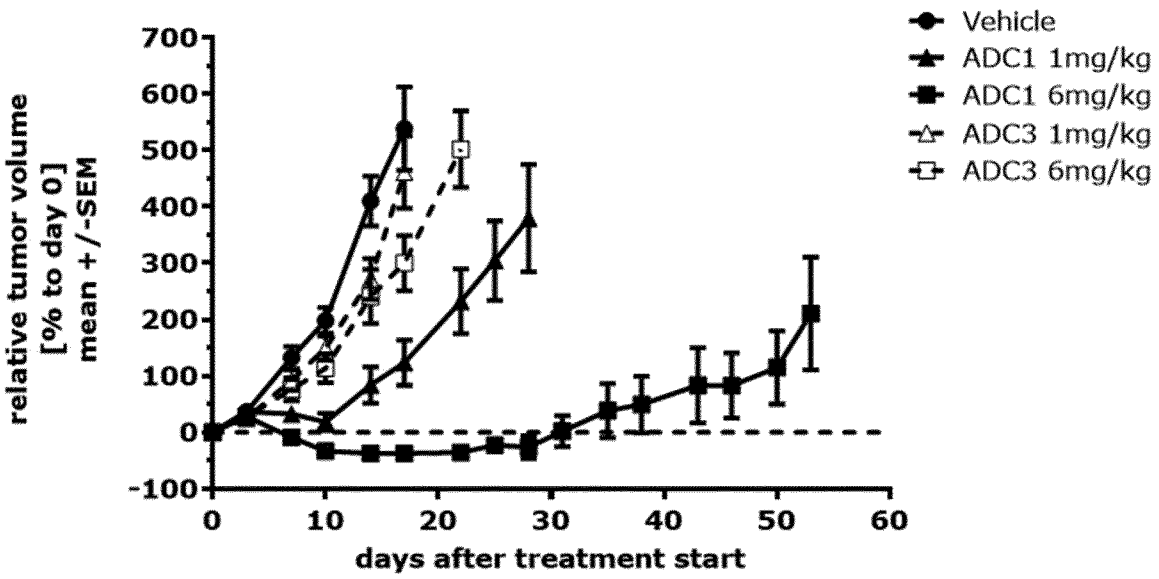

relative tumor volume
[% to day 0]
mean +/-SEM
700
600
500
400
300
200
100
0
-100
0
10
20
30
40
50
60
days after treatment start
Vehicle
ADC1 1mg/kg
ADC1 6mg/kg
ADC3 1mg/kg
ADC3 6mg/kg

Fig. 33:

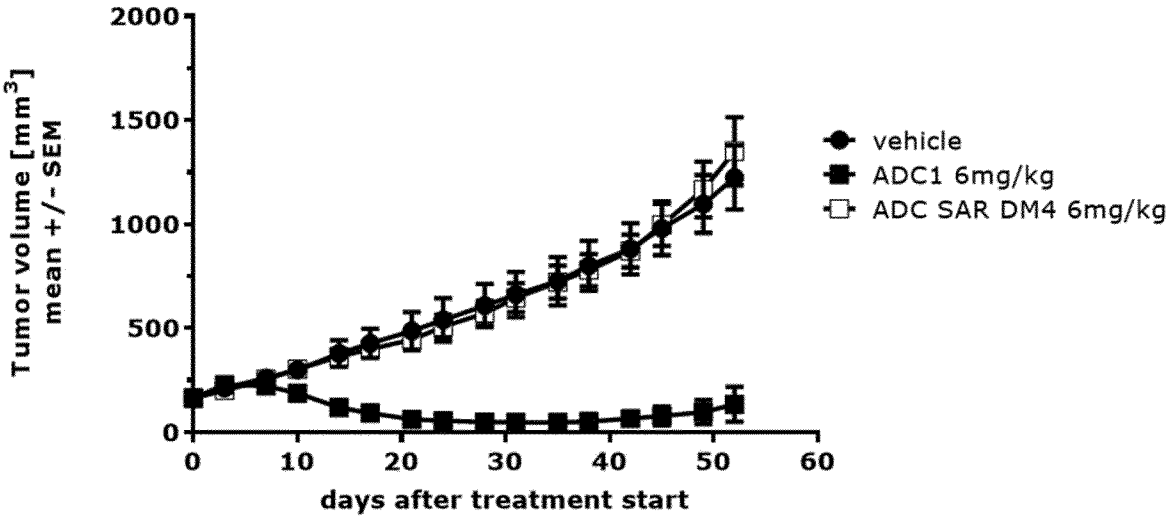

Tumor volume [mm3]
mean +/- SEM
2000
1500
1000
500
0
0
10
20
30
40
50
60
days after treatment start
vehicle
ADC1 6mg/kg
ADC SAR DM4 6mg/kg

Fig. 34:

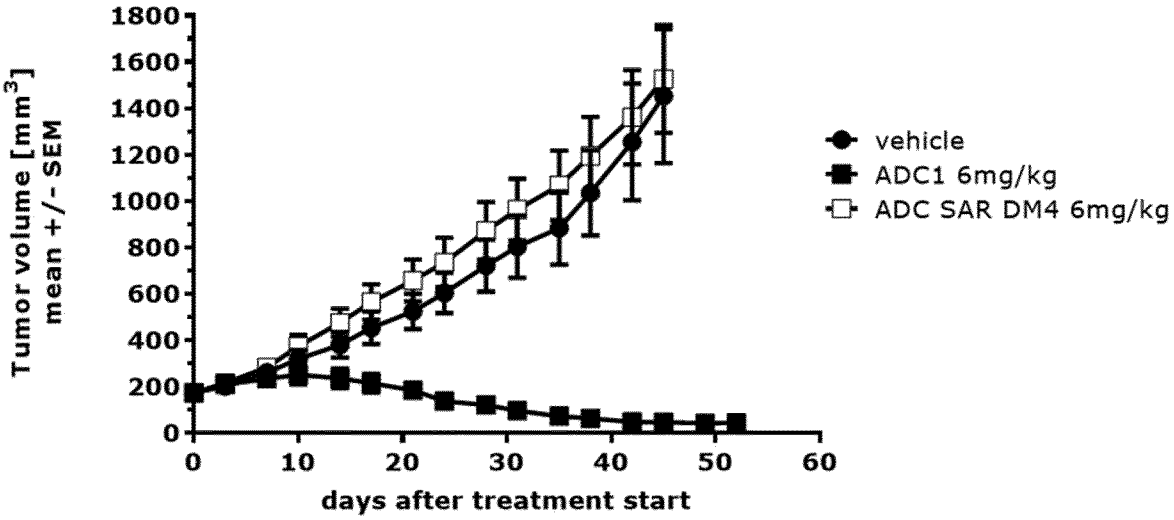

Tumor volume [mm3] mean +/- SEM
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
days after treatment start
vehicle
ADC1 6mg/kg
ADC SAR DM4 6mg/kg

Fig. 35:

