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

FIELD OF THE INVENTION

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.

BACKGROUND OF THE PRESENT INVENTION

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.

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.

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.

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.

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.

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.

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 $CuSO_4$ 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).

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.

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.

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 cq. 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).

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

The present invention relates to antibody-drug conjugates (ADCs) wherein a linker drug is site-specifically conjugated to an antibody through an engineered cysteine at one or more specific positions 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

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

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.

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).

The present invention 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).

In one embodiment, the present invention 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, 152, 153, 155, 171, 247, 297, 339 and 375, and light chain 40, 41, and 165.

In a particularly preferred embodiment, the present invention relates to an antibody-drug conjugate 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 and 89 (according to Kabat numbering) and light chain 40 and 41 (according to Kabat numbering).

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.

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

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.

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.

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).

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.

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.

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)).

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.

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.

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.

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 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.

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 claimed 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 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.

In one particular embodiment, the present invention 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, 152, 153, 247, 339 and 375, and light chain 40, 41, and 165.

In another embodiment, the present invention 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, 247, 297 and 376, and light chain 40 and 41.

In one embodiment, the present invention 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, 152, 153, 155 and 171, and light chain 40, 41, 165 and 168 in the Fab part of said antibody.

In another embodiment, the present invention 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, 152 and 153, and light chain 40, 41 and 165 in the Fab part of said antibody.

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, in a particularly preferred embodiment, the present invention 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, most preferably 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.

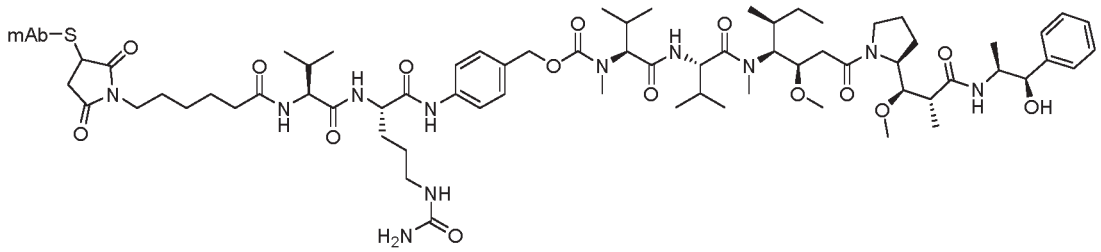
In a specific embodiment, the antibody-drug conjugate (ADC) compound of the above preferred embodiment may further comprise an additional engineered cysteine at one or more positions of the antibody selected from heavy chain 152, 153, 155, 171, 339 and 375, and light chain 165 and 168. Preferably said further engineered cysteine is at heavy chain position 375 in the Fc part of said antibody.

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.

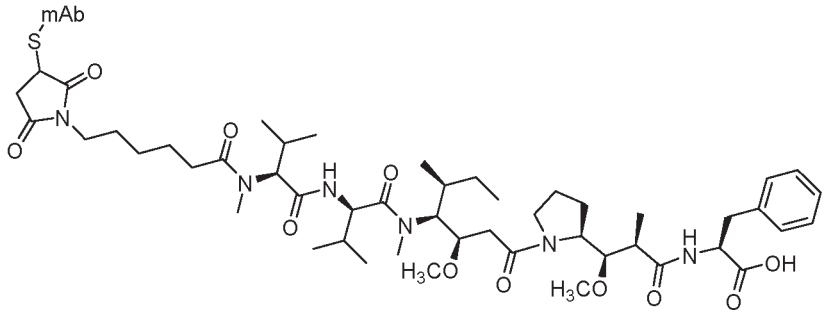
In accordance with the present invention, any linker drug known in the art of ADCs can be used for site-specific conjugation to an antibody, provided it has a chemical group which can react with the thiol group of an engineered cysteine, typically a maleimide or haloacetyl group. Suitable linker drugs 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 in accordance with the present invention. Suitable examples of maytansinoid drugs include DM1 and DM4. Suitable examples of auristatin drugs include MMAE and MMAF.

