

(19) **DANMARK**

(10) **DK/EP 3151865 T3**



(12) **Oversættelse af
europæisk patentskrift**

Patent- og
Varemærkestyrelsen

-
- (51) Int.Cl.: **A 61 K 47/68 (2017.01)**
- (45) Oversættelsen bekendtgjort den: **2021-10-25**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2021-09-01**
- (86) Europæisk ansøgning nr.: **15724638.0**
- (86) Europæisk indleveringsdag: **2015-05-22**
- (87) Den europæiske ansøgnings publiceringsdag: **2017-04-12**
- (86) International ansøgning nr.: **EP2015061456**
- (87) Internationalt publikationsnr.: **WO2015177360**
- (30) Prioritet: **2014-05-22 EP 14169493**
- (84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**
- (73) Patenthaver: **Byondis B.V., Microweg 22, 6545 CM Nijmegen, Holland**
- (72) Opfinder: **ARIAANS, Gerardus Joseph Andreas, Synthron Biopharmaceuticals B.V., Microweg 22, NL-6545 CM Nijmegen, Holland**
COUMANS, Rudy Gerardus Elisabeth, Synthron Biopharmaceuticals B.V., Microweg 22, NL-6545 CM Nijmegen, Holland
- (74) Fuldmægtig i Danmark: **Gotapatent AB, Box 3127, S-550 03 Jönköping, Sverige**
- (54) Benævnelse: **STEDSSPECIFIK KONJUGATION AF LINKER MEDICIN TIL ANTISTOF OG RESULTERENDE ADCS**
- (56) Fremdragne publikationer:
WO-A1-2013/093809
WO-A2-2006/034488
WO-A2-2007/038658
WO-A2-2011/133039
US-A1- 2004 120 958
JAGATH R JUNUTULA ET AL: "Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, UNITED STATES, vol. 26, no. 8, 1 August 2008 (2008-08-01) , pages 925-932, XP002727747, ISSN: 1546-1696, DOI: 10.1038/NBT.1480 [retrieved on 2008-07-20]
None

Description**FIELD OF THE INVENTION**

5 **[0001]** The present invention relates to antibody-drug conjugates (ADCs) wherein a linker drug is site-specifically conjugated to an antibody through an engineered cysteine, and their use in the treatment of human solid tumours and haematological malignancies, in particular breast cancer, gastric cancer, colorectal cancer, urothelial cancer, ovarian cancer, uterine cancer, lung cancer, mesothelioma, liver cancer, pancreatic cancer, prostate cancer, and leukaemia.

10 BACKGROUND OF THE PRESENT INVENTION

[0002] Antibody-drug conjugates (ADCs) are an emerging class of targeted therapeutics having an improved therapeutic index over traditional chemotherapy. Drugs and linkers have been the focus of ADC development, in addition to (monoclonal) antibody (mAb) and target selection. Recently, however, the importance of conjugate homogeneity was realized. The conventional methods for drug attachment to an antibody lead to a heterogeneous mixture, and some individual constituents of that mixture can have poor *in vivo* performance. Newer methods for site-specific drug attachment lead to more homogeneous conjugates and allow control of the site of drug attachment. These subtle improvements can have profound effects on *in vivo* efficacy and/or *in vivo* safety and thereby on the therapeutic index. Methods for site-specific drug conjugation to antibodies are comprehensively reviewed by C.R. Behrens and B. Liu in mAbs, Vol. 6, Issue 1, 2014, pages 1-8.

[0003] Conventional ADCs are typically produced by conjugating the linker drug to the antibody through the side chains of either surface-exposed lysines or free cysteines generated through reduction of interchain disulfide bonds. Because antibodies contain many lysine residues and cysteine disulfide bonds, conventional conjugation typically produces heterogeneous mixtures that present challenges with respect to analytical characterization and manufacturing. Furthermore, the individual constituents of these mixtures exhibit different physicochemical properties and pharmacology with respect to their pharmacokinetic, efficacy, and safety profiles, hindering a rational approach to optimizing this modality.

[0004] These two conventional techniques for chemical modification of antibodies were used to construct the two ADCs with current FDA marketing approvals. Brentuximab vedotin (Adcetris™, Seattle Genetics) consists of an anti-CD30 monoclonal antibody conjugated to the highly cytotoxic drug monomethyl auristatin E (MMAE) via modification of native cysteine side chain thiols. The manufacture involves partial reduction of the solvent-exposed interchain disulfides followed by modification of the resulting thiols with maleimide-containing linker drugs. For brentuximab vedotin, the thiols were modified with mc-vc-PAB-MMAE, which incorporates a cathepsin B protease cleavage site (vc, valine-citrulline) and a self-immolative linker (PAB, para-aminobenzyloxycarbonyl) between the maleimide group (mc, maleimidocaproyl) and the cytotoxic drug (MMAE). The cysteine attachment strategy results in maximally two drugs per reduced disulfide. Most human IgG molecules have four solvent-exposed disulfide bonds, and so a range of from zero to eight drugs per antibody is possible. The exact number of drugs per antibody is determined by the extent of disulfide reduction and the number of molar equivalents of linker drug used in the ensuing conjugation reaction. Full reduction of all four disulfide bonds gives a homogeneous construct with eight drugs per antibody, while a partial reduction typically results in a heterogeneous mixture with zero, two, four, six, or eight drugs per antibody. Brentuximab vedotin has an average of about 4 drugs per antibody.

[0005] The other ADC with current FDA approval is ado-trastuzumab emtansine (T-DM1, Kadcyla™, Roche / Genentech), which was constructed by coupling the anti-HER2 monoclonal antibody trastuzumab to the cytotoxic drug maytansine through modification of lysine side chain amines. This version of maytansine (DM1) was modified to include a thiol that could be attached to a maleimide linker. A bifunctional linker (SMCC, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) with a maleimide at one end and an N-hydroxysuccinimidyl (NHS) ester at the other end was reacted with lysine primary amine side chains to form a stable amide bond. The modified maytansine (DM1) was then attached to the antibody through conjugation to the maleimide end of the bifunctional linker. In contrast to the linker utilized in brentuximab vedotin, this linker has no (protease) cleavage site and thus requires lysosomal degradation of the antibody part of the ADC to liberate the active DM1-linker-lysine metabolite. The attachment method resulted in a heterogeneous mixture of conjugates with an average of 3.5 drugs per antibody. Compared with the cysteine method described above, this strategy gave a more heterogeneous mixture because 20 to 40 lysine residues were found to be modified, whereas only maximally 8 different cysteine residues are modified using the native cysteine modification method.

[0006] Recently, it was reported that the pharmacological profile of ADCs may be improved by applying site-specific conjugation technologies that make use of surface-exposed cysteine residues engineered into antibodies that are then conjugated to a linker drug, resulting in site-specifically conjugated ADCs with defined drug-to-antibody ratios (DARs). Relative to the heterogeneous mixtures created using conventional lysine and cysteine conjugation methodologies, site-specifically conjugated ADCs have generally demonstrated at least equivalent *in vivo* potency, improved PK, and an expanded therapeutic window.

[0007] The first site-specific conjugation approach was developed at Genentech by introducing a cysteine residue using site-directed mutagenesis at positions showing high thiol reactivity as elaborated in WO2006/034488. This common practice in protein modification was more complicated in an antibody because of the various native cysteine residues already present. Introducing the extra cysteine residue in an unsuitable position could result in improper formation of interchain disulfide bonds and therefore improper folding of the antibody. Engineered cysteine residues in suitable positions in the mutated antibody are often capped by other thiols, such as cysteine or glutathione, to form disulfides.

[0008] Drug attachment to the mutant residues was achieved by reducing both the native interchain and mutant disulfides, then re-oxidizing the native interchain cysteines using a mild oxidant such as CuSO₄ or dehydroascorbic acid, followed by standard conjugation of the liberated mutant cysteine with a linker drug. Under optimal conditions, two drugs per antibody will be attached (if one cysteine is engineered into the heavy chain or light chain of the mAb). The engineered cysteine method proved to be suitable for developing the site-specific ADC SGN-CD33A (Seattle Genetics), which recently entered a Phase I dose-escalation clinical study as a treatment for acute myeloid leukaemia (AML), as well as a Phase Ib clinical trial in combination with standard of care chemotherapy, including cytarabine and daunorubicin. This ADC comprises a cleavable dipeptide linker (i.e., valine-alanine) and a DNA-cross-linking, pyrrolobenzodiazepine (PBD) dimer as the drug linked to heavy chain position S239C in the Fc part of IgG1 mAb h2H12 (DAR 1.9; Sutherland et al. Blood 2013; 122(8):1455-1463).

[0009] Whereas in WO2006/034488 specifically surface accessible valine, alanine and serine residues not involved in antigen binding interactions and distant from the existing interchain disulfide bonds were substituted to obtain engineered cysteine residues with high thiol reactivity, WO2014/124316 from Novartis specifically focuses on the identification of surface accessible sites in the constant regions of the antibody heavy and light chains, at which sites substitution for a cysteine residue enables efficient conjugation of payloads and provides conjugates with high stability.

[0010] In addition to the engineered cysteine conjugation strategy, other methods for site-specific attachment of drugs have been developed. Pfizer demonstrated a new technique for conjugation using microbial transglutaminase to couple an amine-containing drug to an engineered glutamine on the antibody. Transglutaminase is an enzyme that catalyzes amide bond formation between the acyl group of a glutamine side chain and the primary amine of a lysine side chain.

[0011] In addition to enzymatic conjugation, orthogonal chemistry conjugation has also been used to site-specifically modify a wide variety of proteins using non-natural amino acids (notably technologies from Ambrx and Sutro Biopharma). In particular, p-acetylphenyl-alanine and p-azidomethyl-L-phenylalanine were chosen as the non-natural amino acids, because they, respectively, contain a ketone and an azide functional group that is not found in any of the 20 natural amino acid side chains. This allows for specific modification of the ketone or azide groups without interference from other amino acids. This method provided an additional route for constructing ADCs with a maximum of two drugs per antibody (per one such non-natural amino acid).

[0012] In all of the prior art methods disclosed thus far, the emphasis was put on site-specifically conjugating linker drugs at surface/solvent-exposed positions, at positions showing high thiol reactivity, and at positions in specifically the constant regions of monoclonal antibodies, with the aim of improving homogeneity and pharmacokinetic properties. Even though the above-described conventional lysine and cysteine conjugation methods have led to FDA-approved antibody-drug conjugates and they are being used for constructing most of a large number of ADCs currently in preclinical and clinical trials, there is still a need for new conjugation strategies with the aim to (further) improve the physicochemical, pharmacokinetic, pharmacological, and/or toxicological properties of ADCs to obtain ADCs having acceptable antigen binding properties, *in vivo* efficacy, therapeutic index, and/or stability.

BRIEF DESCRIPTION OF THE PRESENT INVENTION

[0013] The scope of the invention is defined by the claims. The present invention relates to antibody-drug conjugates (ADCs) wherein a duocarmycin derivative linker drug is site-specifically conjugated to an IgG1 antibody through an engineered cysteine at heavy chain position 41 of said antibody, and their use in the treatment of human solid tumours and haematological malignancies, in particular breast cancer, gastric cancer, colorectal cancer, urothelial cancer, ovarian cancer, uterine cancer, lung cancer, mesothelioma, liver cancer, pancreatic cancer, prostate cancer, and leukaemia.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

Figure 1A. Identification of suitable linker drug conjugation positions in the Fab part of an antibody

Figure 1B. Docking of duocarmycin linker drug vc-seco-DUBA in the Fab cavity of an antibody (overlay of multiple vc-seco-DUBA dockings)

Figure 1C. Identification of suitable linker drug conjugation positions in the Fc part of an antibody

Figure 1D. Docking of duocarmycin linker drug vc-seco-DUBA in the Fc cavity of an antibody (overlay of multiple vc-

seco-DUBA dockings)

Figure 2A. *In vivo* efficacy of engineered cysteine anti-PSMA (VH S41C) ADC (SYD1091) *versus* vehicle control, comparator engineered cysteine anti-PSMA (CH T120C) ADC (SYD1035), and non-engineered anti-PSMA (wild-type) wt ADC (SYD998) at 2 mg/kg each

Figure 2B. *In vivo* efficacy of engineered cysteine anti-PSMA (VH S41C) ADC (SYD1091) *versus* vehicle control, comparator engineered cysteine anti-PSMA (CH T120C) ADC (SYD1035), and non-engineered anti-PSMA wt ADC (SYD998) at 10 mg/kg each

Figure 3. Effect on body weight of engineered cysteine anti-PSMA (VH S41C) ADC (SYD1091) *versus* vehicle control, comparator engineered cysteine anti-PSMA (CH T120C) ADC (SYD1035), and non-engineered anti-PSMA wt ADC (SYD998) at 10 mg/kg each

Figure 4A. *In vivo* efficacy of engineered cysteine anti-5T4 (VH P41C) H8 ADC (H8-41C-vc-*seco*-DUBA) *versus* vehicle control, and non-engineered anti-5T4 wt H8 ADC (H8-vc-*seco*-DUBA) at 3 mg/kg each

Figure 4B. *In vivo* efficacy of engineered cysteine anti-5T4 (VH P41C) H8 ADC (H8-41C-vc-*seco*-DUBA) *versus* vehicle control, and non-engineered anti-5T4 wt H8 ADC (H8-vc-*seco*-DUBA) at 10 mg/kg each

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0015] Antibody-drug conjugates (ADCs) are emerging as a new class of anticancer therapeutics that combine the efficacy of small-molecule therapeutics with the targeting ability of antibodies. By combining these two components into a single new molecular entity, highly cytotoxic small molecule drugs can be delivered to cancerous target tissues, thereby enhancing efficacy while reducing the potential systemic toxic side effects of the small molecule.

[0016] Antibodies have been conjugated to a variety of cytotoxic drugs, including small molecules that bind DNA (e.g. anthracyclines), alkylate or crosslink DNA (e.g. duocarmycins or pyrrolobenzodiazepine dimers, respectively), cause DNA strand breaks (e.g. calicheamicins) or disrupt microtubules (e.g. maytansinoids and auristatins).

[0017] The application relates to an antibody-drug conjugate (ADC) compound wherein a linker drug is site-specifically conjugated to an antibody through an engineered cysteine at one or more positions of said antibody selected from heavy chain 40, 41, 89 (Kabat numbering), 152, 153, 155, 171, 247, 297, 339, 375 and 376 (Eu numbering), and light chain 40, 41 (Kabat numbering), 165 and 168 (Eu numbering).

[0018] As the focus in earlier work on site-specific ADCs was on finding conjugation positions that show good reactivity with the linker drug, and at the same time have a low risk of forming disulfide bonds between antibodies (leading to aggregation) or disturbing the antibody structure (so-called disulfide bridge shuffling), the effects on hydrophobicity of the conjugates in relation to the conjugation site have not been evaluated. In addition, the focus has primarily been on finding suitable sites in the constant regions of the antibody, as modification of the variable regions of an antibody is generally thought to be associated with a high risk of partial or complete loss of antigen binding.

[0019] The current inventors, however, have focused on influencing the hydrophobicity characteristics of site-specific ADCs.

[0020] An *in silico* method, employing the YASARA software package (www.yasara.org, see: Krieger et al. *Proteins* 2009; 77 Suppl 9: 114-122), was used to identify sites of strong interaction of the linker drug with the antibody. Suitable locations show a minimal increase in the hydrophobic surface. In the vicinity of the thus-identified interaction sites suitable residues (i.e., with sufficient accessibility) to convert to cysteines were identified. In this approach no limitation was made to the constant regions of the antibody, also the variable region amino acids were considered if not in the vicinity of antigen binding sites. Locations in the variable domain of the Fab part turned out to be preferable.

[0021] Docking of linker drugs into the Fab and Fc models of various antibodies was simulated with the commonly used VINA algorithm (Trott O and Olson AJ. *J. Comput. Chem.* 2010; 31: 455-461) as implemented in YASARA. The antibody Fab and Fc models used were obtained from X-ray structures or by homology modeling using YASARA.

[0022] The duocarmycin type linker drugs, e.g. vc-*seco*-DUBA (i.e., SYD980; an ADC compound thereof is depicted in formula II), were shown to have a strong preference for binding in cavities which are present in all antibody structures (see Figures 1B and 1D for the Fab and the Fc part of an antibody, respectively). Multiple suitable conjugation positions for linker drug attachment were identified in and in close proximity to these cavities, i.e., with good accessibility of engineered cysteines at these locations (see Figures 1A and 1C for the Fab and the Fc part of an antibody, respectively).

[0023] In the context of the present invention, Kabat numbering is used for indicating the amino acid positions of engineered cysteines in the heavy chain (HC) and light chain (LC) variable regions and Eu numbering is used for indicating the positions in the heavy chain and light chain constant regions of the antibody. In view of the sequence variability in the variable regions of antibodies, the exact amino acid to be substituted by cysteine can be different for different antibodies. For most antibodies, in particular IgG antibodies, in the heavy chain of the variable region (VH), there usually is an A or S at position 40, a P at position 41 and a V at position 89 and in the light chain of the variable region (VL), there usually is a P at position 40 and a G at position 41. In the heavy chain of the constant regions (CH1, CH2 and CH3), there is normally an E at position 152, a P at position 153, a T at position 155, a P at position 171, a P

at position 247, an N at position 297, an A at position 339, an S at position 375 and a D at position 376, and in the light chain of the κ constant region (CL), there is normally an E at position 165 and an S at position 168. In the five λ light chain isotype constant regions (CL), there is normally an S at position 165 and an S at position 168.

[0024] The expression "Kabat numbering" refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a framework region (FR) or complementary determining region (CDR) of the variable domain. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

[0025] The expression "Eu numbering" refers to the Eu index as in Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD., NIH publication no. 91-3242, pp. 662, 680, 689 (1991). The "Eu index as in Kabat" refers to the residue numbering of the human IgG1 Eu antibody (Edelman, G.M. et al., Proc. Natl. Acad. Sci. USA, 63, 78-85 (1969)).

[0026] Heavy chain positions 40, 41 and 89 are located in the variable region and positions 152, 153, 155, 171, 247, 297, 339, 375 and 376 are located in the constant region of the antibody. Light chain positions 40 and 41 are located in the variable region and positions 165 and 168 are located in the constant region of the antibody.

[0027] Heavy chain positions 40, 41, 89, 152, 153, 155 and 171 and light chain positions 40, 41, 165 and 168 are located in the Fab part and heavy chain positions 247, 297, 339, 375 and 376 are located in the Fc part of the antibody.

[0028] In accordance with the present invention, the term "engineered cysteine" means replacing a non-cysteine amino acid in the heavy chain or light chain of an antibody by a cysteine. As is known by the person skilled in the art, this can be done either at the amino acid level or at the DNA level, e.g. by using site-directed mutagenesis.