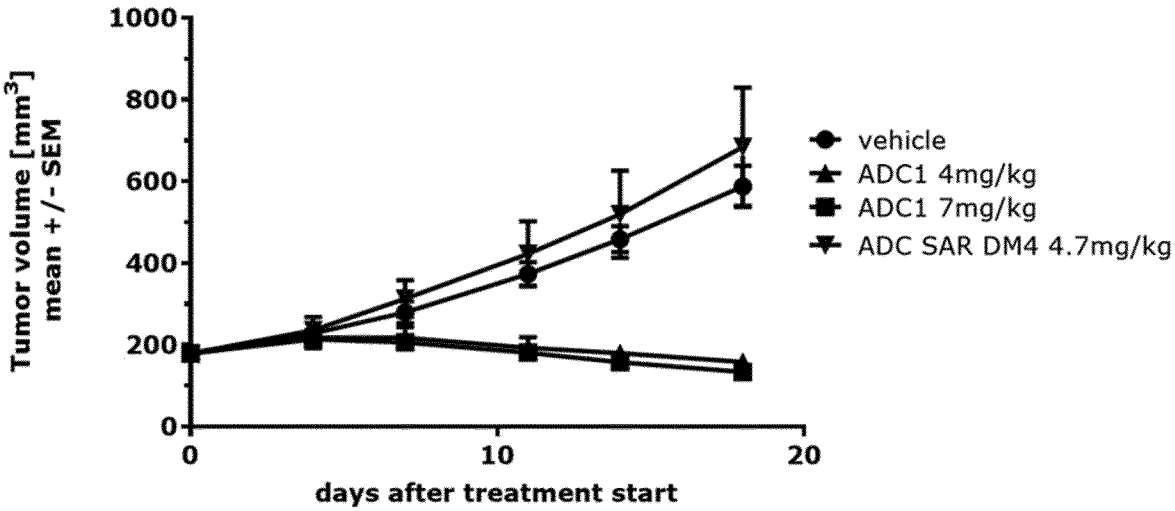
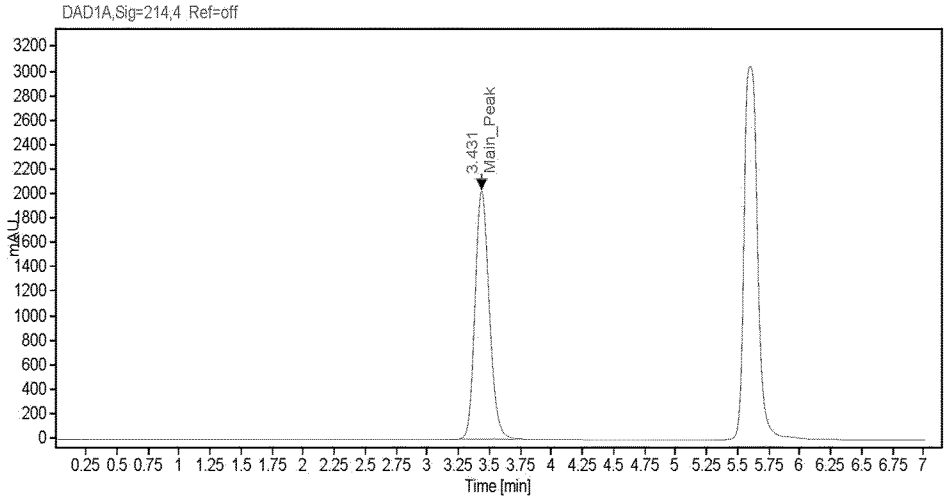

Tumor volume [mm3] mean +/- SEM
1000
800
600
400
200
0
0
10
20
days after treatment start
vehicle
ADC1 4mg/kg
ADC1 7mg/kg
ADC SAR DM4 4.7mg/kg

Fig. 36:

Typical SEC chromatograms showing the purity of the input mAb and the final BDS:

Input mAb

Purity: 100.0%



ADC BDS

Purity: 98.9%

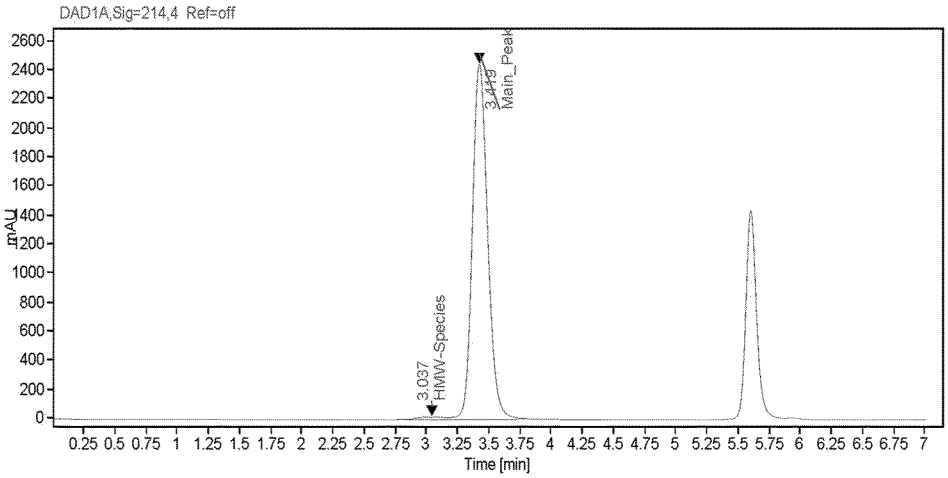


Fig. 37:

Typical RP-HPLC chromatograms illustrating DAR determination of the final BDS:

ADC BDS

DAR= 8.16

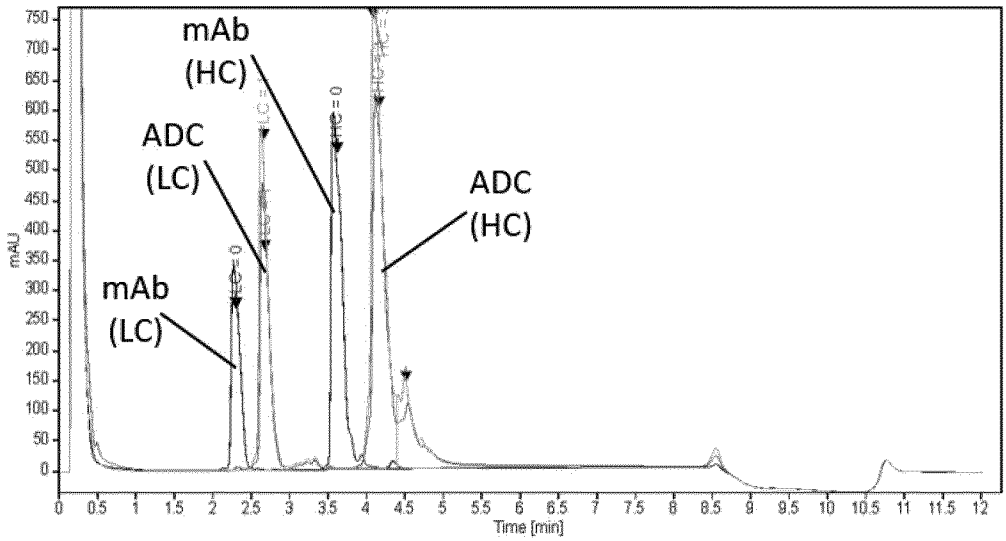
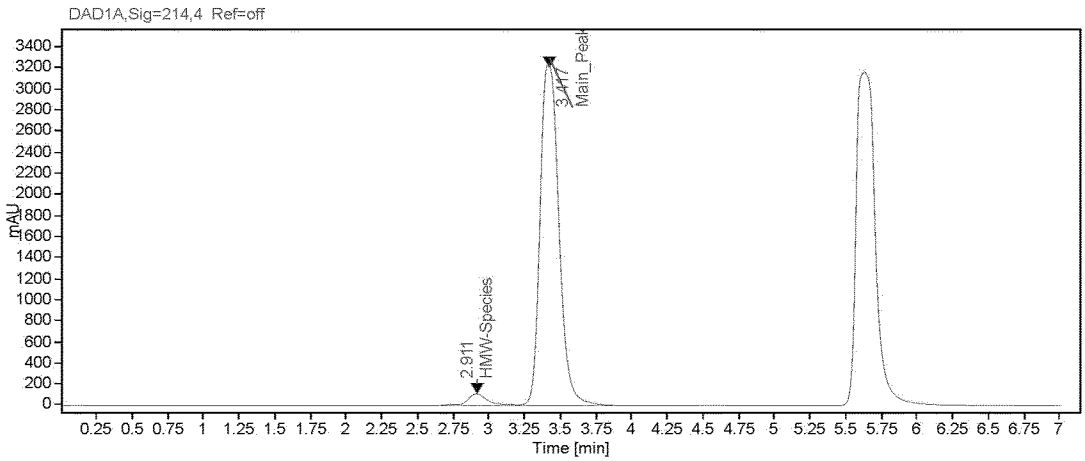


Fig. 38:

Typical SEC chromatograms showing the purity of the input mAb and the final BDS:

Input mAb

Purity: 96,3%



ADC BDS

Purity: 99.9

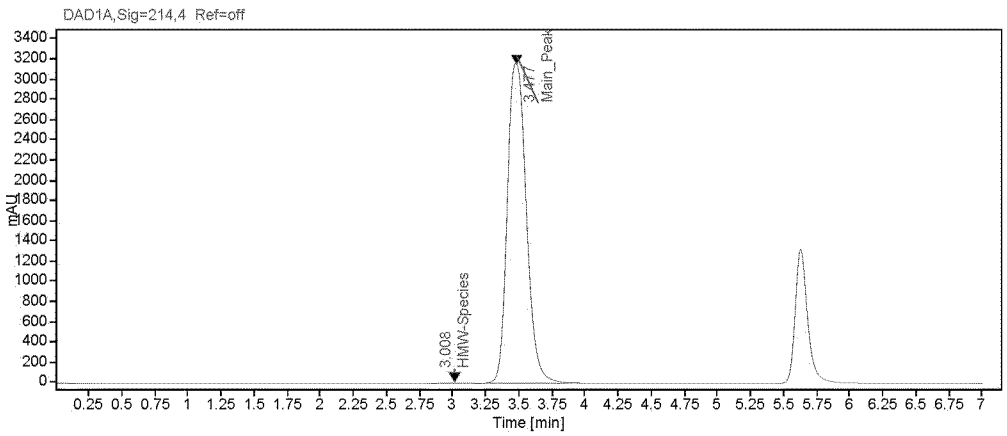
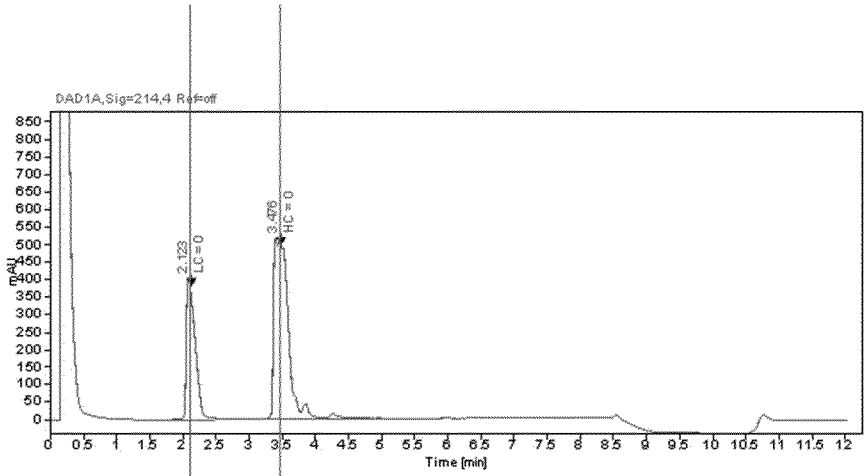


Fig. 39:

Typical RP-HPLC chromatograms illustrating DAR determination of the final BDS:

Input mAb



ADC BDS

DAR= 3.87

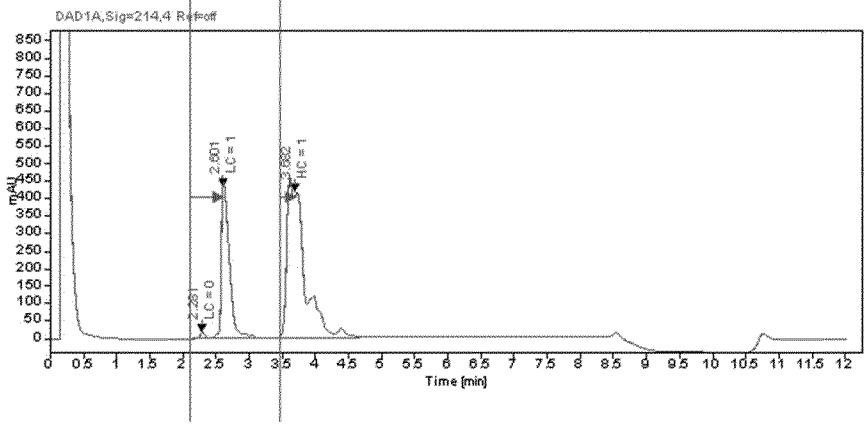
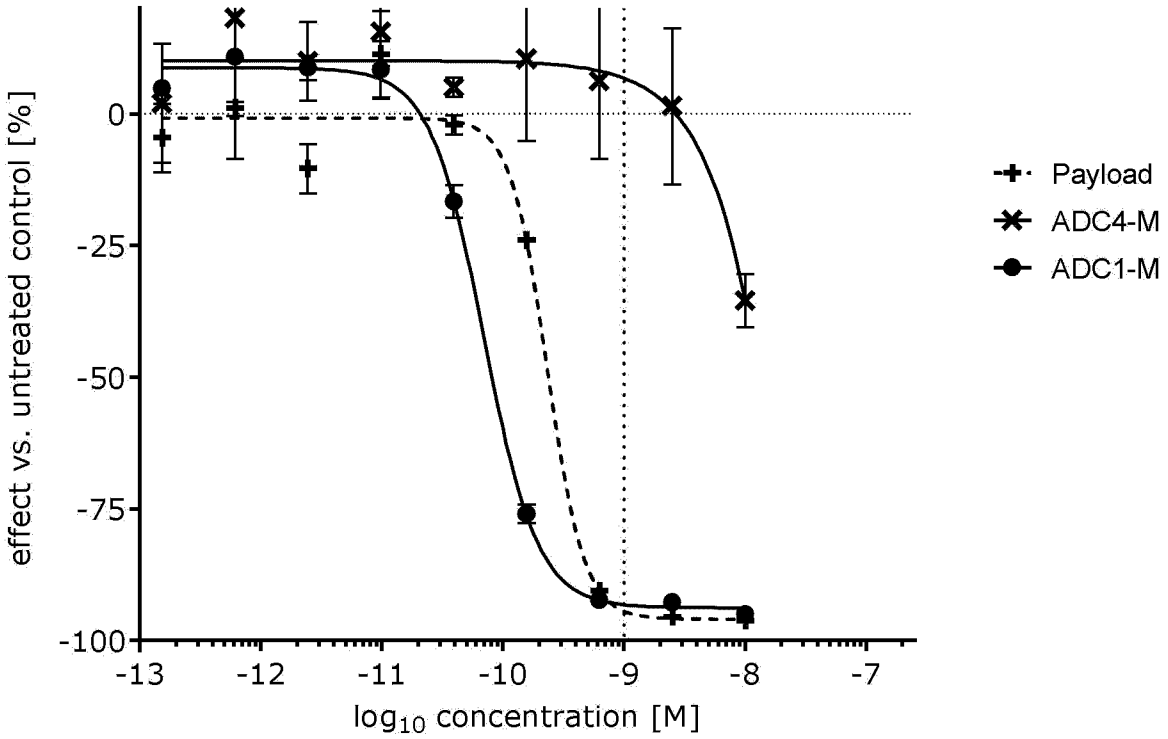
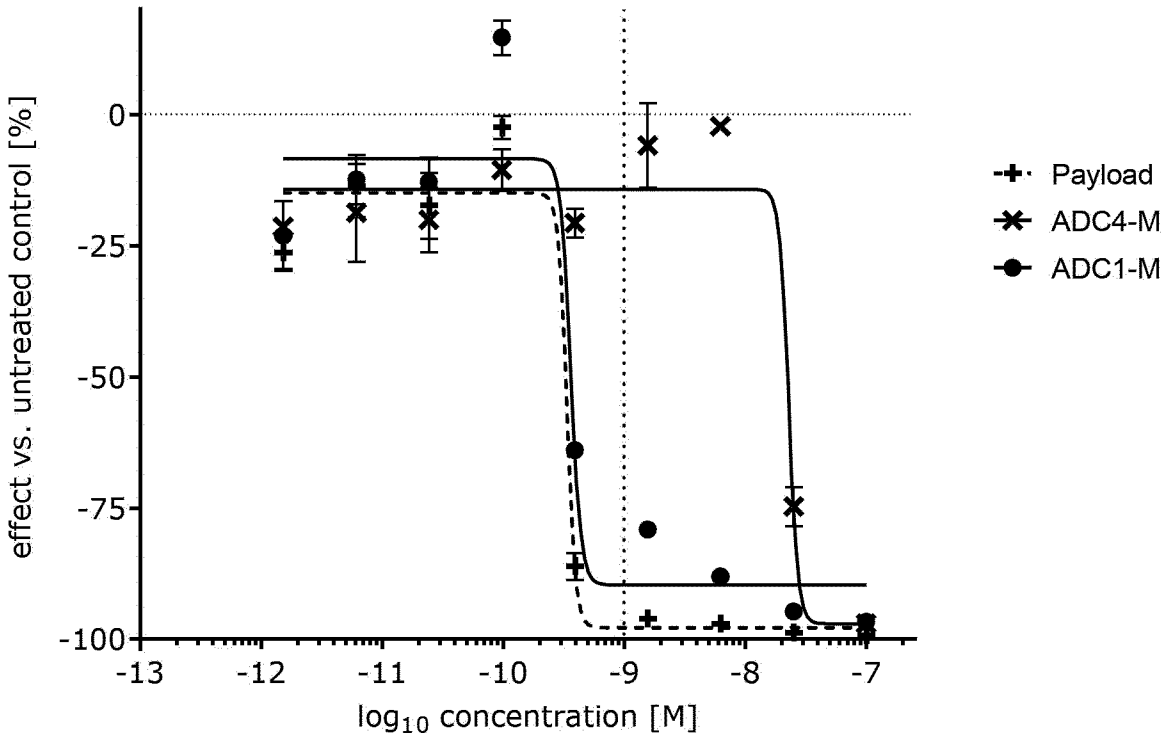