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).

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



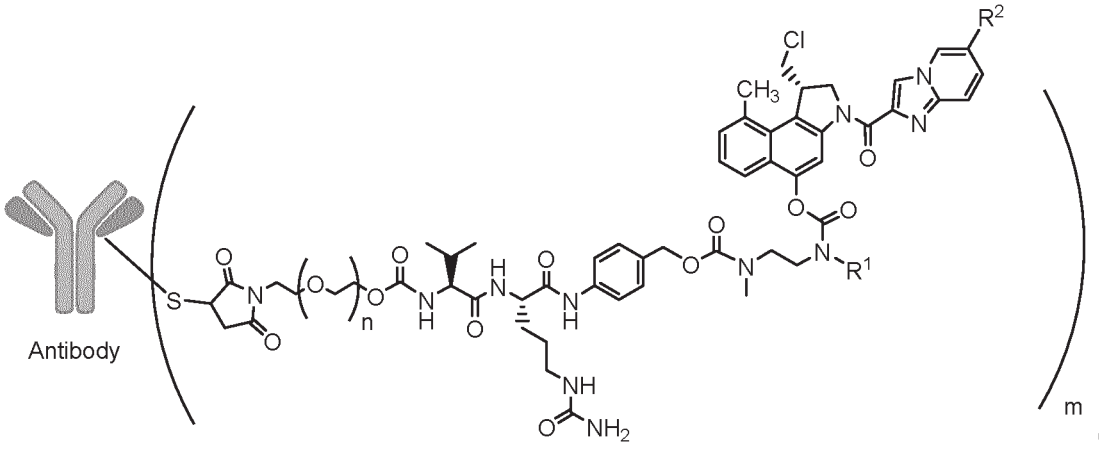
Molecular structure of mc-MMAF linked to a mAb

In one embodiment, the present invention relates to an ADC compound wherein said linker drug comprises a duocarmycin derivative.

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.

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.

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



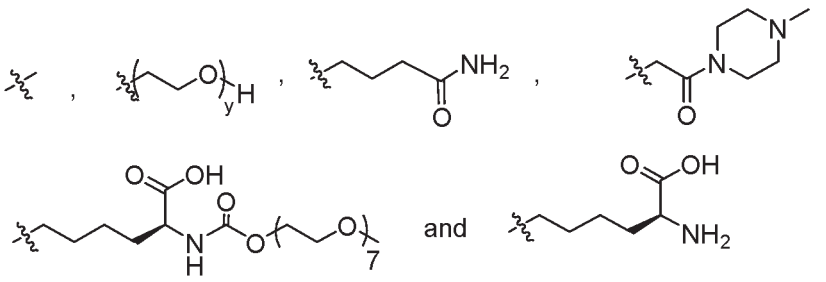
(I)

wherein

n is 0-3, preferably 0-1,

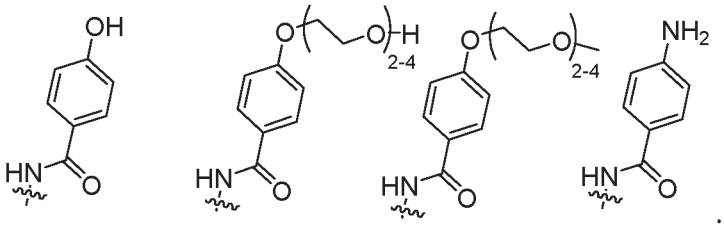
m represents an average DAR of from 1 to 6, preferably of from 1 to 4,

R^1 is selected from



y is 1-16, and

R^2 is selected from



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.

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.

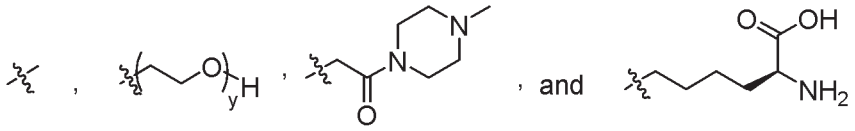
Conjugation to two or more of the engineered cysteine sites of the present invention allows for the preparation of ADCs comprising hydrophobic drug classes with a higher DAR, notably DAR 4, without getting too much aggregate.