[0029] The present inventors surprisingly have found that the site-specifically conjugated ADC compounds of the present invention show improved physicochemical, pharmacological and/or pharmacokinetic properties, as compared to ADCs wherein the linker drug is conjugated through native interchain disulfide bonds of the antibody and, moreover, as compared to engineered cysteine ADCs wherein the linker drug is conjugated at positions disclosed in the prior art from the ones specifically disclosed or claimed in this patent application. The ADC compounds in accordance with the present invention have binding properties similar to the naked antibodies, good *in vivo* efficacy, an increased therapeutic index and/or improved stability. Notably, it was found that the ADC compounds are generally less hydrophobic and less susceptible to cathepsin B cleavage and therefore likely also to other intra- or extracellular enzymes/proteases in the tumour mass (tumour microenvironment) than ADCs that are site-specifically conjugated at different positions, but still show similar *in vitro* cytotoxicity. Unexpectedly, ADCs in accordance with the present invention show improved *in vivo* efficacy in a tumour xenograft animal model as compared to ADCs that are site-specifically conjugated at other positions.

[0030] Without wishing to be bound by any theory, the present inventors have found that when linker drugs are conjugated at the specific positions of the antibody as disclosed herein, said linker drug fits into either the Fab cavity that is formed by the CH1, VH, VL and CL domains of the antibody or the Fc cavity that is formed by the two CH2 and two CH3 domains of the antibody. In an IgG1 antibody the top of the Fc cavity is formed by the glycoside/carbohydrate that is attached to the heavy chain position N297. As a result, the linker drug (which typically is more hydrophobic than the antibody) is shielded from the hydrophilic aqueous environment surrounding the antibody and the ADC as such is less hydrophobic as compared to ADCs wherein the linker drug is conjugated through native disulfide bonds of the antibody and is much less hydrophobic as compared to ADCs wherein the linker drug is site-specifically conjugated at different positions that are not presently disclosed or claimed and where the linker drug is forced to the outside of the antibody, i.e., is pointed in a direction away from the antibody.

[0031] Modification of the variable part of an antibody is generally avoided as it can lead to partial or complete loss of antigen binding properties. However, contrary to the general expectations, it was found that specific residues in the framework regions of the heavy and light chains of the antibody are both suitable for conjugation and do not lead to (significant) reduction of antigen binding after conjugation of the linker drug. Therefore, the application relates to an antibody-drug conjugate (ADC) compound wherein said engineered cysteine is at one or more positions of said antibody selected from heavy chain 40, 41 and 89 and light chain 40 and 41 in the Fab part of said antibody. Preferably, said engineered cysteine is at heavy chain position 40 or 41 and/or light chain position 40 or 41, more preferably at heavy chain position 41 and/or light chain position 40 or 41. In accordance with the present invention, said engineered cysteine is at heavy chain position 41. As it is known from the literature that tumour-associated proteases in the tumour microenvironment can partially cleave the Fc constant domains, under the hinge region, conjugation in the Fab part is preferred over conjugation in the Fc part. Cleavage of the Fc constant domains would result in loss of Fc-conjugated linker drugs, which in turn could lead to a decreased activity of the ADC *in vivo*. (Fan et al. Breast Cancer Res. 2012; 14: R116 and Brezsky et al. PNAS 2009; 106: 17864-17869). Moreover, conjugation to these positions in the Fab part also enables the use of antigen binding fragments.

[0032] In a specific embodiment, the antibody-drug conjugate (ADC) compound may further comprise an additional

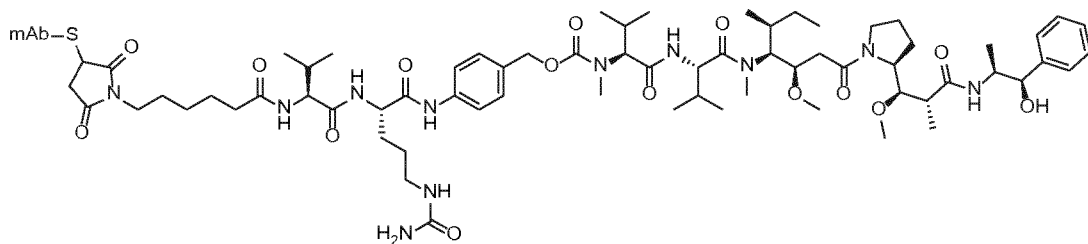
engineered cysteine at one or more positions of the antibody selected from heavy chain 152, 153, 155, 171, 339 and 375, and light chain 165 and 168. Preferably said further engineered cysteine is at heavy chain position 375 in the Fc part of said antibody.

[0033] In accordance with the present invention, the one or more cysteine residues can be engineered into the antibody by using conventional molecular cloning techniques or the heavy chain or light chain domain(s) of the antibody carrying the cysteine mutation(s) can be synthesized as such using known (peptide or DNA) synthesis equipment and procedures.

[0034] Any linker drug known in the art of ADCs can be used for site-specific conjugation to an antibody, provided it has a chemical group which can react with the thiol group of an engineered cysteine, typically a maleimide or haloacetyl group. Suitable linker drugs may comprise a duocarmycin, calicheamicin, pyrrolobenzodiazepine (PBD) dimer, maytansinoid or auristatin derivative as a cytotoxic drug. Either a cleavable or a non-cleavable linker may be used. Suitable examples of maytansinoid drugs include DM1 and DM4. Suitable examples of auristatin drugs include MMAE and MMAF.

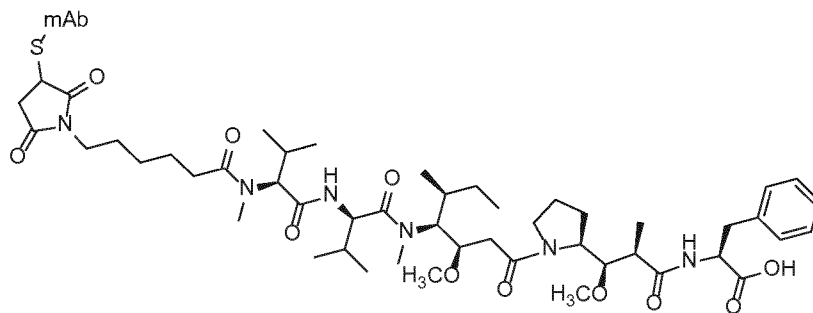
[0035] These abbreviations are well-known to the skilled artisan. Examples of suitable linker drugs known to the person skilled in the art include mc-vc-PAB-MMAE (also abbreviated as mc-vc-MMAE and vc-MMAE), mc-MMAF, and mc-vc-MMAF. Preferably, the linker used is a cleavable linker, such as valine-citrulline (vc) or valine-alanine (va).

[0036] The generic molecular structures of a vc-MMAE ADC and mc-MMAF ADC are depicted below.



Molecular structure of vc-MMAE linked to a mAb

[0037]



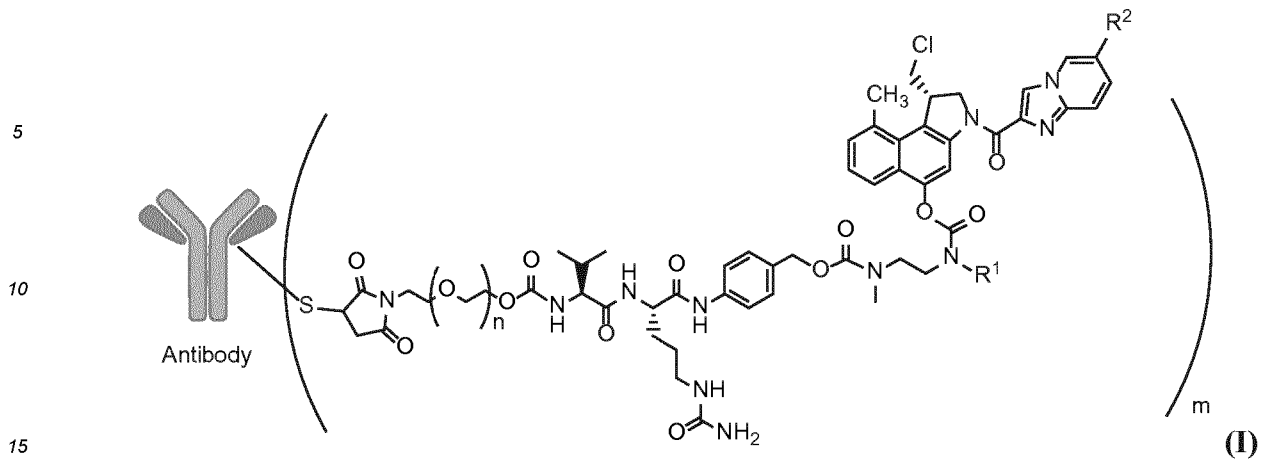
Molecular structure of mc-MMAF linked to a mAb

[0038] In accordance with the present invention the linker drug in the ADC compound of the invention comprises a duocarmycin derivative.

[0039] Duocarmycins, first isolated from a culture broth of *Streptomyces* species, are members of a family of antitumour antibiotics that include duocarmycin A, duocarmycin SA, and CC-1065. These extremely potent agents allegedly derive their biological activity from an ability to sequence-selectively alkylate DNA at the N3 position of adenine in the minor groove, which initiates a cascade of events that terminates in an apoptotic cell death mechanism.

[0040] WO2011/133039 discloses a series of linker drugs comprising a duocarmycin derivative of CC-1065. Suitable linker-duocarmycin derivatives to be used in accordance with the present invention are disclosed on pages 182-197. The chemical synthesis of a number of these linker drugs is described in Examples 1-12 of WO2011/133039.

[0041] In one embodiment, the present invention relates to a compound of formula (I)

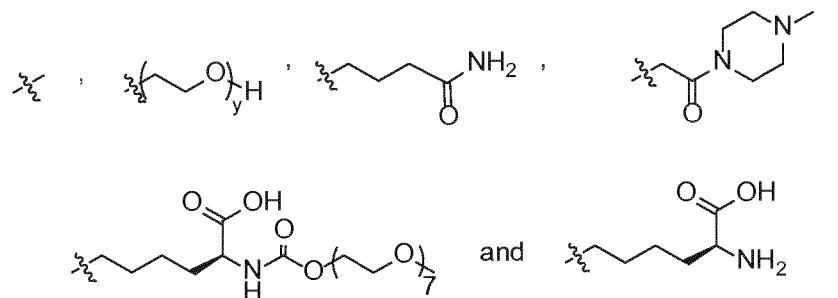


wherein

n is 0-3, preferably 0-1,

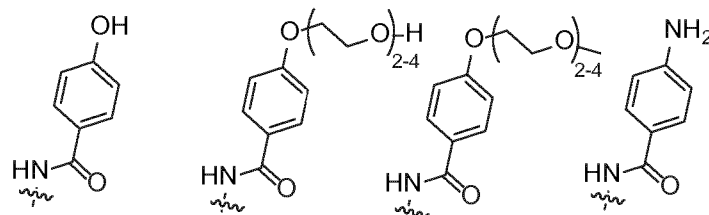
m represents an average DAR of from 1 to 6, preferably of from 1 to 4,

R¹ is selected from



y is 1-16, and

R² is selected from



[0042] In the structural formulae shown in the present specification, n represents an integer from 0 to 3, while m represents an average drug-to-antibody ratio (DAR) of from 1 to 6. As is well-known in the art, the DAR and drug load distribution can be determined, for example, by using hydrophobic interaction chromatography (HIC) or reversed phase high-performance liquid chromatography (RP-HPLC). HIC is particularly suitable for determining the average DAR.

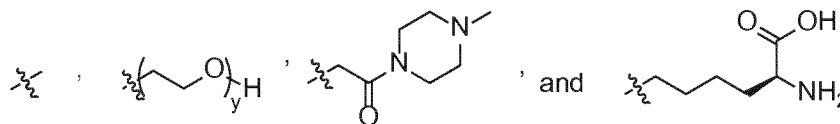
[0043] Compounds of the formula (I) in accordance with the present invention can be obtained according to methods and procedures that are well known to a person skilled in the art. Suitable methods for site-specifically conjugating linker drugs can for example be found in Examples 7 and 8 of WO2005/084390, which describe complete reduction strategies for (partial) loading of antibodies with the linker drug vc-MMAE, in Examples 11 and 12 of WO2006/034488, which describe site-specific conjugation of a maytansine (DM1)-comprising linker drug, and in Doronina et al. Bioconjugate Chem. 17 (2006): 114-124, which describes the conjugation with mc-MMAF.

[0044] Conjugation to two or more of the engineered cysteine sites of the present invention allows for the preparation of ADCs comprising hydrophobic drug classes with a higher DAR, notably DAR 4, without getting too much aggregate.

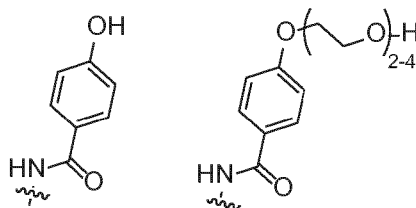
[0045] In accordance with the application, one or two engineered cysteines can be incorporated into the heavy chain

and/or light chain of the antibody, under optimal reaction conditions resulting in an ADC compound having a DAR of 2 or 4, respectively. When one engineered cysteine is introduced, it can be located either in the Fab or in the Fc part of the antibody. It is preferred to introduce said cysteine in the Fab part of the antibody at position HC 40, 41, 89, 152 or 153 or LC 40, 41 or 165, preferably HC 40, 41 or 89 or LC 40, 41 or 165, more preferably HC 40 or 41 or LC 40 or 41, even more preferably HC 41 or LC 40 or 41, most preferably HC 41. When two engineered cysteines are introduced, these two cysteines can both be located in the Fab or in the Fc part of the antibody or, preferably, one can be in the Fab part, preferably HC 40, 41, 152 or 153 or LC 40, 41 or 165, more preferably HC 40 or 41 or LC 40 or 41, even more preferably HC 41 or LC 40 or 41, most preferably HC 41, and the other can be in the Fc part of the antibody, preferably HC 247, 297, 339 or 375, more preferably HC 339 or 375, most preferably HC 375. When two engineered cysteines are introduced in the Fab part of the antibody, one cysteine residue may be introduced in the heavy chain and the other cysteine is introduced in the light chain of the antibody, e.g. HC 40 or 41 and LC 40 or 41. In addition, when two engineered cysteines are introduced in the Fab part of the antibody, one cysteine residue may be introduced at one of the specific positions as identified in the present invention, e.g. HC 40 or 41 or LC 40 or 41, and the other may be located at a surface-exposed (i.e., not herein claimed) engineered cysteine position leading to a higher DAR and still acceptable hydrophobicity.

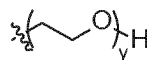
[0046] In a particular embodiment, the present invention relates to a compound of the formula (I) as disclosed hereinabove, wherein n is 0-1, m represents an average DAR of from 1 to 6, preferably of from 1 to 4, more preferably of from 1 to 2, most preferably of from 1.5 to 2, R¹ is selected from



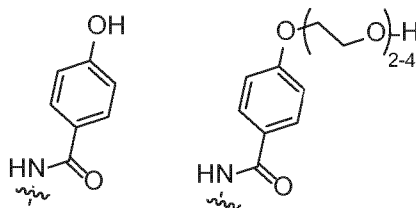
y is 1-16, preferably 1-4, and R² is selected from



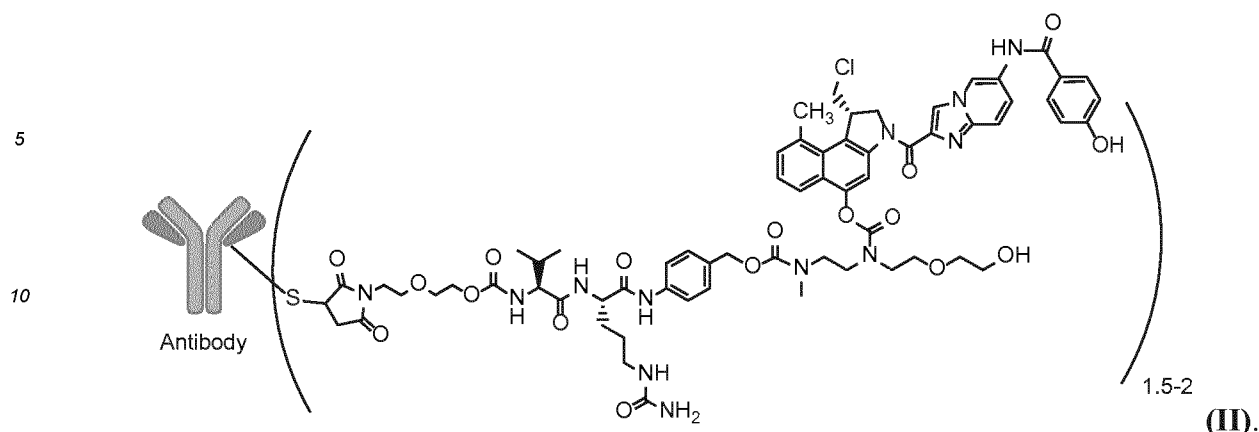
[0047] In a specific embodiment, the present invention relates to a compound of the structural formula (I) as disclosed hereinabove, wherein n is 0-1, m represents an average DAR of from 1.5 to 2, R¹ is



y is 1-4, and R² is selected from



[0048] In a particularly preferred embodiment, the present invention relates to a compound of formula (II)



[0049] Any antibody - particularly any antibody known to have therapeutic activity or any antibody known in the art of ADCs - or any antigen binding fragment thereof can be used for site-specific conjugation of a linker drug at the specific antibody positions claimed herein. Said antibody can be an IgA, IgD, IgE, IgG or IgM antibody. Said antibody can have κ (kappa) light chains or λ (lambda) light chains. Said IgG antibody can be an IgG1, IgG2, IgG3 or IgG4 antibody. Preferably, the antibody binds to a(n) antigen target that is expressed in or on the cell membrane (e.g., on the cell surface) of a tumour cell, more preferably, the antibody is internalised by the cell after binding to the (antigen) target, after which the toxin is released intracellularly. Preferably, the antibody is an IgG antibody. In accordance with the present invention, the antibody is an IgG1 antibody, most preferably an IgG1 antibody having κ light chains. Preferably, the IgG1 antibody carries a native glycoside/carbohydrate moiety attached at N297 of the heavy chain of the antibody.

[0050] Suitable antibodies include an anti-annexin A1 antibody, an anti-CD 19 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD37 antibody, an anti-CD38 antibody, an anti-CD44 antibody, an anti-CD47 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CD74 antibody, an anti-CD79 antibody, an anti-CD115 antibody, an anti-CD 123 antibody, an anti-CD 138 antibody, an anti-CD203c antibody, an anti-CD303 antibody, an anti-CEACAM antibody, an anti-CLL-1 antibody, an anti-c-MET (or anti-HGFR) antibody, an anti-Cripto antibody, an anti-DLL3 antibody, an anti-EGFR antibody, an anti-EPCAM antibody, an anti-EphA2 antibody, an anti-EphB3 antibody, an anti-ETBR antibody, an anti-FcRL5 antibody, an anti-FOLR1 antibody, an anti-GCC antibody, an anti-GPNMB antibody, an anti-Her2 antibody, an anti-HMW-MAA antibody, an anti-integrin antibody, an anti-Lewis A like carbohydrate antibody, an anti-Lewis Y antibody, an anti-LIV 1 antibody, an anti-mesothelin antibody, an anti-MN antibody, an anti-MUC1 antibody, an anti-MUC16 antibody, an anti-NaPi2b antibody, an anti-Nectin-4 antibody, an anti-PSMA antibody, an anti-SIRP α antibody, an anti-SLC44A4 antibody, an anti-STEAP-1 antibody, an anti-5T4 (or anti-TPBG, trophoblast glycoprotein) antibody, an anti-Tag72 antibody, an anti-TF (or anti-tissue factor) antibody, an anti-TROP2 antibody and an anti-VLA antibody.