Fig. 40:

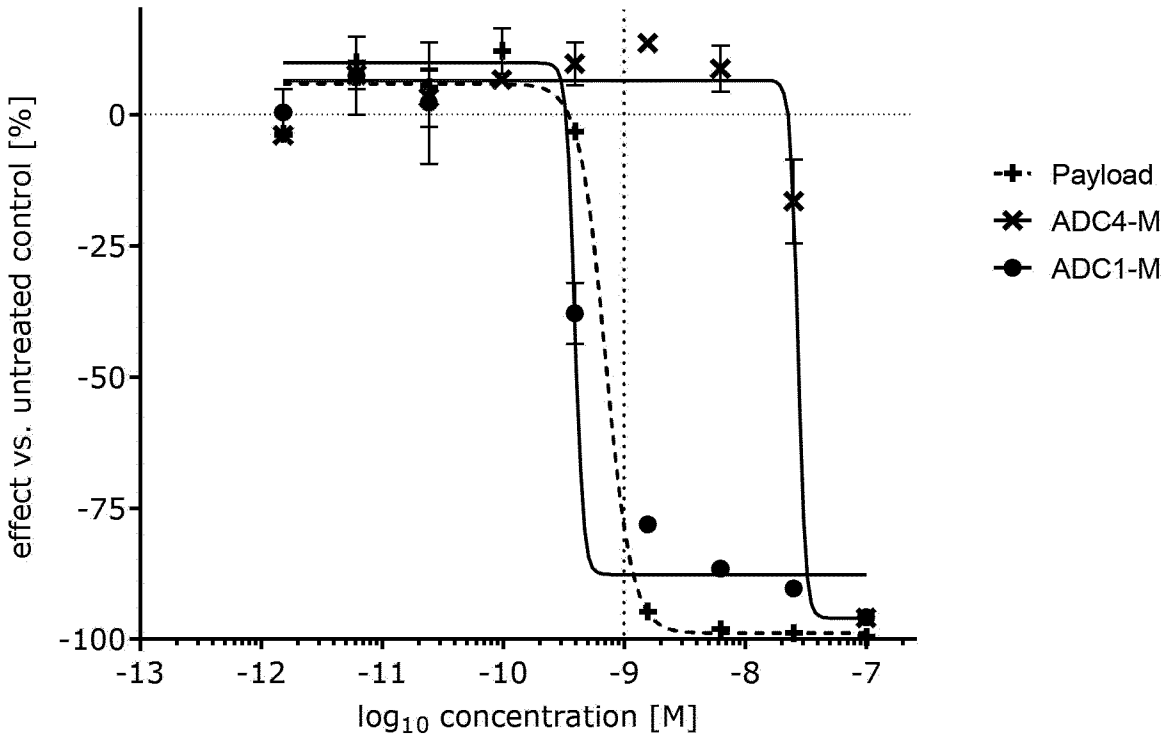
(a)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC4-M
ADC1-M

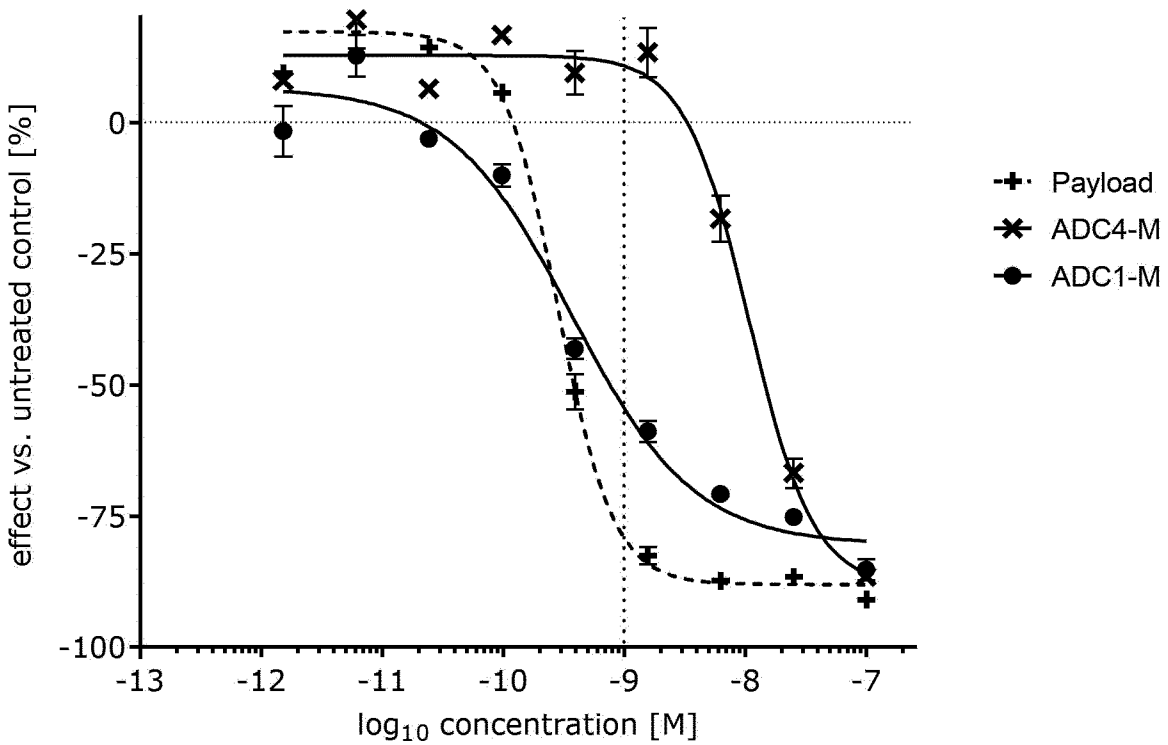
(b)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC4-M
ADC1-M

(c)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC4-M
ADC1-M

(d)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC4-M
ADC1-M

(e)