In accordance with a particular embodiment of the present invention, 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.

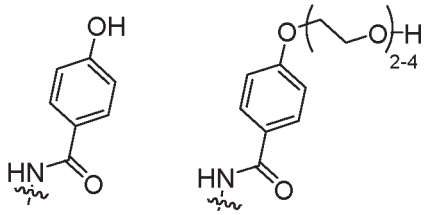
In a particular embodiment, the present invention relates to a compound of the formula (I) as disclosed hereinabove, wherein n is 0-1, m represents an average DAR of from 1 to 6, preferably of from 1 to 4, more preferably of from 1 to 2, most preferably of from 1.5 to 2,

R^1 is selected from



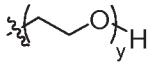
y is 1-16, preferably 1-4, and

R^2 is selected from

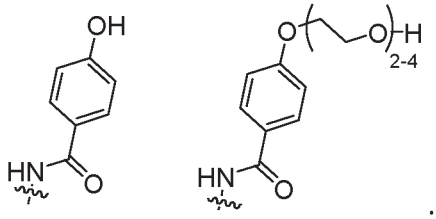


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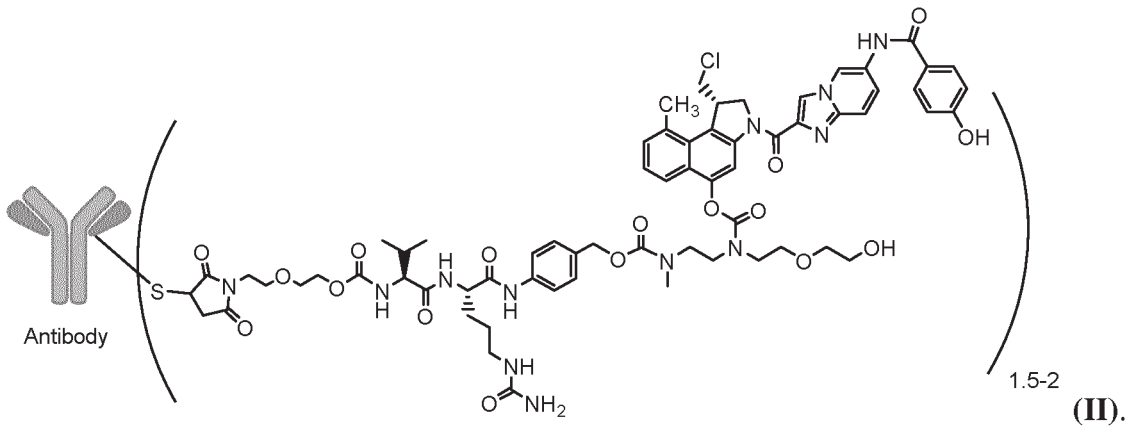
In a specific embodiment, the present invention relates to a compound of the structural formula (I) as disclosed hereinabove, wherein n is 0-1, m represents an average DAR of from 1.5 to 2, R^1 is



y is 1-4, and R^2 is selected from



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



(II).

In accordance with the present invention, 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, more preferably an IgG1 antibody, most preferably an IgG1 antibody having κ light chains. Preferably, the IgG antibody carries a native glycoside/carbohydrate moiety attached at N297 of the heavy chain of the antibody.

Suitable antibodies include an anti-annexin A1 antibody, an anti-CD19 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-CD123 antibody, an anti-CD138 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-LIV1 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-SIRPα 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.

Preferably, the antibody is an anti-annexin A1 antibody, an anti-CD115 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.

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, even more preferably a humanized or human IgG antibody, most preferably a humanized or human IgG1 mAb. Preferably, said antibody has κ (kappa) light chains, i.e., a humanized or human IgG1-κ antibody.