[0051] Preferably, the antibody is an anti-annexin A1 antibody, an anti-CD 115 antibody, an anti-CD123 antibody, an anti-CLL-1 antibody, an anti-c-MET antibody, an anti-MUC1 antibody, an anti-PSMA antibody, an anti-5T4 antibody or an anti-TF antibody. More preferably, the antibody is an anti-PSMA antibody or an anti-5T4 antibody.

[0052] The antibody to be used in accordance with the present invention preferably is a monoclonal antibody (mAb) and can be a chimeric, humanized or human mAb. More preferably, in accordance with the present invention a humanized or human mAb is used. Preferably, said antibody has κ (kappa) light chains, i.e., a humanized or human IgG1- κ antibody.

[0053] In humanized antibodies, the antigen-binding CDRs in the variable regions are derived from antibodies from a non-human species, commonly mouse, rat or rabbit. These non-human CDRs are placed within a human framework (FR1, FR2, FR3 and FR4) of the variable region, and are combined with human constant regions. Like human antibodies, these humanized antibodies can be numbered according to the Kabat numbering system. The application particularly relates to an ADC compound wherein said engineered cysteine is at a position selected from VH 40, VH 41, VH 89, VL 40 or VL 41 in the human framework (i.e., VH 40, VH 41, VL 40 and VL 41 are in the middle of FR2, VH 89 is in FR3) of a humanized antibody, more particularly at VH 40, VH 41, VL 40 or VL 41, even more particularly at VH 41, VL 40 or VL 41, especially at VH 41.

[0054] In accordance with the application, these specific residues in the framework regions are both suitable for conjugation of a linker drug and do not lead to significant reduction of antigen binding properties of the antibody after conjugation of the linker drug. Furthermore, these sites are not only suitable in antibodies, but also in any antigen binding fragments thereof.

[0055] In one particular embodiment, the present invention relates to an ADC compound as described hereinabove wherein said antibody is an anti-annexin A1 antibody, an anti-CD 115 antibody, an anti-CD 123 antibody, an anti-CLL-1 antibody, an anti-c-MET antibody, an anti-MUC1 antibody, an anti-PSMA antibody, an anti-5T4 antibody or an anti-

TF antibody and said linker drug comprises a duocarmycin derivative, preferably an ADC compound in accordance with formula (I) or (II).

[0056] In a further particular embodiment, the present invention relates to an ADC compound as described hereinabove wherein said antibody is an anti-PSMA (monoclonal) antibody or an anti-5T4 (monoclonal) antibody and said linker drug comprises a duocarmycin derivative, preferably an ADC compound in accordance with formula (I) or (II).

[0057] Suitable anti-PSMA antibodies are described in WO98/03873 (e.g., Example 12), WO02/098897 (e.g., Figs. 1-2), WO2007/002222 (e.g., Table 1) and WO2011/069019 (e.g., Fig. 2). Suitable anti-5T4 antibodies include H8, which is described in WO2006/031653, and the A1 and A3 antibodies which are described in WO2007/106744, as well as any antibodies binding to the same epitope as these known antibodies.

[0058] In a preferred embodiment, the heavy chain of the anti-PSMA antibody comprises the amino acid sequence of SEQ ID NO:2 and the light chain of the anti-PSMA antibody comprises the amino acid sequence of SEQ ID NO:5. More preferably, the heavy chain of the anti-PSMA antibody comprises the amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3, and the light chain of the anti-PSMA antibody comprises the amino acid sequences of SEQ ID NO:5 and SEQ ID NO:6.

[0059] In a particularly preferred embodiment, the present invention relates to an ADC compound of formula (II), wherein the antibody is an anti-PSMA antibody, the heavy chain of said anti-PSMA antibody comprising the amino acid sequence of SEQ ID NO:2 and the light chain of said anti-PSMA antibody comprising the amino acid sequence of SEQ ID NO:5. More preferably, the heavy chain of said anti-PSMA antibody comprises the amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3, and the light chain of said anti-PSMA antibody comprises the amino acid sequences of SEQ ID NO:5 and SEQ ID NO:6.

[0060] In another preferred embodiment, the heavy chain of the anti-5T4 antibody comprises the amino acid sequence of SEQ ID NO:8 and the light chain of the anti-5T4 antibody comprises the amino acid sequence of SEQ ID NO:11. More preferably, the heavy chain of the anti-5T4 antibody comprises the amino acid sequences of SEQ ID NO:8 and SEQ ID NO:9, and the light chain of the anti-5T4 antibody comprises the amino acid sequences of SEQ ID NO:11 and SEQ ID NO:6.

[0061] In a particularly preferred embodiment, the present invention relates to an ADC compound of formula (II), wherein the antibody is an anti-5T4 antibody, the heavy chain of said anti-5T4 antibody comprising the amino acid sequence of SEQ ID NO:8 and the light chain of said anti-5T4 antibody comprising the amino acid sequence of SEQ ID NO:11. More preferably, the heavy chain of said anti-5T4 antibody comprises the amino acid sequences of SEQ ID NO:8 and SEQ ID NO:9, and the light chain of said anti-5T4 antibody comprises the amino acid sequences of SEQ ID NO:11 and SEQ ID NO:6.

[0062] The present invention further relates to an ADC compound as described hereinabove for use as a medicament.

[0063] In one embodiment, the present invention relates to an ADC compound as described hereinabove for use in the treatment of human solid tumours and haematological malignancies.

[0064] In a further embodiment, the present invention relates to an ADC compound as described hereinabove for use in the treatment of human solid tumours selected from the group consisting of breast cancer, gastric cancer, colorectal cancer, urothelial cancer (e.g. bladder cancer), ovarian cancer, uterine cancer, lung cancer (especially non-small cell lung cancer and small-cell lung cancer), mesothelioma (especially malignant pleural mesothelioma), liver cancer, pancreatic cancer, and prostate cancer.

[0065] In a preferred embodiment, the present invention relates to an ADC compound as described hereinabove, particularly a compound comprising an anti-PSMA (monoclonal) antibody and a duocarmycin derivative linker drug, for use in the treatment of prostate cancer.

[0066] In another preferred embodiment the present invention relates to an ADC compound as described hereinabove, particularly a compound comprising an anti-5T4 (monoclonal) antibody and a duocarmycin derivative linker drug, for use in the treatment of human solid tumours selected from the group consisting of breast cancer, gastric cancer, colorectal cancer, ovarian cancer, lung cancer (especially non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC)), and malignant pleural mesothelioma.

[0067] In yet a further embodiment, the present invention relates to an ADC compound as described hereinabove for use in the treatment of human haematological malignancies, particularly leukaemia, selected from the group consisting of acute lymphoblastic and myeloid leukaemia (ALL and AML, respectively).

[0068] The present invention further relates to a pharmaceutical composition comprising an ADC compound as described hereinabove and one or more pharmaceutically acceptable excipients. Typical pharmaceutical formulations of therapeutic proteins such as monoclonal antibodies and (monoclonal) antibody-drug conjugates take the form of lyophilized cakes (lyophilized powders), which require (aqueous) dissolution (i.e., reconstitution) before intravenous infusion, or frozen (aqueous) solutions, which require thawing before use.

[0069] Typically, the pharmaceutical composition is provided in the form of a lyophilized cake. Suitable pharmaceutically acceptable excipients for inclusion into the pharmaceutical composition (before freeze-drying) in accordance with the present invention include buffer solutions (e.g. citrate, histidine or succinate containing salts in water), lyoprotectants

(e.g. sucrose, trehalose), tonicity modifiers (e.g. sodium chloride), surfactants (e.g. polysorbate), and bulking agents (e.g. mannitol, glycine). Excipients used for freeze-dried protein formulations are selected for their ability to prevent protein denaturation during the freeze-drying process as well as during storage. As an example, the sterile, lyophilized powder single-use formulation of Kadcyla™ (Roche) contains - upon reconstitution with Bacteriostatic or Sterile Water for Injection (BWFI or SWFI) - 20 mg/mL ado-trastuzumab emtansine, 0.02% w/v polysorbate 20, 10 mM sodium succinate, and 6% w/v sucrose with a pH of 5.0.

[0070] The present invention also relates to the use of a compound or a pharmaceutical composition as described hereinabove for the treatment of human solid tumours and haematological malignancies as described hereinabove.

[0071] The present invention further relates to the use of a sequentially or simultaneously administered combination of a compound or a pharmaceutical composition as described hereinabove with a therapeutic antibody and/or a chemotherapeutic agent, for the treatment of human solid tumours and haematological malignancies as described hereinabove.

[0072] In one embodiment of the present invention, the therapeutic antibody is adecatumumab, alemtuzumab, amatuximab, bevacizumab, cetuximab, denosumab, etaracizumab, farletuzumab, gemtuzumab, labetuzumab, mapatumumab, minretumomab, nimotuzumab, nivolumab, oregovomab, panitumumab, pentumomab, pertuzumab, ramucirumab, sibrotuzumab, trastuzumab or volociximab and the chemotherapeutic agent is i) an alkylating agent, particularly nitrogen mustards, such as mechlorethamine, chlorambucil, cyclophosphamide, ifosfamide and melphalan, nitrosoureas, such as streptozocin, carmustine and lomustine, alkyl sulfonates, such as busulfan, triazines, such as dacarbazine and temozolomide, ethylenimines, such as thiotepa and altretamine, or platinum drugs, such as cisplatin, carboplatin and oxaliplatin, ii) an anti-metabolite, particularly 5-fluorouracil, 6-mercaptopurine, capecitabine, cytarabine, flouxuridine, fludarabine, gemcitabine, hydroxyurea, methotrexate or pemetrexed, iii) an anti-tumour antibiotic, particularly daunorubicin, doxorubicin, epirubicin, idarubicin, actinomycin D, bleomycin, mitomycin-C or mitoxantrone, iv) a topoisomerase inhibitor, particularly topoisomerase I inhibitors, such as topotecan and irinotecan, or topoisomerase II inhibitors, such as etoposide, teniposide and mitoxantrone, v) a mitotic inhibitor, particularly taxanes, such as paclitaxel, cabazitaxel and docetaxel, epothilones, such as ixabepilone, vinca alkaloids, such as vinblastine, vincristine and vinorelbine, or estramustine, vi) a signalling cascade inhibitor, particularly mTOR (mammalian target of rapamycin) inhibitors, such as temsirolimus and everolimus, or tyrosine kinase inhibitors, such as gefitinib, erlotinib, imatinib, pazopanib, ceritinib, crizotinib, lapatinib and afatinib, vii) a corticosteroid, particularly prednisone, methylprednisolone or dexamethasone, viii) a hormonal therapeutic agent, particularly androgen receptor modulating agents, such as bicalutamide, enzalutamide and abiraterone acetate, anti-oestrogens, such as tamoxifen, or aromatase inhibiting or steroid modifying agents, such as anastrozole, letrozole, fulvestrant and exemestane, ix) a PARP inhibitor, particularly olaparib, or x) another chemotherapy drug, particularly L-asparaginase or bortezomib. The person skilled in the art will have no difficulty in selecting suitable combination therapies for use in the treatment of human solid tumours and haematological malignancies as described hereinabove.

[0073] In another embodiment of the present invention, particularly in case of an anti-PSMA ADC compound comprising a duocarmycin derivative linker drug, the therapeutic antibody is bevacizumab, denosumab, pertuzumab or trastuzumab and the chemotherapeutic agent is a topoisomerase II inhibitor, particularly mitoxantrone, a mitotic inhibitor, particularly a taxane, more particularly cabazitaxel or docetaxel, a corticosteroid, particularly prednisone, or a hormonal therapeutic agent, particularly an androgen receptor modulating agent, more particularly enzalutamide or abiraterone acetate.

[0074] In yet another embodiment of the present invention, particularly in case of an anti-5T4 ADC compound comprising a duocarmycin derivative linker drug, the therapeutic antibody is bevacizumab, cetuximab, nivolumab, or ramucirumab and the chemotherapeutic agent is an alkylating agent, particularly a platinum drug, more particularly cisplatin or carboplatin, an anti-metabolite, particularly gemcitabine or pemetrexed, a topoisomerase II inhibitor, particularly etoposide, a mitotic inhibitor, particularly a taxane or a vinca alkaloid, more particularly paclitaxel or docetaxel, or vinblastine or vinorelbine, or a signalling cascade inhibitor, particularly a tyrosine kinase inhibitor, more particularly erlotinib, ceritinib, crizotinib or afatinib.

[0075] In a further embodiment of the present invention, particularly in case of an anti-5T4 ADC compound comprising a duocarmycin derivative linker drug, the therapeutic antibody is bevacizumab and the chemotherapeutic agent is an alkylating agent, particularly a nitrogen mustard, a platinum drug or a triazine, more particularly cyclophosphamide, ifosfamide, cisplatin, or temozolomide, an anti-tumour antibiotic, particularly doxorubicin, an anti-metabolite, particularly gemcitabine, a topoisomerase I or II inhibitor, particularly topotecan, irinotecan or etoposide, or a mitotic inhibitor, particularly a taxane or a vinca alkaloid, more particularly paclitaxel or docetaxel, or vincristine or vinorelbine.

[0076] In yet a further embodiment of the present invention, particularly in case of an anti-5T4 ADC compound comprising a duocarmycin derivative linker drug, the therapeutic antibody is amatuximab and the chemotherapeutic agent is an alkylating agent, particularly a platinum drug, more particularly cisplatin or carboplatin, an anti-metabolite, particularly gemcitabine or pemetrexed, or a mitotic inhibitor, particularly a vinca alkaloid, more particularly vinorelbine.

[0077] A therapeutically effective amount of the compounds in accordance with the present invention lies in the range of about 0.01 to about 15 mg/kg body weight, particularly in the range of about 0.1 to about 10 mg/kg body weight, more particularly in the range of about 0.3 to about 10 mg/kg body weight. This latter range corresponds roughly to a flat dose

in the range of 20 to 800 mg of the ADC compound. The compound of the present invention may be administered weekly, bi-weekly, three-weekly or monthly, for example weekly for the first 12 weeks and then every three weeks until disease progression. Alternative treatment regimens may be used depending upon the severity of the disease, the age of the patient, the compound being administered, and such other factors as would be considered by the treating physician.

EXAMPLES

Transient expression of engineered cysteine (mutant) antibodies

1a) Preparation of cDNAs

[0078] The cDNA sequence for the heavy chain was obtained from the amino acid sequences of a leader sequence (SEQ ID NO:1), the heavy chain variable region of an anti-PSMA antibody (SEQ ID NO:2, Kabat numbering, having a cysteine residue at position 41) and the human IgG1 heavy chain constant region (SEQ ID NO:3, sequential numbering, Eu numbering starting at alanine 118) by back-translating the combined amino acid sequences into a cDNA sequence optimized for expression in human cells (*Homo sapiens*) (SEQ ID NO:4).

[0079] Similarly, the cDNA sequence for the light chain was obtained from the amino acid sequences of a secretion signal (SEQ ID NO:1), the light chain variable region of an anti-PSMA antibody (SEQ ID NO:5, Kabat numbering), and the human κ Ig light chain constant region (SEQ ID NO:6, sequential numbering) by back-translating the combined amino acid sequences into a cDNA sequence optimized for expression in human cells (*Homo sapiens*) (SEQ ID NO:7).

[0080] Similarly, the cDNA sequence for the heavy chain of the anti-5T4 antibody H8-HC41 (SEQ ID NO:10) was obtained from the amino acid sequences of a leader sequence (SEQ ID NO:1), the heavy chain variable region of the H8 antibody (SEQ ID NO:8, sequential numbering, having a cysteine residue at position 41) and the human IgG1 heavy chain constant region (SEQ ID NO:9, sequential numbering, Eu numbering starting at alanine 118).

[0081] The cDNA sequence for the H8 antibody light chain (SEQ ID NO:12) was obtained from the amino acid sequences of a leader sequence (SEQ ID NO:1), the light chain variable region of the H8 antibody (SEQ ID NO:11, Kabat numbering), and the human κ Ig light chain constant region (SEQ ID NO:6, sequential numbering).

[0082] The cDNA sequence for the heavy chain of natalizumab (SEQ ID NO:16) was obtained from the amino acid sequences of a leader sequence (SEQ ID NO:13), the heavy chain variable region of natalizumab (SEQ ID NO:14, Kabat numbering) and the human IgG4 heavy chain constant region (SEQ ID NO:15, sequential numbering, Eu numbering starting at alanine 118; having a proline residue at position 225 and a cysteine residue at position 375).

[0083] The cDNA sequence for the natalizumab light chain (SEQ ID NO:19) was obtained from the amino acid sequences of a leader sequence (SEQ ID NO:17), the light chain variable region of natalizumab (SEQ ID NO:18, Kabat numbering), and the human κ Ig light chain constant region (SEQ ID NO:6, sequential numbering).

[0084] The heavy chain and light chain cDNA constructs were chemically synthesized by and obtained from a commercial source (Life Technologies). Cleavage of the leader sequence corresponded to the predicted cleavage site using the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>).

1b) Vector construction and cloning strategy

[0085] For expression of the antibody chains the mammalian expression vector 0098 was constructed as follows. The CMV:BGHpA expression cassette was excised from the pcDNA3.1(-) (Life Technologies) plasmid and re-inserted back into the same original vector (still containing an intact CMV:BGHpA expression cassette), thereby duplicating the CMV:BGHpA expression cassette, to allow expression of both HC and LC cDNAs from a single plasmid vector. Subsequently, an IRES-DHFR fragment was isolated from the vector pOptiVEC-TOPO (Life Technologies) and inserted between the CMV promoter and the BGHpA polyadenylation sequence in one of the CMV:BGHpA expression cassettes.

[0086] The cDNAs for the heavy chain (HC) and the light chain (LC) were ligated into pMA-RQ and pMA-T plasmid vectors (Life Technologies), respectively, using SfiI restriction sites. After transfer to *E. coli* K12 and expansion, the LC cDNA was transferred to the mammalian expression vector 0098 using Ascl and HpaI restriction sites. The resulting vector was digested with BamHI and NheI restriction enzymes, and ligated with the HC cDNA fragment, digested with the same restriction enzymes. The final vector, containing both the HC and LC expression cassettes (CMV:HC:BGHpA and CMV:LC-BGHpA, respectively) was transferred to and expanded in *E. coli* NEB 5-alpha cells (New England Biolabs).

[0087] Large-scale production of the final antibody mutant expression vector for transfection was performed using Maxi- or Megaprep kits (Qiagen).