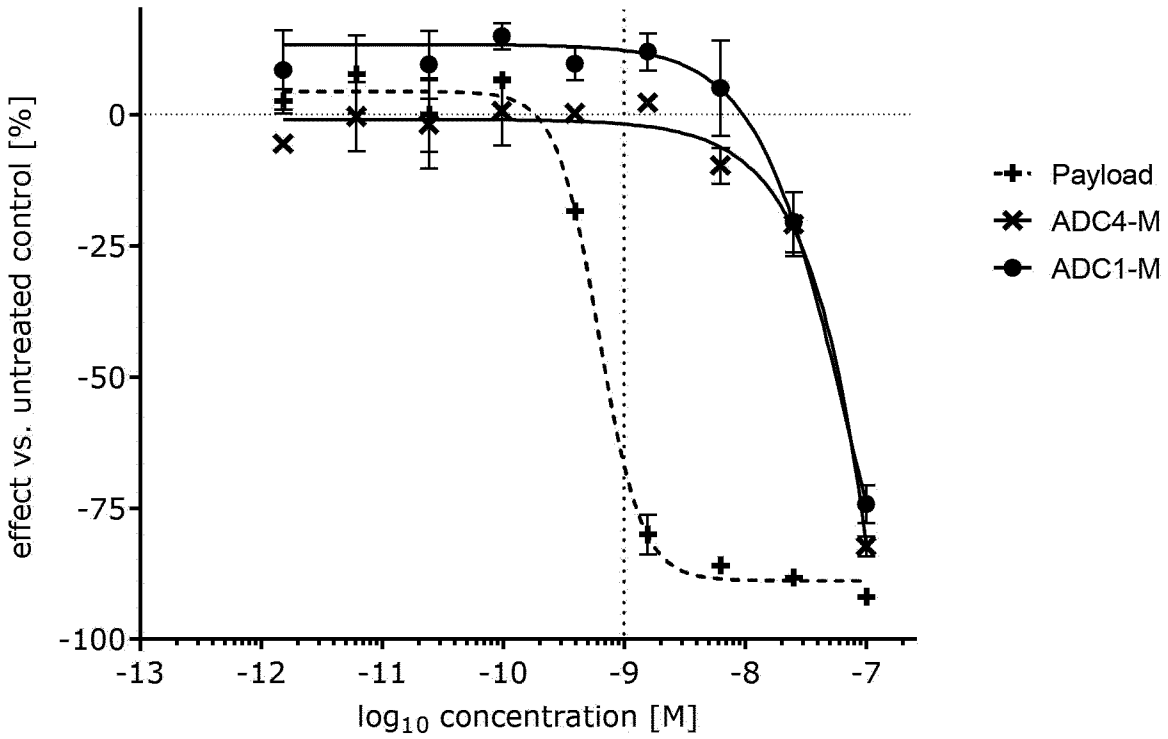
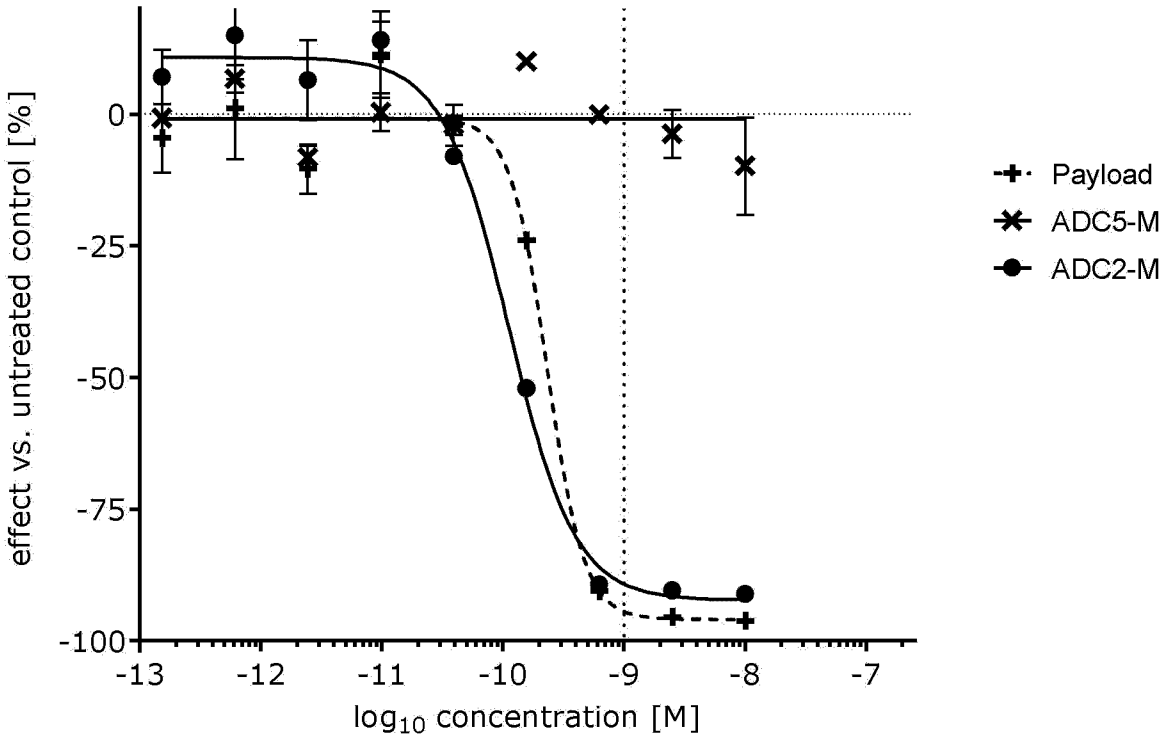
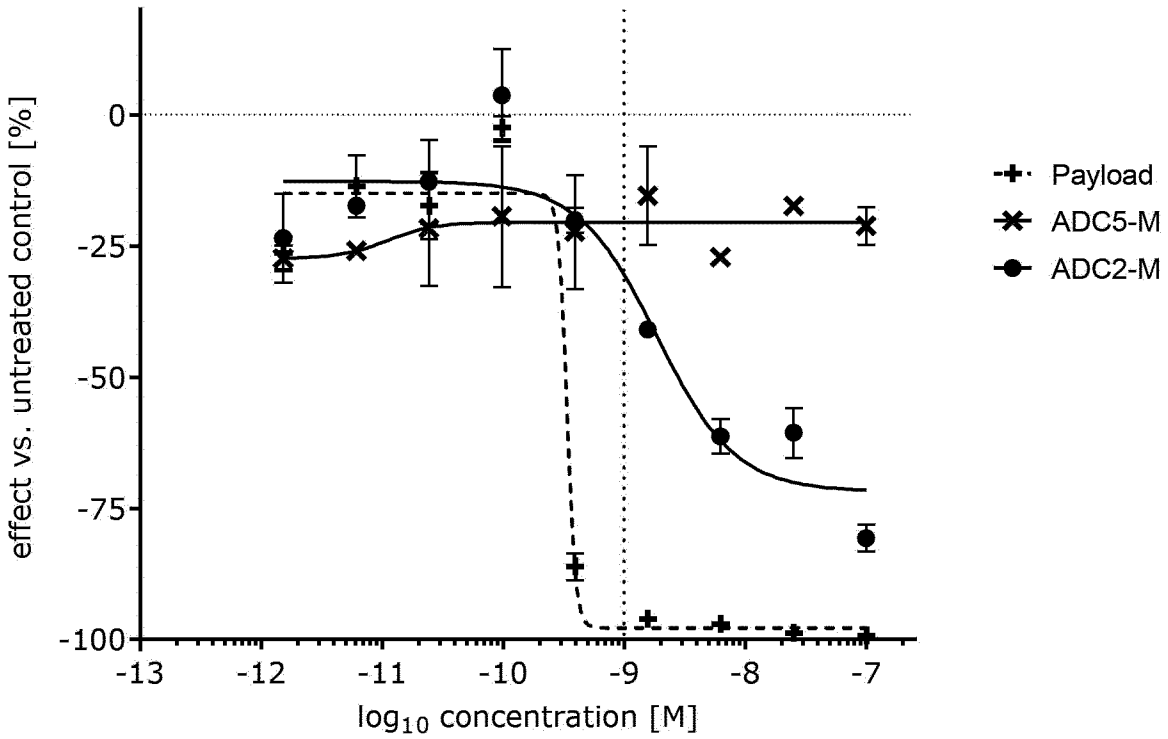

effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC4-M
ADC1-M

Fig. 41:

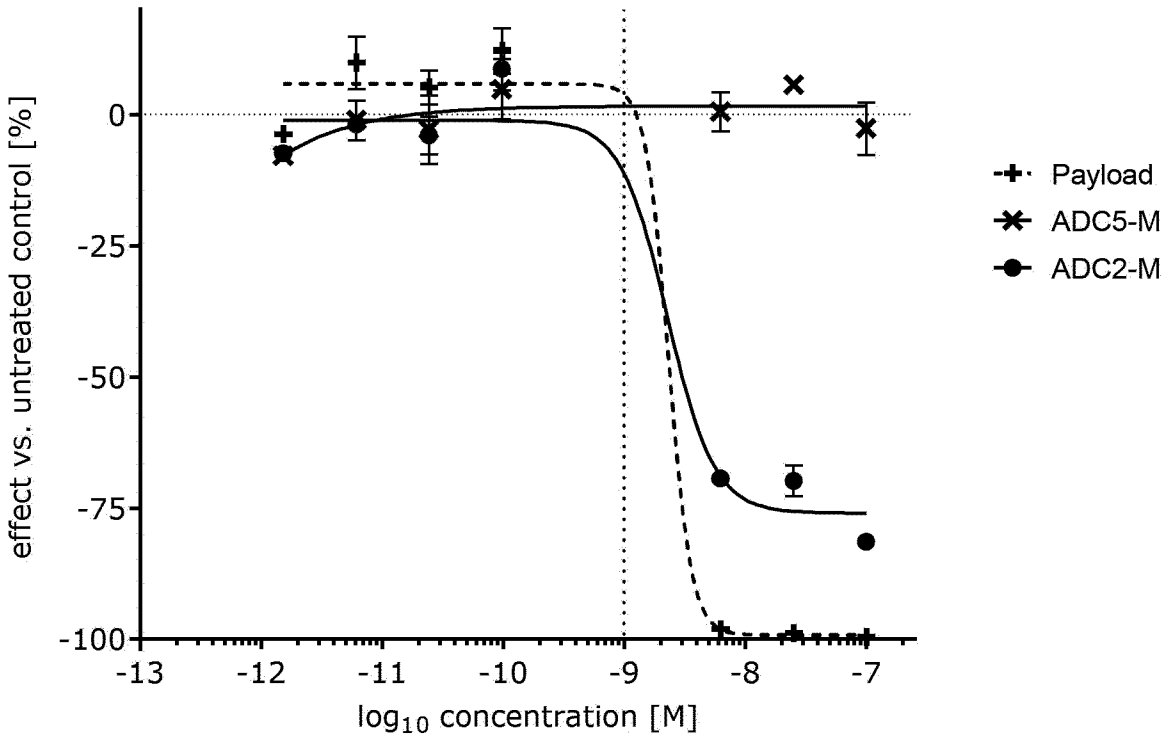
(a)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC5-M
ADC2-M

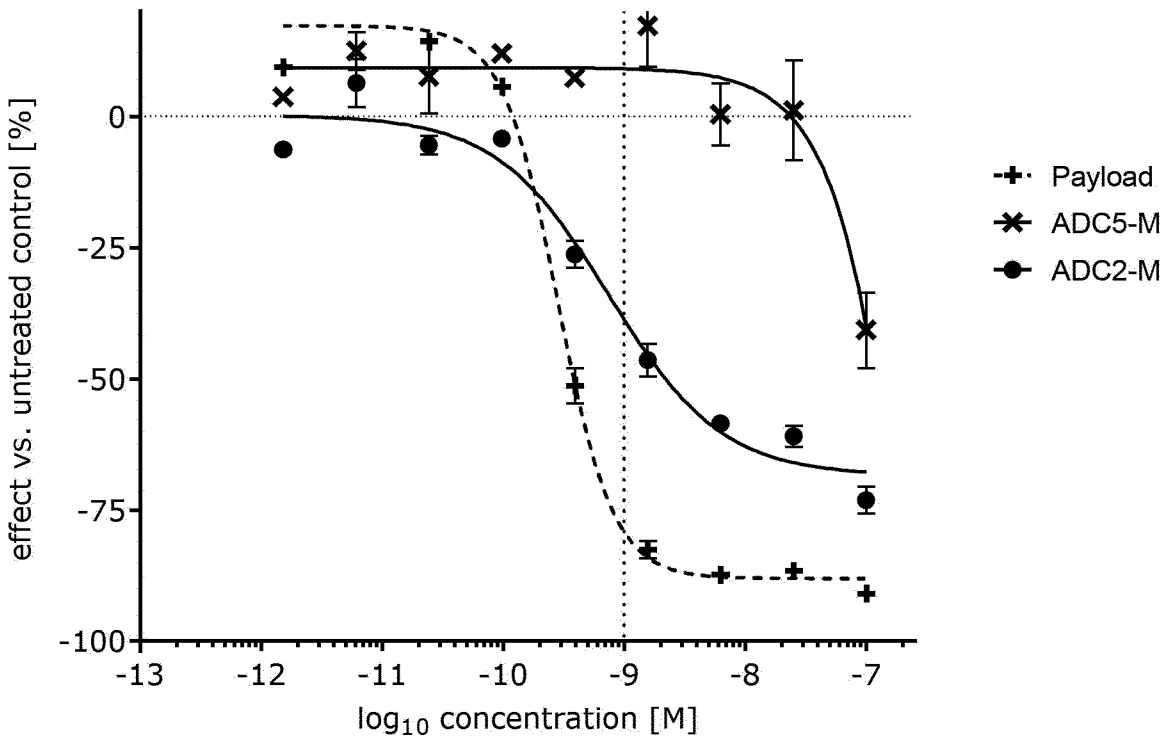
(b)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC5-M
ADC2-M

(c)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC5-M
ADC2-M

(d)


effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC5-M
ADC2-M

(e)

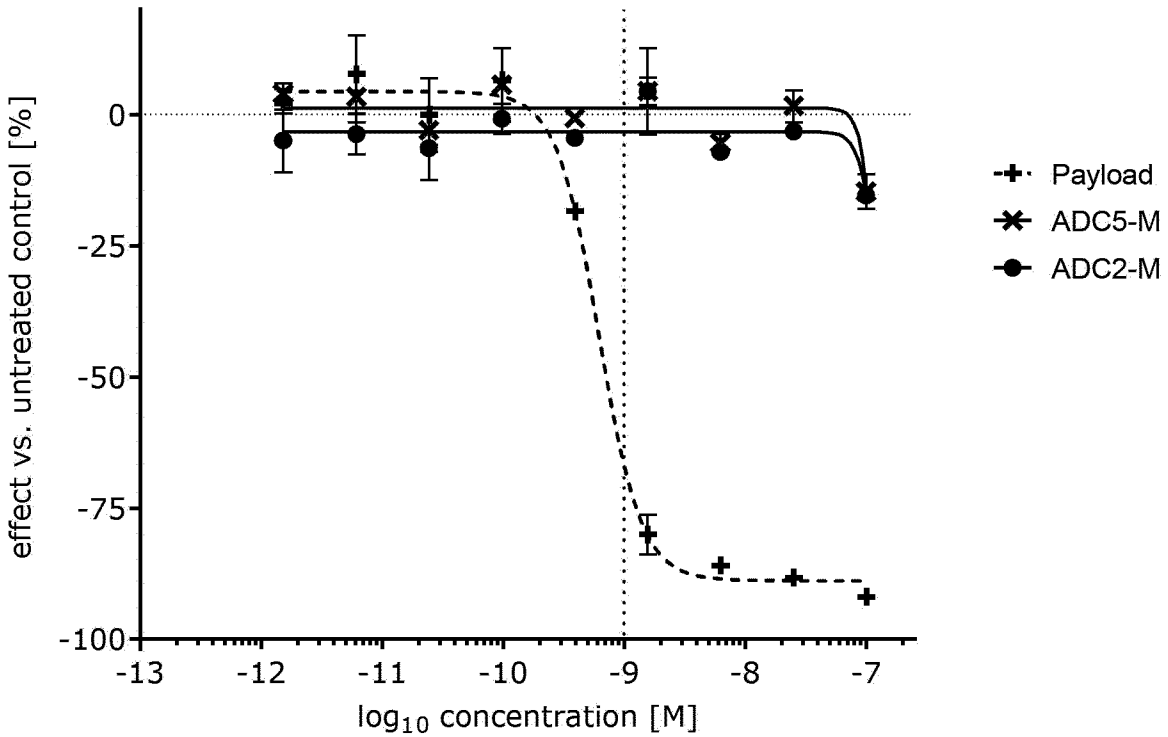

effect vs. untreated control [%]
0
-25
-50
-75
-100
-13
-12
-11
-10
-9
-8
-7
log10 concentration [M]
Payload
ADC5-M
ADC2-M