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 present invention 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.

In accordance with the present invention, 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.

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-CD115 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 and said linker drug comprises a duocarmycin derivative, preferably an ADC compound in accordance with formula (I) or (II).

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).

In a preferred embodiment, the present invention relates to an ADC compound as described hereinabove, wherein said linker drug comprises a duocarmycin derivative and is conjugated at position 41 of the heavy chain variable region of an anti-PSMA (monoclonal) antibody or an anti-5T4 (monoclonal) antibody, most preferably an ADC compound according to formula (II). 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.

In a more 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.

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.

In another more 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.

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.

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

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.

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.

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.

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.

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).

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.

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.

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.

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.

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, pemtumomab, 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, floxuridine, 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, particulary 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.

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.

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 topoisomerease 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.

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 topoisomerease 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.

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.

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

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).

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).

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).

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).

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).

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).

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

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.

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 AscI 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).

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

Commercially available Expi293F cells (Life Technologies) were transfected with the antibody mutant expression vector prepared under 1) above using the ExpiFectamine transfection agent (Life Technologies) according to the manufacturer's instructions as follows: 75 x 10^7 cells were seeded in 300 mL Expi293 Expression medium; 300 μg of the antibody mutant expression vector was combined with 800 μl 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

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.

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

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.

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

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 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.

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

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.

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.

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.

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:

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

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-	S41C,	wt	3.3	40.0	12.3	7.3	0.9

ADC (vc-*seco*-DUBA)	Cys mutations		DAR	HMW (%)[2]	RT_{DAR2}	RT_{DAR0}	Relative hydrophobicity[3]
	HC	LC					
120[7]	T120C						
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
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 $(RT_{DAR2} - RT_{DAR0}) / (RT_{DAR2,\ wt\text{-}ADC} - RT_{DAR0,\ wt\text{-}ADC})$, 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 $(RT_{DAR2} - RT_{DAR0}) / (RT_{DAR2, wt\text{-}ADC} - RT_{DAR0, wt\text{-}ADC})$, 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
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 $(RT_{DAR2} - RT_{DAR0}) / (RT_{DAR2, wt\text{-}ADC} - RT_{DAR0, wt\text{-}ADC})$, RT is retention time

Cellular binding

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 µg/ml) similar to the wild-type antibody, and all three ADCs were unable to bind to PSMA-negative DU-145 cells (EC_{50} >10 µg/ml).

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 µg/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 µg/ml).

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

In vitro cytotoxicity

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.

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 (vc-*seco*-DUBA)	Cys mutations		PSMA-positive cell line LNCaP-C4.2		
	HC	LC	IC_{50} (nM)	95% CI (nM)[1]	% efficacy[2]
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
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.

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 (IC_{50} >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.

The potencies of the engineered anti-5T4 ADCs were equal to the conventionally linked ADC H8-wt on 5T4-expressing MDA-MB-468 cells (IC_{50} between 0.07 and 0.09 nM, Table 6). The anti-5T4 ADCs were inactive on 5T4-negative SK-MEL-30 cells (IC_{50} >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.

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

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*-)DUBA 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.

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 IC_{50} values), while none of the ADCs were cleaved after a short period of 2 minutes exposure with cathepsin B (IC_{50} >10 nM, data not shown in Table).

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.

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

	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					
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
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

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.

Tumours were induced subcutaneously by injecting $1x10^7$ of LnCap C4.2 cells in 200 µL 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.

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).

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).

PA-1 tumours were induced subcutaneously by injecting $1x10^7$ cells in 100 µL 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 mm^3. 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-

41C-vc-*seco*-DUBA was clearly more active when compared to the native, non-engineered anti-5T4 ADC, H8-vc-*seco*-DUBA (Figure 4A).