2) Transient expression in mammalian cells

[0088] Commercially available Expi293F cells (Life Technologies) were transfected with the antibody mutant expres-

sion vector prepared under 1) above using the ExpiFectamine transfection agent (Life Technologies) according to the manufacturer's instructions as follows: 75×10^7 cells were seeded in 300 mL Expi293 Expression medium; 300 μ g of the antibody mutant expression vector was combined with 800 μ l of ExpiFectamine transfection agent and added to the cells. One day after transfection, 1.5 mL Enhancer 1 and 15 mL Enhancer 2 were added to the culture. Six days post transfection, the cell culture supernatant was harvested by centrifugation at 4,000 g for 15 minutes and filtering the clarified harvest over MF 75 filters (Nalgene).

3) Purification of expressed proteins

[0089] Clarified harvests were first checked on expression level using SDS-PAGE electrophoresis. As production was deemed adequate, the antibodies were purified using commercially available protein A resin (MabSelect SuRe, GE Healthcare), using Äkta chromatographic equipment (GE Healthcare). A 20 cm bed height column was used with a maximum load of 25 mg/mL packed resin. The process was performed at RT.

[0090] After equilibration (PBS pH7.4) and loading the purification step employed two wash steps (PBS pH7.4 and NaAc pH5, respectively) and an elution step (25 mM NaAc, pH3) followed by a regeneration, rinse and cleaning step, respectively, before a new cycle was started. During the elution step the target protein was collected in a peak starting and stopping at an absorbance of 0.05-0.1 AU (0.2 cm cell length). After purification the protein was stored at -20°C to -80°C .

4) Concentration and buffer exchange to the ADC conjugation buffer

[0091] Protein A eluates were, if needed, concentrated to 20-25 mg/mL using Vivaspin centrifugal devices (5 or 30 kDa cut-off, Vivaproducts). After obtaining the desired concentrations the concentrated solutions (typically 25 mg/mL) were dialyzed twice using PD10 columns (GE Healthcare) and 4.2 mM L-Histidine + 50 mM Trehalose pH6.0 buffer. Alternatively, when protein A eluate concentrations were approximately 10 mg/mL, no concentration step was employed and the eluate was immediately dialyzed three times using snakeskin dialysis tube (10 kDa cut-off, Thermo Scientific) against 4.2 mM L-Histidine + 50 mM Trehalose pH6.0 buffer. Any precipitate that appeared was removed by filtration after dialysis was completed. Concentrations were measured by UV spectroscopy using either Nanodrop or a cuvette UV spectrophotometer (both Thermo Scientific). Quality analysis showed that the protein had a purity of >95% and contained negligible amounts of dimers or fragments as determined by HPSEC. The isoelectric point of the engineered cysteine mutant was identical to the wild-type.

[0092] Using the similar/same procedure as described hereinabove for the preparation and purification of the engineered cysteine (VH 41C, Kabat numbering) anti-PSMA antibody, the engineered H8-HC41 (VH 41C, Kabat numbering) and the engineered cysteine natalizumab (CH 225P, 375C, Kabat numbering) antibodies, also the other antibodies of the examples were prepared and purified.

General site-specific conjugation protocol

[0093] To a solution of cysteine engineered antibody (5-10 mg/ml in 4.2 mM histidine, 50 mM trehalose, pH 6) EDTA (25 mM in water, 4% v/v) was added. The pH was adjusted to ~ 7.4 using TRIS (1 M in water, pH 8) after which TCEP (10 mM in water, 20 equivalents) was added and the resulting mixture was incubated at room temperature for 1-3 hrs. The excess TCEP was removed by either a PD-10 desalting column or a Vivaspin centrifugal concentrator (30 kDa cut-off, PES) using 4.2 mM histidine, 50 mM trehalose, pH 6. The pH of the resulting antibody solution was raised to ~ 7.4 using TRIS (1 M in water, pH 8) after which dehydroascorbic acid (10 mM in water, 20 equivalents) was added and the resulting mixture was incubated at room temperature for 1-2 hrs. DMA was added followed by a solution of linker drug (10 mM in DMA). The final concentration of DMA was 5-10%. The resulting mixture was incubated at room temperature in the absence of light for 1-16 hrs. In order to remove the excess of linker drug, activated charcoal was added and the mixture was incubated at room temperature for 1 hr. The coal was removed using a 0.2 μ m PES filter and the resulting ADC was formulated in 4.2 mM histidine, 50 mM trehalose, pH 6 using a Vivaspin centrifugal concentrator (30 kDa cut-off, PES). Finally, the ADC solution was sterile filtered using a 0.22 μ m PES filter.

General conjugation protocol for conjugation via partially reduced endogenous disulfides (wt conjugation)

[0094] To a solution of antibody (5-10 mg/ml in 4.2 mM histidine, 50 mM trehalose, pH 6) EDTA (25 mM in water, 4% v/v) was added. The pH was adjusted to ~ 7.4 using TRIS (1 M in water, pH 8) after which TCEP (10 mM in water, 1-3 equivalents depending on the antibody and the desired DAR) was added and the resulting mixture was incubated at room temperature for 1-3 hrs. DMA was added followed by a solution of linker drug (10 mM in DMA). The final concentration of DMA was 5-10%. The resulting mixture was incubated at room temperature in the absence of light for 1-16 hrs. In

order to remove the excess of linker drug, activated charcoal was added and the mixture was incubated at room temperature for 1 hr. The coal was removed using a 0.2 μm PES filter and the resulting ADC was formulated in 4.2 mM histidine, 50 mM trehalose, pH 6 using a Vivaspin centrifugal concentrator (30 kDa cut-off, PES). Finally, the ADC solution was sterile filtered using a 0.22 μm PES filter.

[0095] Using the above general procedures, cysteine engineered and wild-type ADCs based on vc-seco-DUBA (SYD980; i.e., compound **18b**, n=1 in Example 10 on page 209 of WO2011/133039), vc-MMAE and mc-MMAF linker drugs were synthesized and characterized using analytical Hydrophobic Interaction Chromatography (HIC), Size Exclusion Chromatography (SEC), Shielded Hydrophobic Phase Chromatography (SHPC), RP-HPLC and LAL endotoxin-testing.

[0096] For analytical HIC, 5-10 μL of sample (1 mg/ml) was injected onto a TSKgel Butyl-NPR column (4.6 mm ID x 3.5 cm L, Tosoh Bioscience, cat. nr. 14947). The elution method consisted of a linear gradient from 100% Buffer A (25 mM sodium phosphate, 1.5 M ammonium sulphate, pH 6.95) to 100% of Buffer B (25 mM sodium phosphate, pH 6.95, 20% isopropanol) at 0.4 ml/min over 20 minutes. A Waters Acquity H-Class UPLC system equipped with PDA-detector and Empower software was used. Absorbance was measured at 214 nm and the retention time of ADCs was determined.

[0097] As made apparent by analytical HIC, there were differences in the retention times (RTs) for the DAR2 species of the different cysteine engineered ADCs *versus* the wt conjugates (Tables 1, 2 and 3). Most interestingly, conjugating the linker drug at specific sites inside the Fab cavity or Fc cavity (as predicted by the molecular modelling algorithm) gave rise to a (dramatic) decrease in the retention time as compared to the ADCs that were conjugated via partially reduced endogenous disulfides, leading the present inventors to conclude that based on the HIC data, the engineered ADCs in which the linker drug is conjugated to specific sites in the Fab or Fc cavity are less hydrophobic than the wt conjugates. To further quantify this effect, the term relative hydrophobicity is introduced, which is defined as:

$$(RT_{\text{DAR2}} - RT_{\text{DAR0}}) / (RT_{\text{DAR2, wt-ADC}} - RT_{\text{DAR0, wt-ADC}}).$$

[0098] In essence, the relative hydrophobicity is a measure that allows a facile comparison between the hydrophobicity of the engineered ADCs *versus* the wt-conjugated counterparts based on HIC data. The data are summarized in Tables 1, 2 and 3.

Table 1. The relative hydrophobicity of vc-seco-DUBA ADCs on the previously specified analytical HIC column:

ADC (vc-seco-DUBA)	Cys mutations		DAR	HMW (%) ²	RT _{DAR2}	RT _{DAR0}	Relative hydrophobicity ³
	HC	LC					
ADC-wt (SYD998) ¹	wt	wt	1.8	7.7	9.7	6.9	1.0
ADC-HC41 (SYD1091)	S41C	wt	1.7	1.4	8.5	6.8	0.6
ADC-HC120 (SYD1035) ⁶	T120C	wt	1.8	0.9	11.3	6.8	1.6
ADC-HC152	E152C	wt	1.5	1.2	8.5	6.5	0.7
ADC-HC153	P153C	wt	1.5	2.4	8.7	6.5	0.8
ADC-HC236 ⁶	G236C	wt	1.0	1.1	10.4	6.5	1.4
ADC-HC247	P247C	wt	1.3	1.3	9.2	7.3	0.7
ADC-HC339	A339C	wt	1.7	0.5	8.6	7.3	0.5
ADC-HC375	S375C	wt	1.8	1.0	7.5	6.6	0.3
ADC-HC376	D376C	wt	1.4	3.1	9.8	6.6	1.1
ADC-HC41-120 ⁷	S41C, T120C	wt	3.3	40.0	12.3	7.3	0.9
ADC-HC41-375	S41C, S375C	wt	3.0-4.3 ⁴	1.9	9.3 ⁵	7.3	0.4
ADC-LC40	wt	P40C	1.8	0.5	9.5	6.9	0.9

(continued)

ADC (vc-seco-DUBA)	Cys mutations		DAR	HMW (%) ²	RT _{DAR2}	RT _{DAR0}	Relative hydrophobicity ³
	HC	LC					
ADC-LC41	wt	G41C	1.8	0.6	8.7	6.9	0.6
ADC-LC109 ⁶	wt	T109C	1.0	-	12.4	7.2	1.9
ADC-LC154 ⁶	wt	L154C	1.7	6.4	12.4	6.8	2.0
ADC-LC157 ⁶	wt	G157C	1.1	-	12.5	7.1	1.9
ADC-LC165	wt	E165C	1.5	2.3	8.4	6.6	0.6
ADC-LC205 ⁶	wt	V205C	1.8	1.0	10.6	6.9	1.3
H8-wt ¹	wt	wt	2.0	4.4	9.9	6.4	1.0
H8-HC40	S40C	wt	1.7	1.2	8.8	6.2	0.7
H8-HC41	P41C	wt	1.7	0.4	7.4	6.2	0.3
Natalizumab-HC375	S375C ⁸	wt	1.7	26.0	7.8	6.8	0.4

¹ Random - non-site specific - attachment
² HMW are high molecular weight species, reflecting the amount of aggregates formed
³ Defined as $(RT_{DAR2} - RT_{DAR0}) / (RT_{DAR2, wt-ADC} - RT_{DAR0, wt-ADC})$, RT is retention time
⁴ Based on MS-data
⁵ RT, wt DAR4 species = 12.2
⁶ Comparator ADCs with linker drug conjugated to a cysteine residue pointing outwards
⁷ ADC with linker drug conjugated to one cysteine in the Fab cavity and one cysteine residue pointing outwards; process not yet optimised
⁸ Also 225P mutation to prevent dimerisation of IgG4

Table 2. The relative hydrophobicity of vc-MMAE ADCs on the previously specified analytical HIC column:

ADC (vc-MMAE)	Cys mutations		DAR	HMW (%) ¹	RT _{DAR2}	RT _{DAR0}	Relative hydrophobicity ²
	HC	LC					
ADC-wt	wt	wt	1.7	0.6	9.6	7.2	1.0
ADC-HC41	S41C	wt	1.8	0.5	8.1	7.2	0.4
ADC-LC40	wt	P40C	1.8	0.6	8.5	7.2	0.5
ADC-LC41	wt	G41C	1.9	0.9	8.4	7.3	0.5
H8-HC40	S40C	wt	1.7	1.4	8.1	6.5	ND ³

¹ HMW are high molecular weight species, reflecting the amount of aggregates formed
² Defined as $(RT_{DAR2} - RT_{DAR0}) / (RT_{DAR2, wt-ADC} - RT_{DAR0, wt-ADC})$, RT is retention time
³ ND is not determined; wild-type H8-vc-MMAE was not prepared

Table 3. The relative hydrophobicity of mc-MMAF ADCs on the previously specified analytical HIC column:

ADC (mc-MMAF)	Cys mutations		DAR	HMW (%) ¹	RT _{DAR2}	RT _{DAR0}	Relative hydrophobicity ²
	HC	LC					
ADC-wt	wt	wt	1.8	0.6	8.0	7.2	1.0
ADC-HC41	S41C	wt	1.8	0.5	7.4	7.2	0.3
ADC-LC40	wt	P40C	1.8	0.5	7.6	7.2	0.5

(continued)

ADC (mc-MMAF)	Cys mutations		DAR	HMW (%) ¹	RTDAR2	RT _{DAR0}	Relative hydrophobicity ²
	HC	LC					
ADC-LC41	wt	G41C	1.8	0.6	7.5	7.3	0.3
H8-wt	wt	wt	4.2	0.4	7.2	6.2	1.0
H8-HC40	S40C	wt	1.4	1.2	6.9	6.5	0.4

¹ HMW are high molecular weight species, reflecting the amount of aggregates formed
² Defined as $(RT_{DAR2} - RT_{DAR0}) / (RT_{DAR2, wt-ADC} - RT_{DAR0, wt-ADC})$, RT is retention time

Cellular binding

[0099] Three anti-PSMA ADCs SYD998 (wt), SYD1091 (HC41) and comparator SYD1035 (HC120) had equal binding affinities on PSMA-expressing LNCaP-C4.2 cells (EC_{50} in the range of 0.1-0.2 μ g/ml) similar to the wild-type antibody, and all three ADCs were unable to bind to PSMA-negative DU-145 cells ($EC_{50} > 10 \mu$ g/ml).

[0100] Two anti-5T4 ADCs H8-wt and H8-HC40 had equal binding affinities on 5T4-expressing MDA-MB-468 cells (EC_{50} in the range of 0.1-1.2 μ g/ml) similar to the wild-type H8 antibody, and both ADCs were unable to bind to 5T4-negative SK-MEL-30 cells ($EC_{50} > 10 \mu$ g/ml).

[0101] The antigen binding properties of the ADCs were thus unaffected by the attached duocarmycin derivative linker drug.

In vitro cytotoxicity

[0102] The potencies of the site-specifically conjugated anti-PSMA ADCs were similar to the conventionally linked wild-type ADC (SYD998) on PSMA-expressing LNCaP-C4.2 cells (IC_{50} in the range of 0.1-0.5 nM, based on drug equivalents, see Table 4 below). All ADCs were inactive on PSMA-negative DU-145 cells ($IC_{50} > 70$ nM) indicating selective killing of tumour cells through PSMA.

[0103] The two non-binding control ADCs were at least 50-times less potent than the anti-PSMA ADCs on each of the cell lines evaluated.

Table 4. *In vitro* cytotoxicity of the anti-PSMA-vc-seco-DUBA ADCs in human tumour cells expressing PSMA

ADC (vc-seco-DUBA)	Cys mutations		PSMA-positive cell line LNCaP-C4.2		
	HC	LC	IC_{50} (nM)	95% CI (nM) ¹	% efficacy ²
ADC-wt (SYD998)	wt	wt	0.23	0.20 - 0.27	82
ADC-HC41 (SYD1091)	S41C	wt	0.25	0.21 - 0.28	78
ADC-HC120 (SYD1035)	T120C	wt	0.14	0.13 - 0.16	82
ADC-HC152	E152C	wt	0.44	0.36 - 0.55	78
ADC-HC153	P153C	wt	0.34	0.28 - 0.41	79
ADC-HC236	G236C	wt	0.22	0.19 - 0.26	76
ADC-HC247	P247C	wt	0.10	0.09 - 0.12	82
ADC-HC339	A339C	wt	0.12	0.11 - 0.13	83
ADC-HC375	S375C	wt	0.25	0.22 - 0.28	81
ADC-HC376	D376C	wt	0.20	0.18 - 0.22	82
ADC-LC40	wt	P40C	0.30	0.23 - 0.37	80
ADC-LC41	wt	G41C	0.31	0.25 - 0.38	80
ADC-LC154	wt	L154C	0.24	0.19 - 0.29	82
ADC-LC165	wt	E165C	0.51	0.40 - 0.65	79
ADC-LC205	wt	V205C	0.17	0.15 - 0.19	83

(continued)

ADC (vc-seco-DUBA)	Cys mutations		PSMA-positive cell line LNCaP-C4.2		
	HC	LC	IC ₅₀ (nM)	95% CI (nM) ¹	% efficacy ²
Non-binding control-wt	wt	wt	28.86	24.76 - 36.02	96
Non-binding control-HC41	P41C	wt	>100	n.a.	n.a.
Free linker drug			0.02	0.02 - 0.03	98

¹ 95% CI is 95% confidence interval
² Percentage efficacy was given at the highest concentration tested (~ 100 nM) and calculated by dividing the measured luminescence for each drug or ADC with the average mean of untreated cells (only growth medium) multiplied by 100.

[0104] Conjugation of vc-MMAE to the HC41, LC40 and LC41 positions on anti-PSMA antibodies resulted in cytotoxic potencies in PSMA-positive LNCaP-C4.2 cells similar to anti-PSMA-vc-seco-DUBA linked on these cysteine sites (Tables 4 and 5). The anti-PSMA-vc-MMAE ADCs lacked activity on PSMA-negative DU-145 cells (IC₅₀ >70 nM).

Table 5. *In vitro* cytotoxicity of the anti-PSMA-vc-MMAE ADCs in human tumour cells expressing PSMA

ADC (vc-MMAE)	Cys mutations		PSMA-positive cell line LNCaP-C4.2		
	HC	LC	IC ₅₀ (nM)	95% CI (nM) ¹	% efficacy ²
ADC-wt	wt	wt	0.34	0.31 - 0.38	89
ADC-HC41	S41C	wt	0.39	0.35 - 0.43	91
ADC-LC40	wt	P40C	0.31	0.27 - 0.35	90
ADC-LC41	wt	G41C	0.33	0.29 - 0.37	90
Non-binding control-wt	wt	wt	>100	n.a.	90
Free linker drug			0.23	0.18 - 0.28	94

¹ 95% CI is 95% confidence interval
² Percentage efficacy was given at the highest concentration tested (~ 100 nM) and calculated by dividing the measured luminescence for each drug or ADC with the average mean of untreated cells (only growth medium) multiplied by 100.

[0105] The potencies of the engineered anti-5T4 ADCs were equal to the conventionally linked ADC H8-wt on 5T4-expressing MDA-MB-468 cells (IC₅₀ between 0.07 and 0.09 nM, Table 6). The anti-5T4 ADCs were inactive on 5T4-negative SK-MEL-30 cells (IC₅₀ >90nM).

Table 6. *In vitro* cytotoxicity of the anti-5T4-vc-seco-DUBA ADCs in human tumour cells expressing 5T4

ADC (vc-seco-DUBA)	Cys mutations		vc-seco-DUBA 5T4-positive cell line MDA-MB-468		
	HC	LC	IC ₅₀ (nM)	95% CI (nM) ¹	% efficacy ²
H8-wt	wt	wt	0.09	0.08 - 0.10	91
H8-HC40	S40C	wt	0.07	0.07 - 0.08	88
H8-HC41	P41C	wt	0.07	0.06 - 0.08	88
Non-binding control-HC41	P41C	wt	40.18	34.24 - 47.16	85
Free linker drug			0.02	0.01 - 0.02	94

¹ 95% CI is 95% confidence interval
² Percentage efficacy was given at the highest concentration tested (~ 100 nM) and calculated by dividing the measured luminescence for each drug or ADC with the average mean of untreated cells (only growth medium) multiplied by 100.