Fig. 42

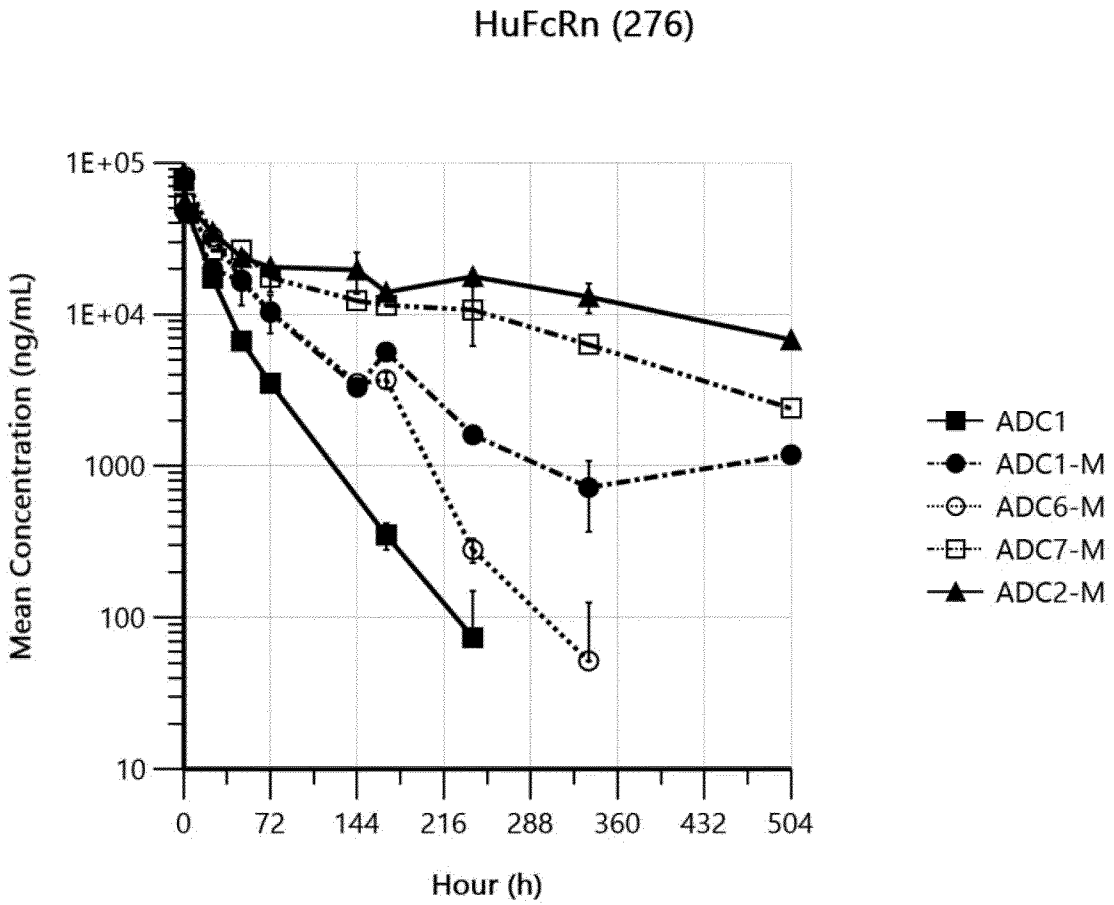

HuFcRn (276)
Mean Concentration (ng/mL)
1E+05
1E+04
1000
100
10
0
72
144
216
288
360
432
504
Hour (h)
ADC1
ADC1-M
ADC6-M
ADC7-M
ADC2-M

Fig. 43

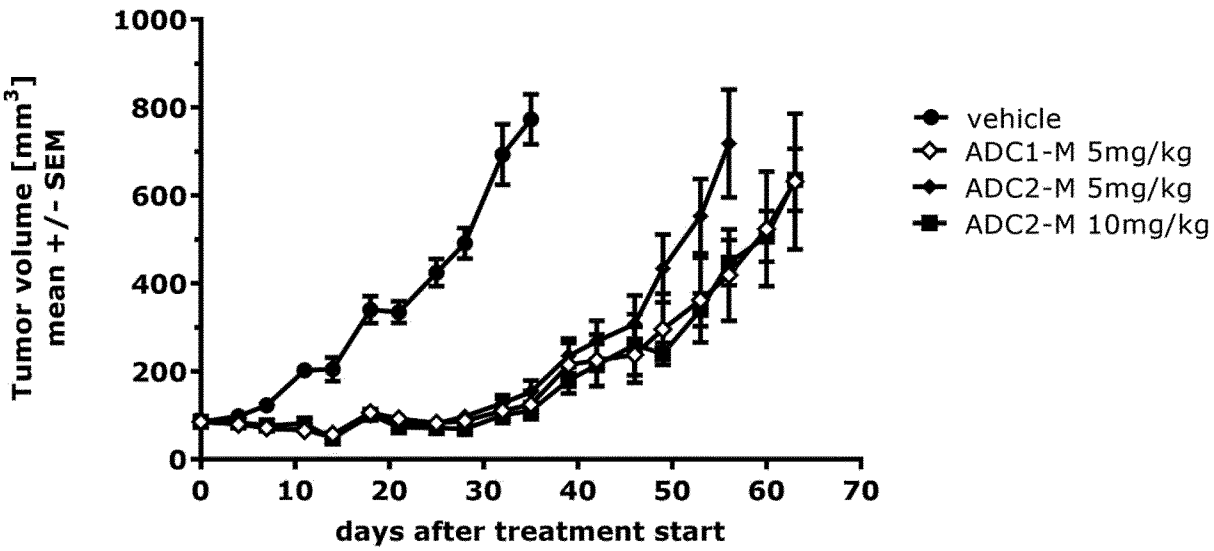

Tumor volume [mm3]
mean +/- SEM
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
days after treatment start
vehicle
ADC1-M 5mg/kg
ADC2-M 5mg/kg
ADC2-M 10mg/kg