[0106] Together, these data show that the tested cysteine positions for site-specific conjugation did not have an impact on the tumour cell killing potency of ADCs comprising two different linker drugs. Moreover, site-specific linkage of linker

drugs in the variable region of the Fab part of different antibodies is generally applicable.

Enzymatic cleavage by cathepsin B

[0107] The valine-citrulline moiety present in the linker of the ADCs with *vc-seco*-DUBA (SYD980) and *vc*-MMAE can be cleaved by cysteine proteases, such as cathepsin B, which results in subsequent intracellular release of the (*seco*-)DU-BA or MMAE drug inside the tumour lysosomes or extracellular in the tumour microenvironment. To assess the sensitivity towards cathepsin B, the ADCs were treated for 2 minutes and 4 hours with activated cathepsin B (Calbiochem). The cytotoxic activity of the released drug from the anti-PSMA ADCs was measured on PSMA-negative DU-145 cells. The cytotoxic activity of the released drug from the anti-5T4 ADCs was measured on 5T4-negative SK-MEL-30 cells. During the pre-incubation step at 37°C, 1 mg/ml of each ADC was mixed with 5 µg/ml cathepsin B (0.04 units/well) in 0.1M Na-acetate pH 5 containing 4 mM DTT. As a control, 1 mg/ml of each ADC was directly diluted in culture medium (RPMI 1640, 10% qualified FBS). Serial dilutions were made from these ADC solutions in culture medium. To measure release of the respective free toxins DUBA or MMAE, PSMA-negative DU-145 cells (1,000 cells/well) and 5T4-negative SK-MEL-30 cells (2,000 cells/well) were cultured with the ADCs for 6 days, and the cell viability was measured after 6 days using the CellTiter-Glo™ (CTG) assay kit.

[0108] Differences in potency of the released drug on PSMA-negative DU-145 cells and 5T4-negative SK-MEL-30 cells reflect the amount of drug that is cleaved from the ADC, and thereby the accessibility of the valine-citrulline cleavage site for cathepsin B. As shown in Table 7, the sensitivity for proteolytic cleavage differed amongst the ADCs after four hours of exposure to activated cathepsin B (see IC₅₀ values), while none of the ADCs were cleaved after a short period of 2 minutes exposure with cathepsin B (IC₅₀ >10 nM, data not shown in Table).

[0109] Together these data show that the site of conjugation influences the accessibility of the linker drug for enzymatic cleavage, and that the *vc-seco*-DUBA (SYD980) linker drug in the anti-PSMA ADCs ADC-HC41, ADC-HC152, ADC-HC339, ADC-HC375, ADC-LC41, and ADC-LC165 are most shielded from cleavage by said enzyme. Conjugation of *vc*-MMAE to the HC41 and LC41 positions of the anti-PSMA antibodies resulted in similar shielding of the valine-citrulline cleavage site (Table 7). A similar trend was also seen for the anti-5T4 antibody H8-HC41 conjugated to *vc-seco*-DUBA (SYD980) via the same HC41 position.

[0110] These data together show that particularly the 41C position is a suitable position for site-specific conjugation of linker-drugs to various antibodies.

Table 7 Cytotoxicity of the free drug cleaved by cathepsin B

ADC	Cys mutations		4 hours pre-incubation with cathepsin B		
	HC	LC	IC ₅₀ (nM)	95% CI (nM)	% efficacy
<i>anti-PSMA antibodies conjugated to vc-seco-DUBA</i>					
ADC-wt (SYD998)	wt	wt	0.70	0.62 - 0.79	97
ADC-HC41 (SYD1091)	S41C	wt	~ 5.00	n.a.	59
ADC-HC120 (SYD1035)	T120C	wt	0.38	0.34 - 0.42	96
ADC-HC152	E152C	wt	>10	n.a.	50
ADC-HC153	P153C	wt	0.76	0.69 - 0.84	98
ADC-HC236	G236C	wt	2.08	1.64 - 2.65	100
ADC-HC247	P247C	wt	2.01	1.69 - 2.39	99
ADC-HC339	A339C	wt	5.00	3.50 - 7.15	99
ADC-HC375	S375C	wt	>10	n.a.	45
ADC-HC376	D376C	wt	0.60	0.52 - 0.68	98
ADC-LC40	wt	P40C	2.11	1.91 - 2.34	96
ADC-LC41	wt	G41C	>10	n.a.	n.a.
ADC-LC154	wt	L154C	0.26	0.22 - 0.30	98
ADC-LC165	wt	E165C	>10	n.a.	50
ADC-LC205	wt	V205C	0.48	0.41 - 0.58	97

(continued)

	Cys mutations		4 hours pre-incubation with cathepsin B		
ADC	HC	LC	IC ₅₀ (nM)	95% CI (nM)	% efficacy
anti-PSMA antibodies conjugated to vc-seco-DUBA					
Non-binding control-wt	wt	wt	0.32	0.28 - 0.35	97
Non-binding control-HC41	P41C	wt	1.56	1.36 - 1.79	98
anti-PSMA antibodies conjugated to vc-MMAE					
ADC-wt	wt	wt	0.63	0.31 - 0.38	96
ADC-HC41	S41C	wt	2.28	2.11 - 2.46	97
ADC-LC40	wt	P40C	0.60	0.55 - 0.64	96
ADC-LC41	wt	G41C	4.28	3.65 - 5.02	96
	Cys mutations		4 hours pre-incubation with cathepsin B		
ADC	HC	LC	IC ₅₀ (nM)	95% CI (nM)	% efficacy
Non-binding control-wt	wt	wt	0.64	0.57 - 0.72	97
anti-5T4 antibodies conjugated to vc-seco-DUBA					
H8-wt	wt	wt	0.35*	0.30 - 0.40	98
H8-HC40	S40C	wt	0.98	0.83 - 1.15	93
H8-HC41	P41C	wt	1.27*	0.98 - 1.67	98
Non-binding control-HC41	P41C	wt	1.86	1.42 - 2.45	85
* LNCaP-C4.2 was used as the 5T4-negative cell line.					

Tumour xenograft animal model

[0111] The *in vivo* efficacy of three anti-PSMA ADCs was evaluated in the LNCaP C4-2 prostate cancer xenograft model. The LNCaP-C4.2 cell line is a human prostate carcinoma epithelial cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent LNCaP-FGC xenograft cell line.

[0112] Tumours were induced subcutaneously by injecting 1×10^7 of LNCaP C4.2 cells in 200 μ L of RPMI 1640 containing matrigel (50:50, v:v) into the right flank of male CB17-SCID mice. LNCaP-C4.2 tumour cell implantation was performed 24 to 72 hours after a whole body irradiation with a γ -source (1.44 Gy, ^{60}Co , BioMep, Bretenières, France). Treatments were started when the tumours reached a mean volume of 100-200 mm^3 . Mice were randomized according to their individual tumour volume into groups and received a single i.v. injection of anti-PSMA ADC (2 or 10 mg/kg) or vehicle in the tail vein. Changes in tumour volumes (Figure 2) and body weight (Figure 3) were monitored. All three ADCs have an average DAR of approximately 1.8.

[0113] Figure 2A demonstrates that at 2 mg/kg the comparator engineered cysteine anti-PSMA ADC SYD1035 is less active compared to the native, non-engineered cysteine SYD998. However, the efficacy of SYD1091, an engineered cysteine ADC in accordance with the present invention, is significantly better than the comparator SYD1035 and is better than the native, non-engineered SYD998. The difference between the comparator SYD1035 and SYD1091 is even more pronounced at 10 mg/kg as shown in Figure 2B. Mice bearing LNCaP C4.2 tumours develop cachexia as illustrated in Figure 3. This loss of body weight is often restored after administration of efficacious treatments and is considered a sensitive efficacy biomarker. Treatment with SYD1091 resulted in much faster restoration of the body weights than was seen with the comparator SYD1035 or native, non-engineered SYD998 (Figure 3).

[0114] The *in vivo* efficacy of two anti-5T4 ADCs, i.e. the native H8-vc-seco-DUBA (average DAR 2.0) and the engineered cysteine (VH P41C) ADC H8-41C-vc-seco-DUBA (average DAR 1.7), was evaluated in the PA-1 ovarian cancer xenograft model. The PA-1 cell line was established from cells taken from ascitic fluid collected from a woman with ovarian carcinoma (Zeuthen J. et al. Int. J. Cancer 1980; 25(1): 19-32).


[0115] PA-1 tumours were induced subcutaneously by injecting 1×10^7 cells in 100 μ L RPMI 1640 medium containing matrigel (50/50, v/v) into the right flank of female Balb/c nude mice. PA-1 tumour cell injection was performed 24 to 72

hours after a whole body irradiation with a γ -source (2 Gy, ^{60}Co , BioMep, Bretenières, France). Treatments were started when the tumours reached a mean volume of 200-300 mm³. Mice were randomized according to their individual tumour volume into groups and received a single i.v. injection of anti-5T4 ADC (3 or 10 mg/kg) or vehicle in the tail vein and changes in tumour volumes (Figures 4A and 4B) were monitored. Even though both variants have similar efficacy at the higher dose 10 mg/kg (Figure 4B), at 3 mg/kg the engineered cysteine anti-5T4 ADC H8-41 C-vc-seco-DUBA was clearly

more active when compared to the native, non-engineered anti-5T4 ADC, H8-vc-seco-DUBA (Figure 4A).
[0116] Together these findings demonstrate that, *in vivo*, the site-specific engineered cysteine ADCs according to the present invention show favourable properties with respect to the efficacy in mouse tumour models.

SEQ ID NO:1 (HAVT20 leader sequence)
 1 MACPGFLWAL VISTCLEFSM A

SEQ ID NO:2 (anti-PSMA antibody HC S41C)

1 EVQLVQSGAE VKKPGASVKI SCKTSGYTFT EYTIHWVKQA GKGLEWIGN
 51 INPNNGGTTY NQKFEDRATL TVDKSTSTAY MELSSLRSED TAVVYCAAGW
 101 NFDYWQGGTT VTVSS

SEQ ID NO:3 (human IgG1 antibody HC constant region)

1 ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSKV
 51 HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP
 101 KSCDKTHTCP PCPAPPELLGG PSVFLFPPKP KDTLMISRTPEVTCVVDVDS
 151 HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLNQDVLNGK
 201 EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTCL
 251 LVKGFYPSDI AVEWESNGQP ENNYKTTTPV LDSDGSFFLY SKLTVDKSRW
 301 QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

SEQ ID NO:4 (anti-PSMA antibody HC S41C cDNA)

1 ATGGCCTGTC CTGGATTTCT GTGGGCCCTC GTGATCAGCA CCTGTCTGGA ATTCAGCATG
 61 GCCGAGGTGC AGCTGGTGCA GTCTGGCGCC GAAGTGAAGA AACCAGGCGC CAGCGTGAAG
 121 ATCAGCTGCA AGACCAGCGG CTACACCTTC ACCGAGTACA CCATCCACTG GGTCAAGCAG
 181 GCCTGTGGCA AGGGCCTGGA ATGGATCGGC AACATCAACC CCAACAACGG CGGCACCACC
 241 TACAACCAGA AGTTCGAGGA CCGGGCCACC CTGACCGTGG ACAAGAGCAC AAGCACCGCC
 301 TACATGGAAC TGAGCAGCCT GCGGAGCGAG GACACCGCCG TGTACTATTG TGCCGCCGGA
 361 TGGAACTTCG ACTACTGGGG CCAGGGCACC ACCGTGACAG TGTCTAGCGC CAGCACAAAAG
 421 GGCCCCAGCG TGTTCCCTCT GGCCCCTAGC AGCAAGTCTA CCTCTGGCGG AACAGCCGCC
 481 CTGGGCTGCC TCGTGAAGGA CTACTTTCCC GAGCCCCTGA CCGTGTCTTG GAACTCTGGC
 541 GCTCTGACAA GCGGCGTGCA CACCTTTCCA GCCGTGCTGC AGAGCAGCGG CCTGTACTCT
 601 CTGAGCAGCG TCGTGAAGT GCCCAGCAGC AGCCTGGGCA CCCAGACCTA CATCTGCAAC
 661 GTGAACCACA AGCCCAGCAA CACCAAGGTG GACAAAAAGG TGGAACCCAA GAGCTGCGAC
 721 AAGACCACA CCTGTCCCC TTGTCTGCC CCTGAACTGC TGGGCGGACC TTCCGTGTTT
 781 CTGTTCCCC CAAAGCCCAA GGACACCCTG ATGATCAGCC GGACCCCCGA AGTGACCTGC
 841 GTGGTGGTGG ATGTGTCCCA CGAGGACCCT GAAGTGAAGT TCAATTGGTA CGTGGACGGC
 901 GTGGAAGTGC ACAACGCCAA GACCAAGCCC AGAGAGGAAC AGTACAACAG CACCTACCGG
 961 GTGGTGTCCG TGCTGACAGT GCTGCACAG GACTGGCTGA ACGGCAAAGA GTACAAGTGC
 1021 AAGGTGTCCA ACAAGGCCCT GCCTGCCCCC ATCGAGAAAA CCATCAGCAA GGCCAAGGGC
 1081 CAGCCCCGCG AACCACAGGT GTACACACTG CCTCCCAGCA GGGACGAGCT GACCAAGAAC
 1141 CAGGTGTCCC TGACATGCCT CGTGAAAGGC TTCTACCCCT CCGATATCGC CGTGGAAATGG
 1201 GAGAGCAACG GCCAGCCCGA GAACAACACT AAGACCACCC CCCCTGTGCT GGACAGCGAC
 1261 GGCTCATTCT TCCTGTACAG CAAGCTGACT GTGGATAAGT CCCGGTGGCA GCAGGGCAAC
 1321 GTGTCAGCT GCAGCGTGAT GCACGAGGCC CTGCACAACC ACTACACCCA GAAAAGCCTG
 1381 TCCTGAGCC CCGGCAAG

SEQ ID NO:5 (anti-PSMA antibody LC)

1 DIVMTQSPSS LSASVGDRTV ITCKASQDVG TAVDWYQQKPK GKAPKLLIYW
 51 ASTRHTGVPD RFTGSGSGTD FTLTISSLQP EDFADYFCQQ YNSYPLTFGG
 101 GTKLEIK

SEQ ID NO:6 (human IgG antibody LC κ constant region)

1 RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG
 51 NSQESVTEQD SKDSTYSLSS TLTLKADYE KHKVYACEVT HQGLSSPVTK
 5 101 SFNRGEC

SEQ ID NO:7 (anti-PSMA antibody LC cDNA)

1 ATGGCTTGTC CTGGATTCT GTGGGCCCTC GTGATCAGCA CCTGTCTGGA ATTCAGCATG
 10 61 GCCGACATCG TGATGACCCA GAGCCCCAGC TCTCTGAGCG CCAGCGTGGG CGACAGAGTG
 121 ACCATCACAT GCAAGGCCAG CCAGGACGTG GGCACCGCCG TGGATTGGTA TCAGCAGAAG
 181 CCTGGCAAGG CCCCCAAGCT GCTGATCTAC TGGGCCAGCA CCAGACACAC CGGCGTGCCC
 241 GATAGATTCA CAGGCAGCGG CTCCGGCACC GACTTCACCC TGACAATCAG CAGCCTGCAG
 301 CCCGAGGACT TCGCCGACTA CTTCTGCCAG CAGTACAACA GCTACCCCTT GACCTTCGGC
 15 361 GGAGGCACCA AGCTGAAAT CAAGCGGACA GTGGCCGCTC CCAGCGTGT CATCTTCCCA
 421 CCTAGCGACG AGCAGCTGAA GTCTGGCACC GCCTCTGTCTG TGTGCCTGCT GAACAACCTC
 481 TACCCCGCG AGGCCAAGGT GCAGTGAAG GTGGACAATG CCCTGCAGAG CGGCAACAGC
 541 CAGGAAAGCG TGACCGAGCA GGACAGCAAG GACTCCACCT ACAGCCTGAG CAGCACCTG
 601 ACCCTGAGCA AGGCCGACTA CGAGAAGCAC AAGGTGTACG CCTGCGAAGT GACCCACCAG
 20 661 GGCCTGTCTA GCCCGTGAC CAAGAGCTT AACC GGGGCG AGTGC

SEQ ID NO:8 (H8 HC P41C)

1 QVQLVQSGAE VKKPGASVKV SCKASGYSFT GYMHVVKQS **Q**GGGLEWIGR
 51 INPNNGVTLY NQKFKDRVIM TRDTSISTAY MELSRLRSDD TAVYYCARST
 25 101 MITNYVMDYW GQGTILVTVSS

SEQ ID NO:9 (human IgG1 antibody HC constant region)

1 ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV
 30 51 HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP
 101 KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTPEVTCVVVDVSD
 151 HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
 201 EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTLC
 251 LVKGFYPSDI AVEWESNGQP ENNYKTTTPV LDSDGSFFLY SKLTVDKSRW
 35 301 QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

SEQ ID NO:10 (H8 HC P41C cDNA)

40

45

50

55

1 ATGGCCTGTC CTGGATTCT GTGGGCCCTC GTGATCAGCA CCTGTCTGGA ATTCAGCATG
 61 GCCCAGGTGC AGCTGGTGCA GTCTGGCGCC GAAGTGAAGA AACCAGGCGC CAGCGTGAAG
 121 GTGTCCTGCA AGGCCAGCGG CTACAGCTTC ACCGGCTACT ACATGCACTG GGTCAAGCAG
 181 AGCTGCGGCC AGGGCCTGGA ATGGATCGG AGAATCAACC CCAACAACGG CGTGACCCTG
 5 241 TACAACCAGA AATTCAAGGA CCGCGTGACC ATGACCCGGG ACACCAGCAT CAGCACCGCC
 301 TACATGGAAC TGAGCCGGCT GAGAAGCGAC GACACCGCCG TGTACTACTG CGCCCGGTCC
 361 ACCATGATCA CCAACTACGT GATGGACTAC TGGGGCCAGG GCACCCTCGT GACAGTGTCT
 421 AGCGCCAGCA CAAAGGGCCC CAGCGTGTTC CCTCTGGCCC CTAGCAGCAA GAGCACATCT
 481 GCGGGAACAG CCGCCCTGGG CTGCCTCGTG AAGGATTACT TCCCCGAGCC CGTGACCCTG
 10 541 TCCTGGAATA GCGGAGCCCT GACAAGCGGC GTGCACACCT TTCCAGCCGT GCTGCAGAGC
 601 AGCGGCCTGT ACTCTCTGAG CAGCGTCTGT ACTGTGCCA GCAGCAGCCT GGGCACCAG
 661 ACCTACATCT GCAACGTGAA CCACAAGCCC AGCAACACCA AGGTGGACAA GAAGTGGAA
 721 CCCAAGAGCT GCGACAAGAC CCACACCTGT CCCCCTTGTC CTGCCCCTGA ACTGCTGGGG
 781 GGACCTTCCG TGTTCTGTG CCCCCAAAG CCCAAGGACA CCCTGATGAT CAGCCGGACC
 841 CCCGAAGTGA CTGCGTGGT GGTGGATGTG TCCCACGAGG ACCCTGAAGT GAAGTCAAT
 15 901 TGGTACGTGG ACGGCGTGGG AGTGACAAC GCCAAGACCA AGCCCAGAGA GGAACAGTAC
 961 AACAGCACCT ACCGGGTGGT GTCCGTGCTG ACAGTGCTGC ACCAGGACTG GCTGAACGGC
 1021 AAAGAGTACA AGTGCAAGGT GTCCAACAAA GCCCTGCCTG CCCCATCGA GAAAACCATC
 1081 AGCAAGGCCA AGGGCCAGCC CCGCGAACCC CAGGTGTACA CACTGCCTCC CAGCCGGGAA
 1141 GAGATGACCA AGAACCAGGT GTCCCTGACA TGCCTCGTGA AAGGCTTCTA CCCCTCCGAT
 20 1201 ATCGCCGTGG AATGGGAGAG CAACGGCCAG CCCGAGAACAA ACTACAAGAC CACCCCCCT
 1261 GTGCTGGACA GCGACGGCTC ATTCTTCTG TACAGCAAGC TGACCGTGGG CAAGTCCCGG
 1321 TGGCAGCAGG GCAACGTGTT CAGCTGCAGC GTGATGCAGG AGGCCCTGCA CAACCACTAC
 1381 ACCCAGAAGT CCCTGAGCCT GAGCCCCGGC AAA

SEQ ID NO:11 (H8 LC)

25 1 DIVMTQSPDS LAVSLGERAT INCKASQSVS NDVAWYQQKP GQSPKLLISY
 51 TSSRYAGVPD RFSGSGSGTD FTLTISSLQA EDVAVYFCQQ DYNSPPTFGG
 101 GTKLEIK

30 SEQ ID NO:12 (H8 LC cDNA)

1 ATGGCCTGTC CTGGATTCT GTGGGCCCTC GTGATCAGCA CCTGTCTGGA ATTCAGCATG
 61 GCCGACATCG TGATGACCCA GAGCCCCGAT AGCCTGGCCG TGTCTCTGGG AGAGAGAGCC
 121 ACCATCAACT GCAAGGCCAG CCAGAGCGTG TCCAACGACG TGGCCTGGTA TCAGCAGAAG
 35 181 CCCGGCCAGA GCCCTAAGCT GCTGATCTCC TACACCAGCA GCAGATATGC CGGCGTGCCC
 241 GACAGATTTT CCGGCAGCGG CTCTGGCACC GACTTCACCC TGACAATCAG CTCCCTGCAG
 301 GCCGAGGACG TGGCCGTGTA CTTCTGTCAG CAAGACTACA ACAGCCCCC CACCTTCGGC
 361 GGAGGCACCA AGCTGGAAT CAAGCGGACA GTGGCCGCTC CCAGCGTGT CATCTTCCCA
 421 CCTAGCGACG AGCAGCTGAA GTCCGGCACA GCCTCTGTG TGTGCTGCT GAACAACCTC
 481 TACCCCGCG AGGCCAAGGT GCAGTGAAG GTGGACAATG CCCTGCAGAG CGGCAACAGC
 40 541 CAGGAAAGCG TGACCGAGCA GGACAGCAAG GACTCCACCT ACAGCCTGAG CAGCACCTG
 601 ACCCTGAGCA AGGCCGACTA CGAGAAGCAC AAGGTGTACG CCTGCGAAGT GACCCACCAG
 661 GGACTGAGCA GCCCTGTGAC CAAGAGCTTC AACCGGGGCG AGTGC

45 SEQ ID NO:13 (germline leader sequence)

1 MDWTWRILFL VAAATGAHS

SEQ ID NO:14 (natalizumab HC)

50 1 QVQLVQSGAE VKKPGASVKV SCKASGFNIK DTYIHWRQA PGQRLEWMGR
 51 IDPANGYTKY DPKFQGRVTI TADTSASTAY MELSSLRSED TAVYYCAREG
 101 YYGNYGVYAM DYWGQTLVLT VSS

SEQ ID NO:15 (natalizumab HC S225P, S375C)

55

1 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSKV
 51 HTFPAVLQSS GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES
 101 KYGPPCPPCP APEFLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSQED
 151 PEVQFNWYVD GVEVHNAKTK PREEQFNSTY RVVSVLTVLH QDWLNGKEYK
 201 CKVSNKGLPS SIEKTISKAK GQPPEPQVYT LPPSQEEMTK NQVSLTCLVK
 251 GFYPCDIAVE WESNGQPENN YKTPPVLDL DGSFFLYSRL TVDKSRWQEG
 301 NVFSCSVME ALHNHYTQKS LSLSLGK

SEQ ID NO:16 (natalizumab HC S225P, S375C cDNA)

10

1 ATGGA CTGGA CCTGGCGCAT CCTGTTTCTG GTGGCCGCTG CTACCGGCGC TCACTCCCAG
 61 GTGCAGCTGG TGCAGTCTGG CGCCGAAGTG AAGAAACCTG GCGCCTCCGT GAAGGTGTCC
 121 TGCAAGGCCT CCGGCTTCAA CATCAAGGAC ACCTACATCC ACTGGGTCCG ACAGGCCCTC
 181 GGACAGCGGC TGGAAATGGAT GGGCAGAATC GACCCCGCCA ACGGCTACAC TAAGTACGAC
 241 CCCAAGTTCC AGGGCAGAGT GACCATCACC GCCGACACCT CCGCCTCCAC AGCCTACATG
 301 GAACTGTCTT CCCTGCGGAG CGAGGACACC GCCGTGTACT ACTGCGCCAG AGAGGGCTAC
 361 TACGGCAACT ACGGCGTGTG CGCCATGGAC TACTGGGGCC AGGGCACCTT GGTACCCGTG
 421 TCCTCCGCTT CCACCAAGGG CCCCTCCGTG TTCCCTCTGG CCCCTTGCTC CCGGTCCACC
 481 TCCGAGTCTA CCGCCGCTCT GGGCTGCCTG GTCAAGGACT ACTTCCCCGA GCCCGTGACC
 541 GTGTCTTGGG ACTCTGGGCG CCTGACCTCT GGCGTGCACA CCTTCCCTGC TGTGTGCGAG
 601 TCCTCCGGCC TGTACTCCCT GTCCTCCGTC GTGACCGTGC CCTCCAGTC CTTGGGCACC
 661 AAGACCTACA CCTGTAACGT GGACCAACG CCCTCCAACA CCAAGGTGGA CAAGCGGGTG
 721 GAATCTAAGT ACGGCCCTCC CTGCCCCCCC TGCCCTGCCC CTGAATTTCT GGGCGGACCT
 781 TCCGTGTTCC TGTTCCCCC AAAGCCCAAG GACACCCTGA TGATCTCCCG GACCCCGAA
 841 GTGACCTGCG TGGTGGTGGG CGTGTCCCAG GAAGATCCCG AGGTCCAGTT CAATTGGTAC
 901 GTGGACGGCG TGGAAATGCA CAACGCCAAG ACCAAGCCCA GAGAGGAACA GTTCAACTCC
 961 ACCTACCGGG TGGTGTCCGT GCTGACCGTG CTGCACCAGG ACTGGCTGAA CGGCAAAGAG
 1021 TACAAGTGCA AGGTGTCCAA CAAGGGCCTG CCCAGCTCCA TCGAAAAGAC CATCTCCAAG
 1081 GCCAAGGGAC AGCCTCGCGA GCCCCAGGTG TACACCCTGC CTCCAAGCCA GGAAGAGATG
 1141 ACCAAGAACC AGGTGTCCCT GACCTGTCTG GTCAAGGGCT TCTACCCCTG CGATATCGCC
 1201 GTGGAATGGG AGTCCAACGG CCAGCCCGAG AACAATAACA AGACCACCC CCGTGTGCTG
 1261 GACTCCGACG GCTCCTTCTT CTGTACTCT CCGGTGACCG TGGACAAGTC CCCTGCGCAG
 1321 GAAGGCAACG TCTTCTCTG CTCCGTGATG CACGAGGCC TGCACAACCA CTACACCCAG
 1381 AAGTCCCTGT CCCTGAGCCT GGGCAAG

15

20

25

30

SEQ ID NO:17 (germline leader sequence)

35

1 MDMRVPAQLL GLLLLWLRGA RC

SEQ ID NO:18 (natalizumab LC)

40

1 DIQMTQSPSS LSASVGDRTV ITCKTSQDIN KYMAWYQQTP GKAPRLLIHY
 51 TSALQPGIPS RFGSGSGGRD YTFTISSLQP EDIATYYCLQ YDNLWTFGQG
 101 TKVEIK

SEQ ID NO:19 (natalizumab LC cDNA)

45

1 ATGGACATGA GAGTGCCCGC CCAGCTGCTG GGACTGCTGC TGCTGTGGCT GAGAGGCGCC
 61 AGATGCGACA TCCAGATGAC CCAGTCCCCC TCCAGCCTGT CCGCCTCCGT GGGCGACAGA
 121 GTGACCATCA CATGCAAGAC CTCCCAGGAC ATCAACAAGT ACATGGCCTG GTATCAGCAG
 181 ACCCCCGGCA AGGCCCTCTG GCTGTGATC CACTACACCT CCGCTCTGCA GCCTGGCATT
 241 CCCTCCAGAT TCTCCGGCTC CGGCTCTGGC CGGGACTATA CCTTACCAT CTCCAGTCTG
 301 CAGCCGAGG ATATCGCCAC CTACTACTGC CTGCACTACG ACAACCTGTG GACCTTCGGC
 361 CAGGGCACCA AGGTGAAAAT CAAGCGGACC GTGGCCGCTC CCTCCGTGTT CATCTTCCCA
 421 CCCTCCGACG AGCAGCTGAA GTCCGGCACC GCCTCCGTCG TGTGCCTGCT GAACAACCTC
 481 TACCCCGCGC AGGCCAAGGT GCAGTGGAAAG GTGGACAACG CCCTGCAGTC CGGCAACTCC
 541 CAGGAATCCG TCACCGAGCA GGACTCCAAG GACAGCACCT ACTCCCTGTC TCCACCCTG
 601 ACCCTGTCCA AGGCCGACTA CGAGAAGCAC AAGGTGTACG CCTGCGAAGT GACCCACCAG
 661 GCCTGTCCA GCCCGTGAC CAAGTCCTT AACCGGGGCG AGTGC

55

SEQUENCE LISTING

[0117]

5 <110> ARIAANS Gerardus Joseph Andreas COUMANS Rudy Gerardus Elisabeth Synthon Biopharmaceuticals B.V.

<120> SITE-SPECIFIC CONJUGATION OF LINKER DRUGS TO ANTIBODIES AND RESULTING ADCS

10 <130> P1664PC00

<150> EP14169493.5

<151> 2014-05-22

15 <160> 19

<170> BiSSAP 1.3

<210> 1

20 <211> 21

<212> PRT

<213> Artificial Sequence

<220>

25 <223> Leader sequence

<400> 1

30 Met Ala Cys Pro Gly Phe Leu Trp Ala Leu Val Ile Ser Thr Cys Leu
 1 5 10 15
 Glu Phe Ser Met Ala
 20

<210> 2

35 <211> 115

<212> PRT

<213> Artificial Sequence

<220>

40 <223> Heavy chain variable region of anti-PSMA antibody

<400> 2

45 Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
 1 5 10 15
 Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr Glu Tyr
 20 25 30
 Thr Ile His Trp Val Lys Gln Ala Cys Gly Lys Gly Leu Glu Trp Ile
 35 40 45
 50 Gly Asn Ile Asn Pro Asn Asn Gly Gly Thr Thr Tyr Asn Gln Lys Phe
 50 55 60
 Glu Asp Arg Ala Thr Leu Thr Val Asp Lys Ser Thr Ser Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 55 Ala Ala Gly Trp Asn Phe Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr
 100 105 110
 Val Ser Ser
 115

<210> 3
 <211> 330
 <212> PRT
 <213> Artificial Sequence

5

<220>
 <223> Heavy chain constant region of anti-PSMA antibody

<400> 3

10

	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe	Pro	Leu	Ala	Pro	Ser	Ser	Lys
	1			5					10					15		
	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr
			20						25					30		
15	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser
			35					40					45			
	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser
		50					55					60				
	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr
20	65					70						75				80
	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys
				85							90				95	
	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys
				100					105					110		
25	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro
			115					120					125			
	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys
		130					135					140				
	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp
							150					155				160
30	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu
					165						170				175	
	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu
				180										190		
	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn
35			195					200					205			
	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly
		210					215					220				
	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu
						230						235				240
40	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr
					245						250				255	
	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn
				260					265					270		
	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe
			275					280					285			
45	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn
							295					300				
	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr
						310					315					320
50	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly	Lys						
					325						330					

55

<210> 4
 <211> 1398
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> cDNA sequence of heavy chain of anti-PSMA antibody

<400> 4

atggcctgtc ctggatttct gtgggccctc gtgatcagca cctgtctgga attcagcatg 60
 5 gccgaggtgc agctggtgca gtctggcgcc gaagtgaaga aaccaggcgc cagcgtgaag 120
 atcagctgca agaccagcgg ctacaccttc accgagtaca ccatccactg ggtcaagcag 180
 10 gcctgtggca agggcctgga atggatcggc aacatcaacc ccaacaacgg cggcaccacc 240
 tacaaccaga agttcgagga ccggggccacc ctgaccgtgg acaagagcac aagcaccgcc 300
 tacatggaac tgagcagcct gcggagcgag gacaccgccg tgtactattg tgccgccgga 360
 15 tggaacttcg actactgggg ccagggcacc accgtgacag tgtctagcgc cagcaciaaag 420
 ggccccagcg tgttcctct ggccccctagc agcaagtcta cctctggcgg aacagccgcc 480
 ctgggctgcc tcgtgaagga ctactttccc gagcccgtga ccgtgtcctg gaactctggc 540
 20 gctctgacaa gcggcgtgca cacctttcca gccgtgctgc agagcagcgg cctgtactct 600
 ctgagcagcg tcgtgactgt gcccagcagc agcctgggca cccagaccta catctgcaac 660
 25 gtgaaccaca agcccagcaa caccaaggtg gacaaaaagg tggaaaccaa gagctgcgac 720
 aagaccaca cctgtcccc ttgtcctgcc cctgaactgc tgggcggacc ttccgtgttc 780
 ctgttcccc caaagccaa ggacaccctg atgatcagcc ggacccccga agtgacctgc 840
 30 gtggtggtgg atgtgtcca cgaggacct gaagtgaagt tcaattggtc cgtggacggc 900
 gtggaagtgc acaacgcaa gaccaagccc agagaggaac agtacaacag cacctaccgg 960
 gtggtgtccg tgctgacagt gctgcaccag gactggctga acggcaaaga gtacaagtgc 1020
 35 aagggtgtcca acaaggccct gcctgcccc atcgagaaaa ccatcagcaa ggccaagggc 1080
 cagccccgcg aaccccaggt gtacacactg cctcccagca gggacgagct gaccaagaac 1140
 40 caggtgtccc tgacatgcct cgtgaaaggc ttctaccct ccgatatcgc cgtggaatgg 1200
 gagagcaacg gccagccga gaacaactac aagaccacc cccctgtgct ggacagcgac 1260
 ggctcattct tcctgtacag caagctgact gtggataagt cccggtggca gcagggcaac 1320
 45 gtgttcagct gcagcgtgat gcacgaggcc ctgcacaacc actacacca gaaaagcctg 1380
 tccctgagcc ccggcaag 1398

<210> 5

50 <211> 107

<212> PRT

<213> Artificial Sequence

<220>

55 <223> Light chain variable region of anti-PSMA antibody

<400> 5

Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ala
 20 25 30
 Val Asp Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45
 Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Phe Ala Asp Tyr Phe Cys Gln Gln Tyr Asn Ser Tyr Pro Leu
 85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
 100 105

<210> 6
 <211> 107
 <212> PRT
 <213> Artificial Sequence
 <220>
 <223> Light chain constant region of anti-PSMA antibody
 <400> 6

Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
 1 5 10 15
 Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
 20 25 30
 Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
 35 40 45
 Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
 50 55 60
 Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
 65 70 75 80
 Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
 85 90 95
 Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
 100 105

<210> 7
 <211> 705
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> cDNA sequence of light chain of anti-PSMA antibody
 <400> 7

atggcttgtc ctggatttct gtgggccctc gtgatcagca cctgtctgga attcagcatg 60
 gccgacatcg tgatgaccca gagccccagc tctctgagcg ccagcgtggg cgacagagtg 120
 5 accatcacat gcaaggccag ccaggacgtg ggcaccgccg tggattggta tcagcagaag 180
 cctggcaagg cccccaagct gctgatctac tgggccagca ccagacacac cggcgtgccc 240
 gatagattca caggcagcgg ctccggcacc gacttcaccc tgacaatcag cagcctgcag 300
 10 cccgaggact tcgccgacta cttctgccag cagtacaaca gctaccccct gaccttcggc 360
 ggaggcacca agctggaaat caagcggaca gtggccgctc ccagcgtggt catcttccca 420
 cctagcgagc agcagctgaa gtctggcacc gcctctgtcg tgtgcctgct gaacaacttc 480
 15 tcccccgcg aggccaaggt gcagtggaag gtggacaatg ccctgcagag cggcaacagc 540
 caggaaagcg tgaccgagca ggacagcaag gactccacct acagcctgag cagcaccctg 600
 20 accctgagca aggccgacta cgagaagcac aaggtgtacg cctgcgaagt gacccaccag 660
 ggctgtcta gccccgtgac caagagcttc aaccggggcg agtgc 705

25 <210> 8
 <211> 120
 <212> PRT
 <213> Artificial Sequence

30 <220>
 <223> H8 HC P41C

<400> 8

35	Gln	Val	Gln	Leu	Val	Gln	Ser	Gly	Ala	Glu	Val	Lys	Lys	Pro	Gly	Ala
	1			5						10					15	
	Ser	Val	Lys	Val	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Ser	Phe	Thr	Gly	Tyr
			20					25						30		
	Tyr	Met	His	Trp	Val	Lys	Gln	Ser	Cys	Gly	Gln	Gly	Leu	Glu	Trp	Ile
			35				40						45			
40	Gly	Arg	Ile	Asn	Pro	Asn	Asn	Gly	Val	Thr	Leu	Tyr	Asn	Gln	Lys	Phe
	50					55						60				
	Lys	Asp	Arg	Val	Thr	Met	Thr	Arg	Asp	Thr	Ser	Ile	Ser	Thr	Ala	Tyr
	65					70					75					80
	Met	Glu	Leu	Ser	Arg	Leu	Arg	Ser	Asp	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
					85					90					95	
45	Ala	Arg	Ser	Thr	Met	Ile	Thr	Asn	Tyr	Val	Met	Asp	Tyr	Trp	Gly	Gln
				100					105						110	
	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser								
			115					120								