Fig. 44

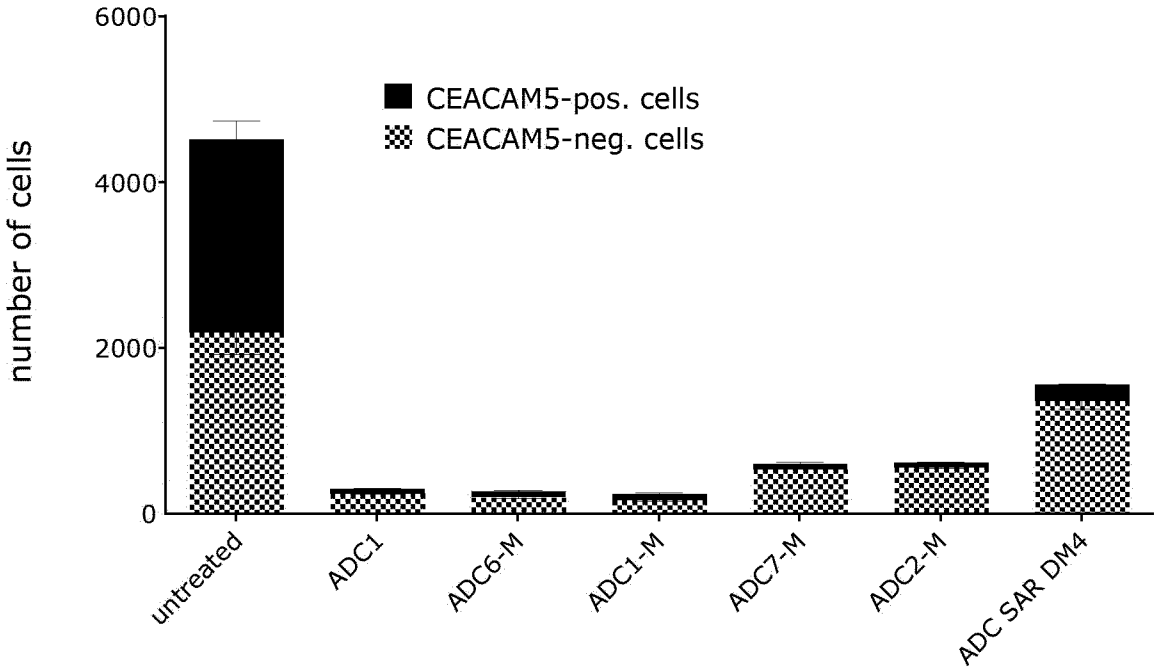

6000
4000
2000
0
number of cells
CEACAM5-pos. cells
CEACAM5-neg. cells
untreated
ADC1
ADC6-M
ADC1-M
ADC7-M
ADC2-M
ADC SAR DM4

Fig. 45

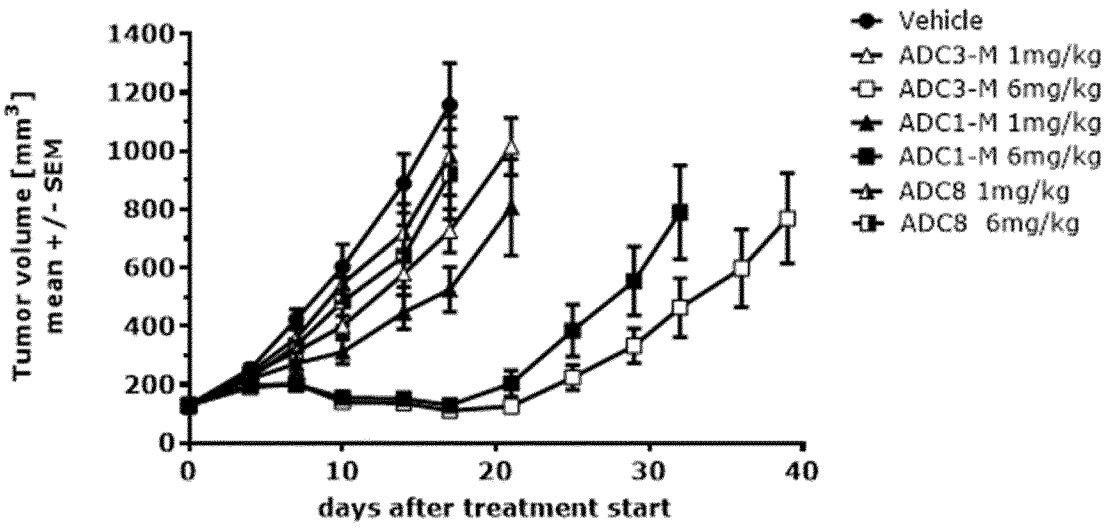

Tumor volume [mm³]
mean +/- SEM
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
days after treatment start
Vehicle
ADC3-M 1mg/kg
ADC3-M 6mg/kg
ADC1-M 1mg/kg
ADC1-M 6mg/kg
ADC8 1mg/kg
ADC8 6mg/kg

Fig. 46

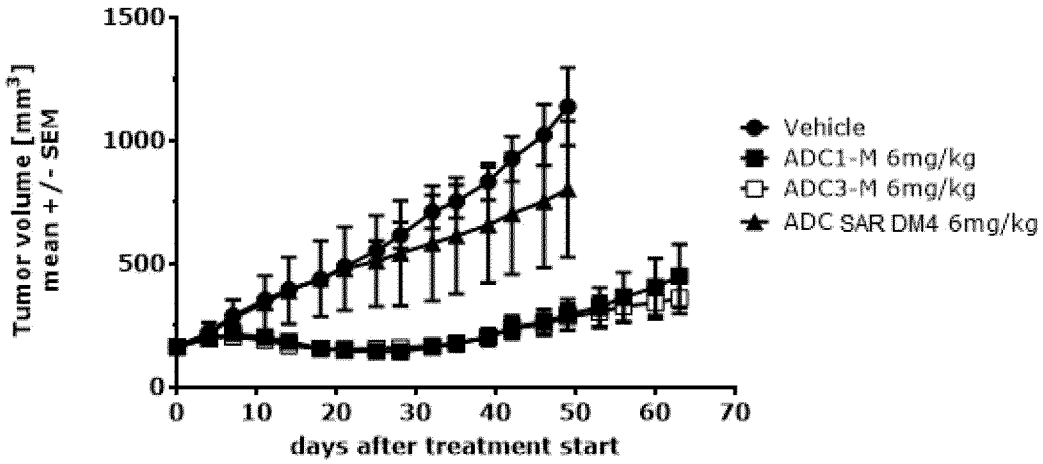
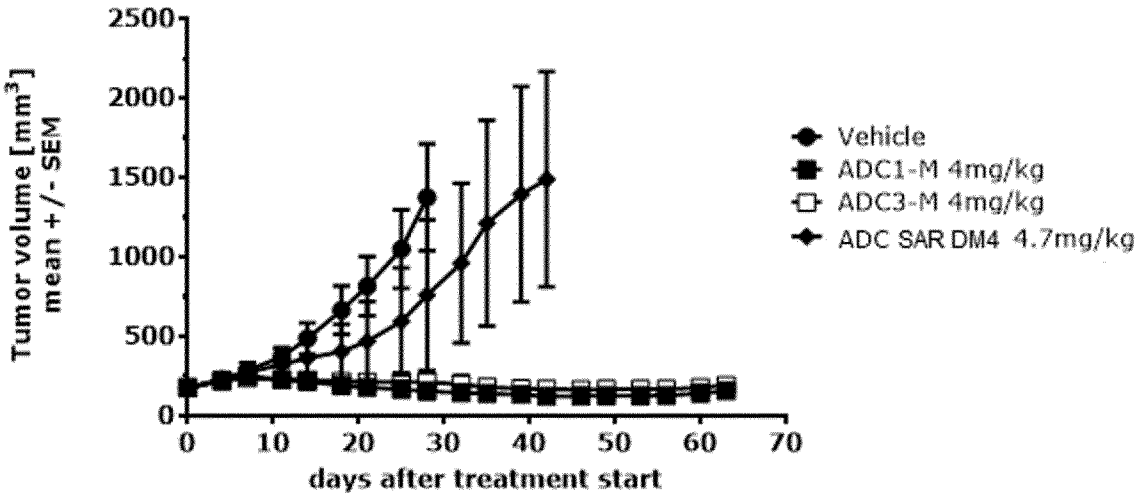

Tumor volume [mm³]
mean +/- SEM
1500
1000
500
0
0
10
20
30
40
50
60
70
days after treatment start
Vehicle
ADC1-M 6mg/kg
ADC3-M 6mg/kg
ADC SAR DM4 6mg/kg

Fig. 47


Tumor volume [mm³]
mean +/- SEM
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
days after treatment start
Vehicle
ADC1-M 4mg/kg
ADC3-M 4mg/kg
ADC SAR DM4 4.7mg/kg

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/056081

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/056081

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P35/00 C07K16/30
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61P C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2021/121204 A1 (JIANGSU HENGRUI MEDICINE CO [CN] ET AL.) 24 June 2021 (2021-06-24) Hu67-14-2-A; claim 20; example 7	1-45
A	WO 2014/079886 A1 (SANOFI SA [FR]) 30 May 2014 (2014-05-30) example all	1-45

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2023	09/06/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fellows, Edward

Form PCT/ISA/210 (second sheet) (April 2005)

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