50 <210> 9
 <211> 330
 <212> PRT
 <213> Artificial Sequence

55 <220>
 <223> human IgG1 antibody HC constant region

<400> 9

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
 1 5 10 15
 Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
 20 25 30
 5 Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
 35 40 45
 Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
 50 55 60
 10 Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
 65 70 75 80
 Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
 85 90 95
 Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
 100 105 110
 15 Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
 115 120 125
 Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
 130 135 140
 Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
 145 150 155 160
 20 Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
 165 170 175
 Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
 180 185 190
 His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
 195 200 205
 25 Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
 210 215 220
 Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
 225 230 235 240
 30
 Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
 245 250 255
 Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
 260 265 270
 35 Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
 275 280 285
 Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
 290 295 300
 Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
 305 310 315 320
 40 Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 325 330

<210> 10
 45 <211> 1413
 <212> DNA
 <213> Artificial Sequence
 <220>
 50 <223> H8 HC P41C cDNA
 <400> 10

	atggcctgtc ctggatttct gtgggccctc gtgatcagca cctgtctgga attcagcatg	60
	gccaggtgc agctggtgca gtctggcgcc gaagtgaaga aaccaggcgc cagcgtgaag	120
5	gtgtcctgca aggccagcgg ctacagcttc accggctact acatgcactg ggtcaagcag	180
	agctgctggc agggcctgga atggatcggc agaatcaacc ccaacaacgg cgtgaccctg	240
	tacaaccaga aattcaagga ccgctgacc atgaccggg acaccagcat cagcaccgcc	300
10	tacatggaac tgagccggct gagaagcgac gacaccgccg tgtactactg cgcccgtcc	360
	accatgatca ccaactacgt gatggactac tggggccagg gcaccctcgt gacagtgtct	420
	agcgcagca caaagggccc cagcgtgttc cctctggccc ctacgagcaa gagcacatct	480
15	ggcggaacag ccgccctggg ctgcctcgtg aaggattact tccccgagcc cgtgaccctg	540
	tcctggaata gcggagccct gacaagcggc gtgcacacct ttccagccgt gctgcagagc	600
20	agcggcctgt actctctgag cagcgtcgtg actgtgccc gcagcagcct gggcaccag	660
	acctacatct gcaacgtgaa ccacaagccc agcaacacca aggtggacaa gaaggtggaa	720
	ccaagagct gcgacaagac ccacacctgt ccccttctc ctgccctga actgctgggc	780
25	ggaccttccg tgttcctggt cccccaaag cccaaggaca ccctgatgat cagccggacc	840
	cccgaagtga cctgcgtggt ggtggatgtg tcccacgagg accctgaagt gaagttcaat	900
30	tggtagctgg acggcgtgga agtgcacaac gccaaagacca agcccagaga ggaacagtac	960
	aacagcacct accgggtggt gtccgtcgtg acagtgtctc accaggactg gctgaacggc	1020
	aaagagtaca agtgaaggt gtccaacaaa gccctgcctg ccccatcga gaaaaccatc	1080
35	agcaaggcca agggccagcc ccggaagccc caggtgtaca cactgcctcc cagccgggaa	1140
	gagatgacca agaaccaggt gtccctgaca tgcctcgtga aaggcttcta cccctccgat	1200
	atcgcctgga aatgggagag caacggccag cccgagaaca actacaagac cccccccct	1260
40	gtgctggaca gcgacggctc attcttctc tacagcaagc tgaccgtgga caagtcccgg	1320
	tggcagcagg gcaacgtggt cagctgcagc gtgatgcacg aggcctgca caaccactac	1380
45	accagaagt ccctgagcct gagccccggc aaa	1413

<210> 11

<211> 107

<212> PRT

50 <213> Artificial Sequence

<220>

<223> H8 LC

55 <400> 11

Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly
 1 5 10 15
 Glu Arg Ala Thr Ile Asn Cys Lys Ala Ser Gln Ser Val Ser Asn Asp
 20 25 30
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
 35 40 45
 Ser Tyr Thr Ser Ser Arg Tyr Ala Gly Val Pro Asp Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala
 65 70 75 80
 Glu Asp Val Ala Val Tyr Phe Cys Gln Gln Asp Tyr Asn Ser Pro Pro
 85 90 95
 Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
 100 105

15 <210> 12
 <211> 705
 <212> DNA
 <213> Artificial Sequence

20 <220>
 <223> H8 LC cDNA

25 <400> 12

atggcctgtc ctggatttct gtgggccctc gtgatcagca cctgtctgga attcagcatg 60
 gccgacatcg tgatgaccga gagccccgat agcctggccg tgtctctggg agagagagcc 120
 30 accatcaact gcaaggccag ccagagcgtg tccaacgacg tggcctggta tcagcagaag
 cccggccaga gccctaagct gctgatctcc tacaccagca gcagatatgc cggcgtgccc 240
 gacagatddd ccggcagcgg ctctggcacc gacttcaccc tgacaatcag ctccctgcag 300
 35 gccgaggacg tggccgtgta cttctgtcag caagactaca acagcccccc caccttcggc
 ggaggcacca agctggaaat caagcggaca gtggccgctc ccagcgtggt catcttccca 420
 cctagcgacg agcagctgaa gtccggcaca gcctctgtcg tgtgcctgct gaacaacttc 480
 40 taccctcgcg aggccaaggt gcagtggaag gtggacaatg ccctgcagag cggcaacagc
 caggaaagcg tgaccgagca ggacagcaag gactccacct acagcctgag cagcaccctg 600
 45 accctgagca aggccgacta cgagaagcac aaggtgtacg cctgcgaagt gaccaccag 660
 ggactgagca gcctgtgac caagagcttc aaccggggcg agtgc 705

50 <210> 13
 <211> 19
 <212> PRT
 <213> Artificial Sequence

55 <220>
 <223> germline leader sequence

<400> 13

Met Asp Trp Thr Trp Arg Ile Leu Phe Leu Val Ala Ala Ala Thr Gly
 1 5 10 15
 Ala His Ser

5 <210> 14
 <211> 123
 <212> PRT
 <213> Artificial Sequence

10 <220>
 <223> natalizumab HC

<400> 14

15 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
 1 5 10 15
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Thr
 20 Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Met
 35 40 45
 Gly Arg Ile Asp Pro Ala Asn Gly Tyr Thr Lys Tyr Asp Pro Lys Phe
 50 55 60
 Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr
 65 70 75 80
 25 Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Glu Gly Tyr Tyr Gly Asn Tyr Gly Val Tyr Ala Met Asp Tyr
 100 105 110
 Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 115 120

30 <210> 15
 <211> 327
 <212> PRT
 <213> Artificial Sequence

35 <220>
 <223> natalizumab HC S225P, S375C

<400> 15

40 Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg
 1 5 10 15
 Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
 20 25 30
 45 Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
 35 40 45
 Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
 50 55 60
 Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Lys Thr
 65 70 75 80
 55 Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys

				85					90				95			
	Arg	Val	Glu	Ser	Lys	Tyr	Gly	Pro	Pro	Cys	Pro	Pro	Cys	Pro	Ala	Pro
				100					105				110			
5	Glu	Phe	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys
			115					120					125			
	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val
			130					135				140				
	Asp	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln	Phe	Asn	Trp	Tyr	Val	Asp
						150					155					160
10	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Phe
					165						170				175	
	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp
				180						185				190		
	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Gly	Leu
			195					200					205			
15	Pro	Ser	Ser	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg
							215						220			
	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Gln	Glu	Glu	Met	Thr	Lys
						230					235					240
	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Cys	Asp
20					245						250				255	
	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys
				260					265					270		
	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser
								280					285			
25	Arg	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Glu	Gly	Asn	Val	Phe	Ser
							295					300				
	Cys	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser
						310					315					320
	Leu	Ser	Leu	Ser	Leu	Gly	Lys									
30					325											

<210> 16
 <211> 1407
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> natalizumab HC S225P, S375C cDNA
 <400> 16

	atggactgga cctggcgcac cctgtttctg gtggccgctg ctaccggcgc tcaactcccag	60
	gtgcagctgg tgcagtctgg cgccgaagtg aagaaacctg ggcctccgt gaaggtgtcc	120
5	tgcaaggcct ccggcttcaa catcaaggac acctacatcc actgggtccg acaggcccct	180
	ggacagcggc tggaatggat gggcagaatc gaccccgcca acggctacac taagtacgac	240
	cccaagttcc agggcagagt gaccatcacc gccgacacct ccgcctccac agcctacatg	300
10	gaactgtcct ccctgcggag cgaggacacc gccgtgtact actgcgccag agagggtac	360
	tacggcaact acggcgtgta cgccatggac tactggggcc agggcaccct ggtcaccgtg	420
	tcctccgctt ccaccaaggg cccctccgtg ttccctctgg ccccttgctc ccggtccacc	480
15	tccgagtcta ccgccgctct gggctgcctg gtcaaggact acttccccga gcccggtgacc	540
	gtgtcctgga actctggcgc cctgacctct ggcgtgcaca ccttccctgc tgtgctgcag	600
20	tcctccggcc tgtactccct gtcctccgtc gtgaccgtgc cctccagctc cctgggcacc	660
	aagacctaca cctgtaacgt ggaccacaag ccctccaaca ccaaggtgga caagcgggtg	720
	gaatctaagt acggccctcc ctgccccccc tgcctgccc ctgaatttct gggcggacct	780
25	tccgtgttcc tgttcccccc aaagcccaag gacaccctga tgatctcccg gacccccgaa	840
	gtgacctgcg tgggtggtgga cgtgtcccag gaagatcccg aggtccagtt caattggtac	900
30	gtggacggcg tggaagtgca caacgccaaag accaagcccc gagaggaaca gttcaactcc	960
	acctaccggg tgggtgtccgt gctgaccgtg ctgcaccagg actggctgaa cggcaaagag	1020
	tacaagtgca aggtgtccaa caagggcctg cccagctcca tcgaaaagac catctccaag	1080
35	gccaagggac agcctcgca gccccaggtg tacaccctgc ctccaagcca ggaagagatg	1140
	accaagaacc aggtgtccct gacctgtctg gtcaagggct tctaccctg cgatatcgcc	1200
	gtggaatggg agtccaacgg ccagcccag aacaactaca agaccacccc ccctgtgctg	1260
40	gactccgacg gtccttctt cctgtactct cggctgaccg tggacaagtc ccggtggcag	1320
	gaaggcaacg tcttctcctg ctccgtgatg cacgaggccc tgcacaacca ctacaccag	1380
45	aagtcctgt ccctgagcct gggcaag	1407

<210> 17

<211> 22

<212> PRT

50 <213> Artificial Sequence

<220>

<223> germline leader sequence

55 <400> 17

Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Leu Trp
 1 5 10 15
 Leu Arg Gly Ala Arg Cys
 20

5

<210> 18
 <211> 106
 <212> PRT
 <213> Artificial Sequence

10

<220>
 <223> natalizumab LC

15

<400> 18

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Thr Ser Gln Asp Ile Asn Lys Tyr
 20 25 30
 Met Ala Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Arg Leu Leu Ile
 35 40 45
 His Tyr Thr Ser Ala Leu Gln Pro Gly Ile Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Arg Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln Tyr Asp Asn Leu Trp Thr
 85 90 95
 Phe Gly Gln Gly Thr Lys Val Glu Ile Lys

30

100 105

<210> 19
 <211> 704
 <212> DNA
 <213> Artificial Sequence

35

<220>
 <223> natalizumab LC cDNA

40

<400> 19

45

50

55

	atggacatga	gagtgcccg	ccagctgctg	ggactgctgc	tgctgtggct	gagaggcgcc	60
	agatgcgaca	tccagatgac	ccagtcccc	tccagcctgt	ccgcctccgt	gggcgacaga	120
5	gtgaccatca	catgcaagac	ctcccaggac	atcaacaagt	acatggcctg	gtatcagcag	180
	acccccggca	aggcccctcg	gctgctgac	cactacacct	ccgctctgca	gcctggcatc	240
	ccctccagat	tctccggctc	cggctctggc	cgggactata	ccttcacccat	ctccagtctg	300
10	cagcccgagg	atatcgccac	ctactactgc	ctgcagtagc	acaacctgtg	gaccttcggc	360
	cagggcacca	aggtggaaat	caagcggacc	gtggccgctc	cctccgtggt	catcttccca	420
15	ccctccgacg	agcagctgaa	gtccggcacc	gcctccgtcg	tgtgcctgct	gaacaacttc	480
	tacccccgcg	aggccaaggt	gcagtggaag	gtggacaacg	ccctgcagtc	cggcaactcc	540
	caggaatccg	tcaccgagca	ggactccaag	gacagcaact	actccctgtc	tccaccctga	600
20	ccctgtccaa	ggccgactac	gagaagcaca	aggtgtacgc	ctgcgaagtg	accaccagg	660
	gcctgtccag	ccccgtgacc	aagtccttca	accggggcga	gtgc		704

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 2006034488 A [0007] [0009] [0043]
- WO 2014124316 A [0009]
- WO 2011133039 A [0040] [0095]
- WO 2005084390 A [0043]
- WO 9803873 A [0057]
- WO 02098897 A [0057]
- WO 2007002222 A [0057]
- WO 2011069019 A [0057]
- WO 2006031653 A [0057]
- WO 2007106744 A [0057]
- EP 14169493 [0117]

Non-patent literature cited in the description

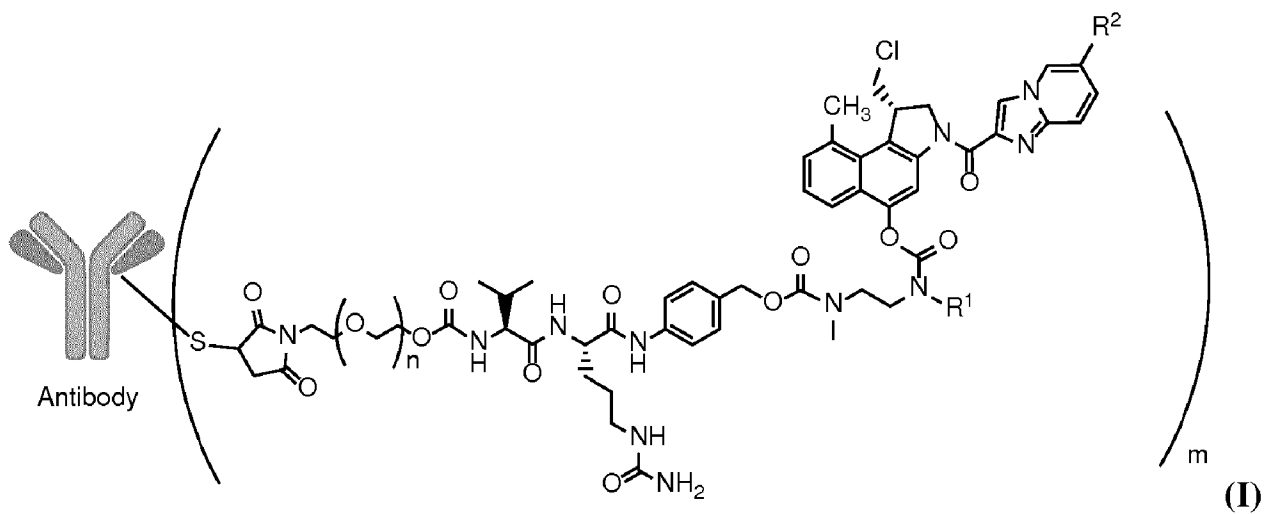
- C.R. BEHRENS ; B. LIU. *mAbs*, 2014, vol. 6 (1), 1-8 [0002]
- SUTHERLAND et al. *Blood*, 2013, vol. 122 (8), 1455-1463 [0008]
- KRIEGER et al. *Proteins*, 2009, vol. 77 (9), 114-122 [0020]
- TROTT O ; OLSON AJ. *J. Comput. Chem.*, 2010, vol. 31, 455-461 [0021]
- KABAT, E.A. et al. Sequences of Proteins of Immunological Interest. Public Health Service, National Institutes of Health, 1991 [0024]
- KABAT, E.A. et al. Sequences of Proteins of Immunological Interest. Public Health Service, National Institutes of Health, 1991, 662, , 680, , 689 [0025]
- EDELMAN, G.M. et al. *Proc. Natl. Acad. Sci. USA*, 1969, vol. 63, 78-85 [0025]
- FAN et al. *Breast Cancer Res.*, 2012, vol. 14, R116 [0031]
- BREZSKY et al. *PNAS*, 2009, vol. 106, 17864-17869 [0031]
- DORONINA et al. *Bioconjugate Chem.*, 2006, vol. 17, 114-124 [0043]
- ZEUTHEN J. et al. *Int. J. Cancer*, 1980, vol. 25 (1), 19-32 [0114]

STEDSSPECIFIK KONJUGATION AF LINKER MEDICIN TIL ANTISTOF OG
 RESULTERENDE ADCS

PATENTKRAV

1. En antistof medicin konjugations forbindelse hvori en
 5 linker medicin er stedsspecifik konjugeret til et IgG1
 antistof gennem en konstrueret cystein i nævnte antistof
 i tung kæde position 41 i henhold til Kabat nummerering,
 hvori nævnte linker medicin indeholder et duocarmycin
 derivat.
- 10 2. Forbindelsen i henhold til patentkrav 1, endvidere
 indeholdende en konstrueret cystein i position 375 i Fc
 delen af nævnte antistof i henhold til EU nummerering.
3. Forbindelsen i henhold til patentkrav 1 eller 2 med
 formelen (I)

15



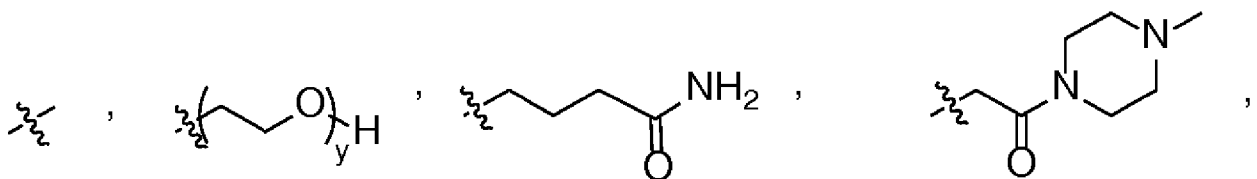
hvor

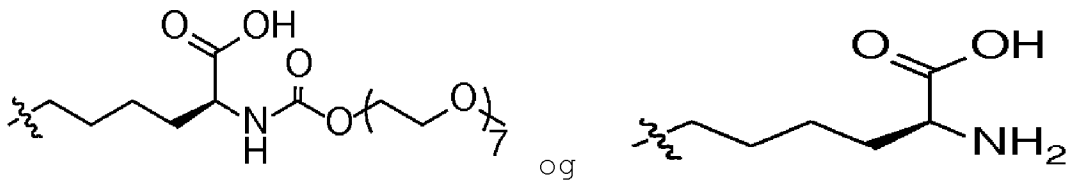
n er 0-3,

m repræsenterer en gennemsnits DAR på fra 1 til 6,

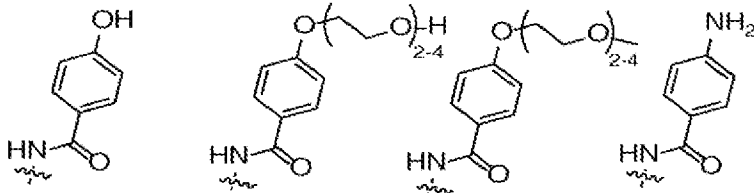
R¹ er udvalgt fra

20

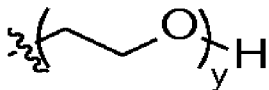




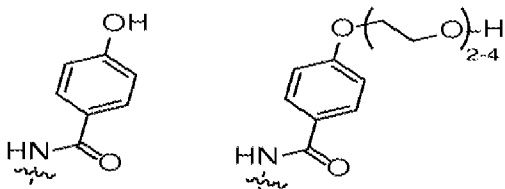
y er 1-16, og
R² er udvalgt fra



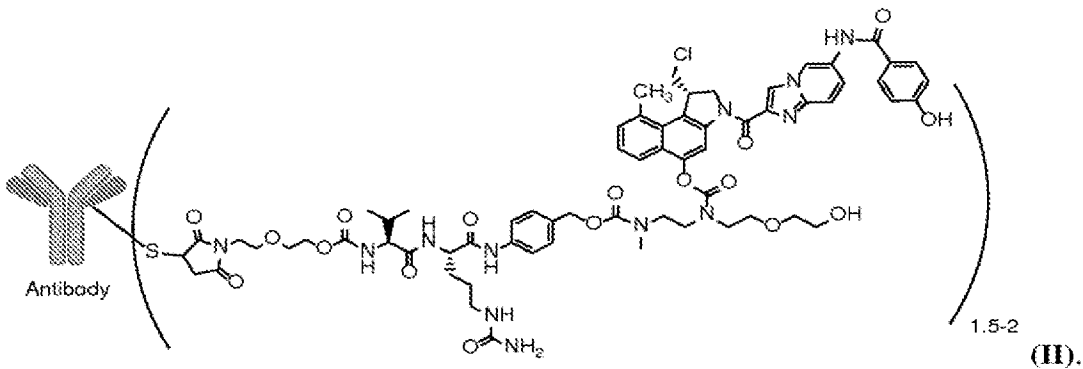
- 5 4. Forbindelsen i henhold til patentkrav 3, hvori
n er 0-1,
m repræsenterer en gennemsnits DAR på fra 1,5 til 2,
R¹ er



- 10 y er 1-4, og
R² er udvalgt fra



5. Forbindelsen i henhold til et af patentkravene 1-4 med
formlen (II)



6. Forbindelsen i henhold til et af patentkravene 1-5, hvori nævnte antistof binder til et antigen target som er udtrykt i eller på en cellemembran af en tumorcelle og hvori nævnte antistof er internaliseret af cellen efter bindingen til nævnte target.
7. Forbindelsen i henhold til et af patentkravene 1-6, hvori nævnte antistof er et anti-annexin A1 antistof, et anti-CD115 antistof, et anti-CD123 antistof, et anti-CLL-1 antistof, et anti-c-MET antistof, et anti-MUC1 antistof, et anti-PSMA antistof, et anti-5T4 antistof eller et anti-TF antistof.
8. Forbindelsen i henhold til et af patentkravene 1 til 7, hvori nævnte antistof er et anti-PSMA monoklonalt antistof eller et anti-5T4 monoklonalt antistof.
9. Forbindelsen i henhold til patentkrav 8, hvori den tunge kæde af nævnte anti-PSMA antistof indeholder aminosyre sekvensen af SEQ ID NR:2 og den lette kæde af nævnte anti-PSMA antistof indeholder aminosyre sekvensen af SEQ ID NR:5.
10. Forbindelsen i henhold til patentkrav 8, hvori den tunge kæde af nævnte anti-5T4 antistof indeholder aminosyre sekvensen af SEQ ID NR:8 og den lette kæde af nævnte anti-5T4 antistof indeholder aminosyre sekvensen af SEQ ID NR:11.
11. En farmaceutisk sammensætning indeholdende en forbindelse i henhold til et af patentkravene 1-10 og en eller flere farmaceutisk acceptable hjælpestoffer, fortrinsvis i form af et frysetørret pulver.
12. Forbindelsen i henhold til et af patentkravene 1-10 eller den farmaceutiske sammensætning i henhold til patentkrav 11 til brug som en medicin.
13. Forbindelsen i henhold til et af patentkravene 1-10 eller den farmaceutiske sammensætning i henhold til patentkrav 11 til brug i behandlingen af humane faste tumorer og hæmatologiske maligniteter.
14. Forbindelsen eller den farmaceutiske sammensætning til brug i henhold til patentkrav 13, hvori de humane faste

tumorer er udvalgt fra gruppen bestående af brystkræft, mavekræft, tyktarmskræft, blærekræft, livmoderhalskræft, livmoderkræft, lungekræft, lungehindkræft, leverkræft, bugspytkirtelkræft og prostatakkræft.

DRAWINGS



Figure 1A

Figure 1B

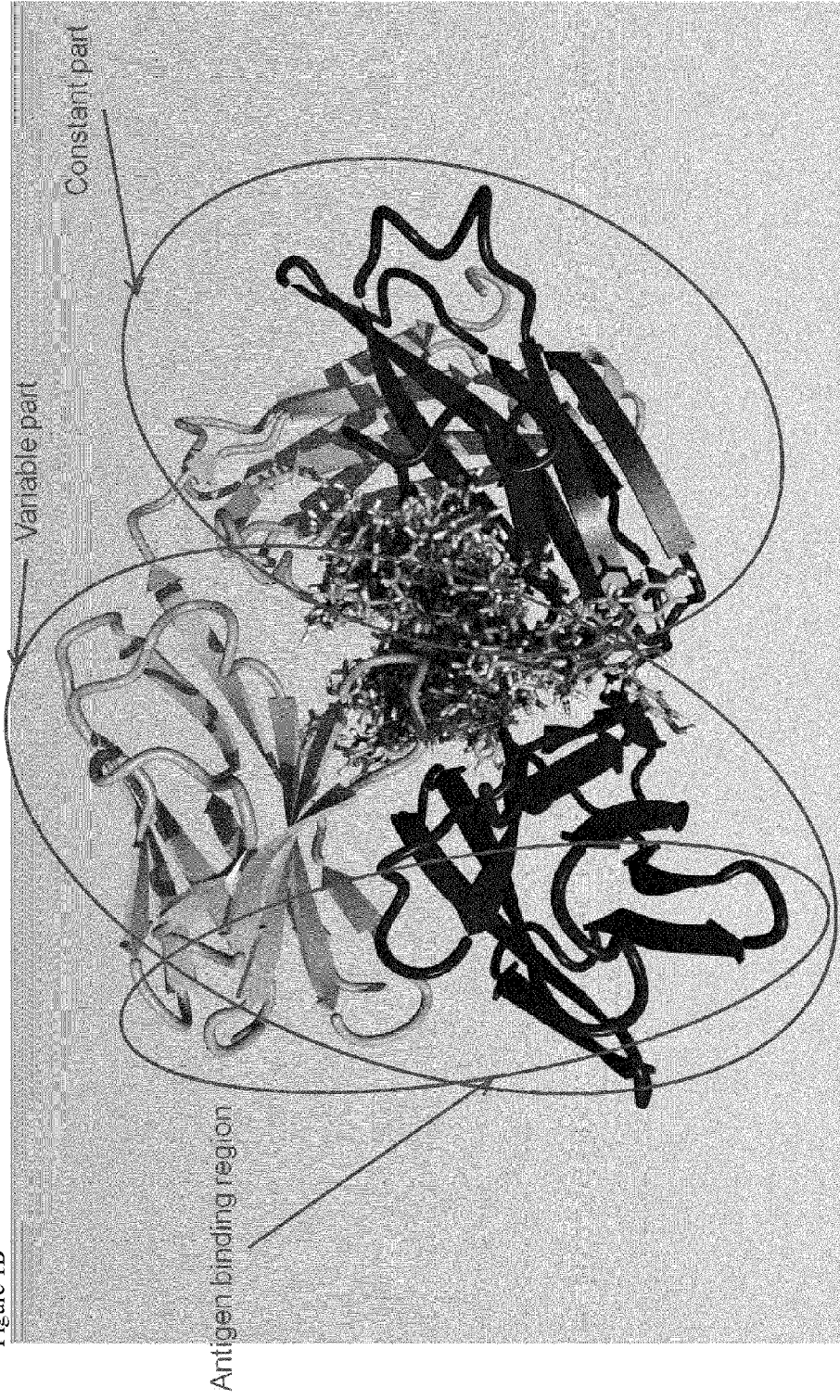


Figure 1C



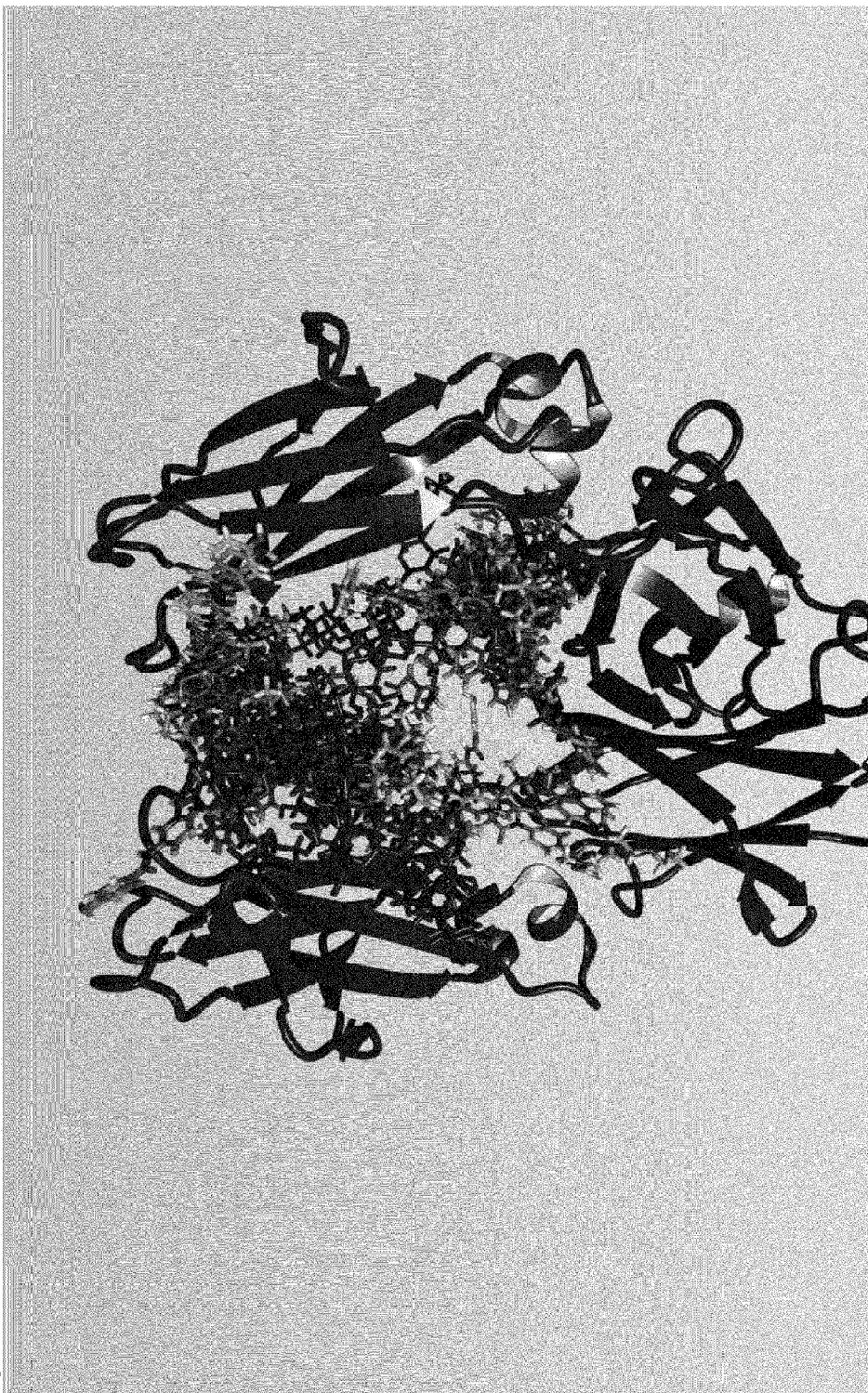


Figure 1D

Figure 2A

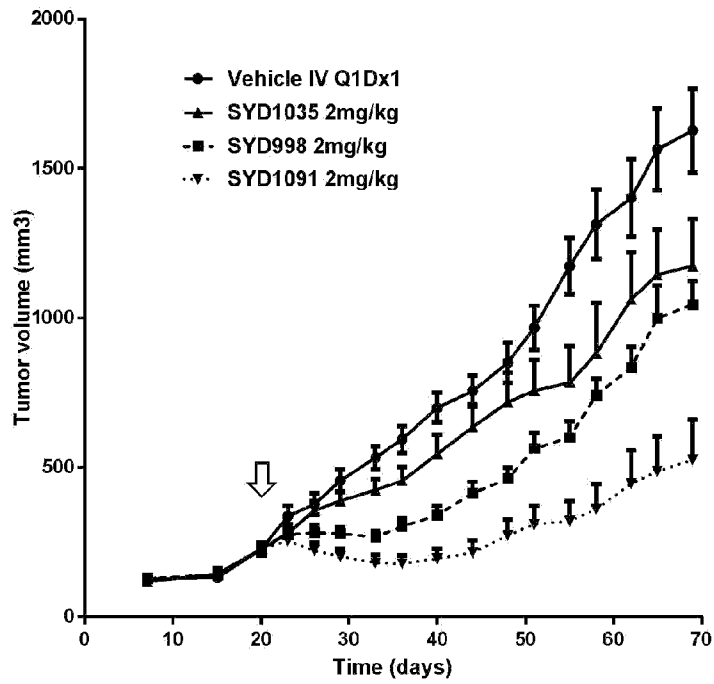


Figure 2B

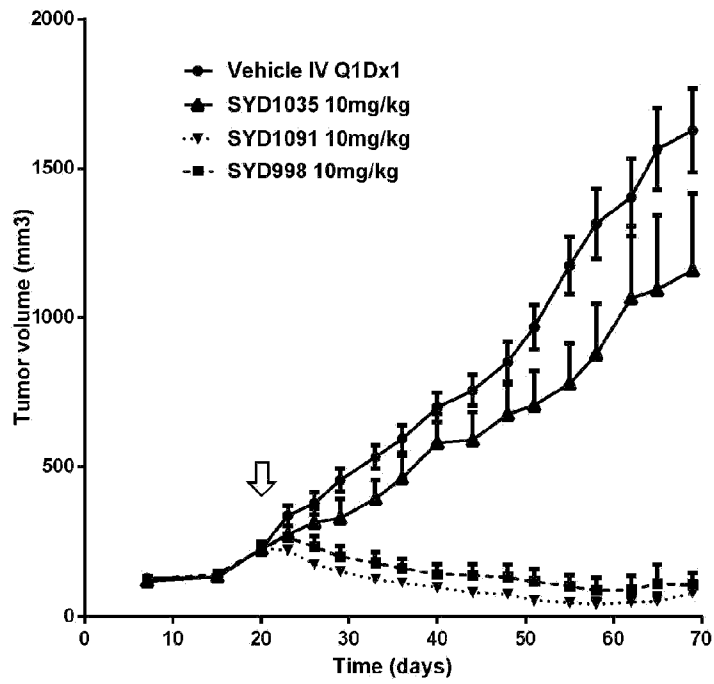


Figure 3

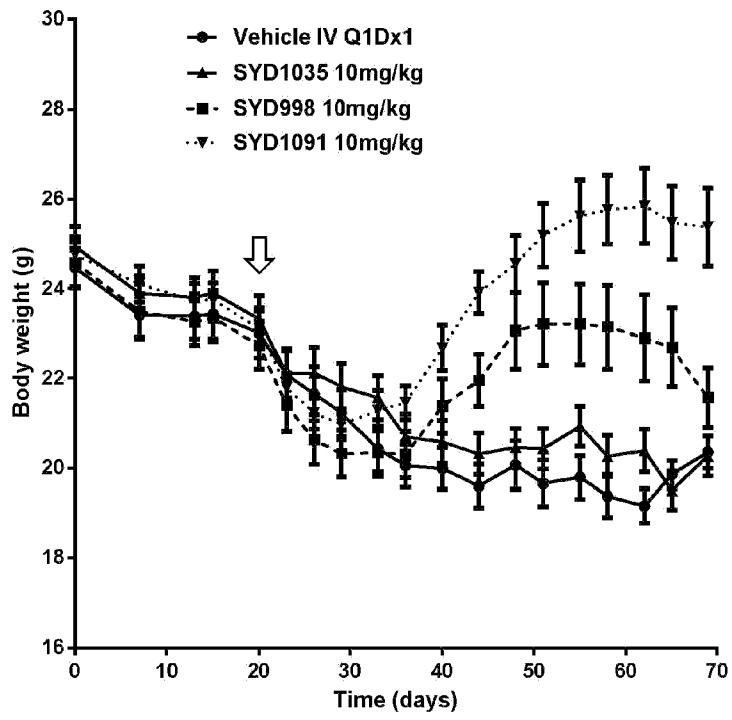


Figure 4A

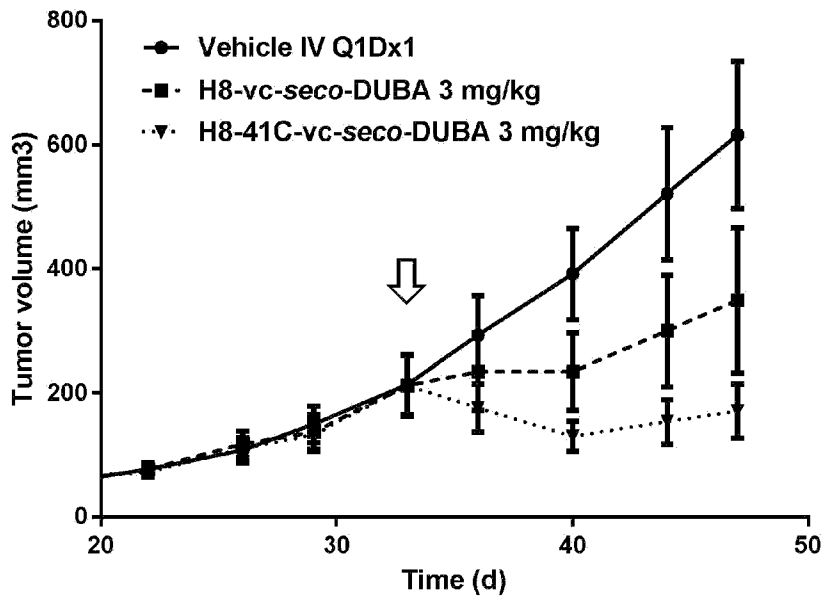


Figure 4B