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 CGKGLEWIGN
 51    INPNNGGTTY NQKFEDRATL TVDKSTSTAY MELSSLRSED TAVYYCAAGW
101    NFDYWGQGTT VTVSS
```

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

```
  1    ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV
 51    HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP
101    KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS
151    HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
201    EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC
251    LVKGFYPSDI AVEWESNGQP ENNYKTTPPV 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 CAGCACAAAG
 421    GGCCCCAGCG TGTTCCCTCT GGCCCCTAGC AGCAAGTCTA CCTCTGGCGG AACAGCCGCC
 481    CTGGGCTGCC TCGTGAAGGA CTACTTTCCC GAGCCCGTGA CCGTGTCCTG GAACTCTGGC
 541    GCTCTGACAA GCGGCGTGCA CACCTTTCCA GCCGTGCTGC AGAGCAGCGG CCTGTACTCT
 601    CTGAGCAGCG TCGTGACTGT GCCCAGCAGC AGCCTGGGCA CCCAGACCTA CATCTGCAAC
 661    GTGAACCACA AGCCCAGCAA CACCAAGGTG GACAAAAAGG TGGAACCCAA GAGCTGCGAC
 721    AAGACCCACA CCTGTCCCCC TTGTCCTGCC CCTGAACTGC TGGGCGGACC TTCCGTGTTC
 781    CTGTTCCCCC CAAAGCCCAA GGACACCCTG ATGATCAGCC GGACCCCCGA AGTGACCTGC
 841    GTGGTGGTGG ATGTGTCCCA CGAGGACCCT GAAGTGAAGT TCAATTGGTA CGTGGACGGC
 901    GTGGAAGTGC ACAACGCCAA GACCAAGCCC AGAGAGGAAC AGTACAACAG CACCTACCGG
 961    GTGGTGTCCG TGCTGACAGT GCTGCACCAG GACTGGCTGA ACGGCAAAGA GTACAAGTGC
1021    AAGGTGTCCA ACAAGGCCCT GCCTGCCCCC ATCGAGAAAA CCATCAGCAA GGCCAAGGGC
1081    CAGCCCCGCG AACCCCAGGT GTACACACTG CCTCCCAGCA GGGACGAGCT GACCAAGAAC
1141    CAGGTGTCCC TGACATGCCT CGTGAAAGGC TTCTACCCCT CCGATATCGC CGTGGAATGG
1201    GAGAGCAACG GCCAGCCCGA GAACAACTAC AAGACCACCC CCCCTGTGCT GGACAGCGAC
1261    GGCTCATTCT TCCTGTACAG CAAGCTGACT GTGGATAAGT CCCGGTGGCA GCAGGGCAAC
1321    GTGTTCAGCT GCAGCGTGAT GCACGAGGCC CTGCACAACC ACTACACCCA GAAAAGCCTG
1381    TCCCTGAGCC CCGGCAAG
```

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

```
  1    DIVMTQSPSS LSASVGDRVT ITCKASQDVG TAVDWYQQKP 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 TLTLSKADYE KHKVYACEVT HQGLSSPVTK
101    SFNRGEC
```

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

```
  1    ATGGCTTGTC CTGGATTTCT GTGGGCCCTC GTGATCAGCA CCTGTCTGGA ATTCAGCATG
 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 GCTACCCCCT GACCTTCGGC
361    GGAGGCACCA AGCTGGAAAT CAAGCGGACA GTGGCCGCTC CCAGCGTGTT CATCTTCCCA
421    CCTAGCGACG AGCAGCTGAA GTCTGGCACC GCCTCTGTCG TGTGCCTGCT GAACAACTTC
481    TACCCCCGCG AGGCCAAGGT GCAGTGGAAG GTGGACAATG CCCTGCAGAG CGGCAACAGC
541    CAGGAAAGCG TGACCGAGCA GGACAGCAAG GACTCCACCT ACAGCCTGAG CAGCACCCTG
601    ACCCTGAGCA AGGCCGACTA CGAGAAGCAC AAGGTGTACG CCTGCGAAGT GACCCACCAG
661    GGCCTGTCTA GCCCCGTGAC CAAGAGCTTC AACCGGGGCG AGTGC
```

SEQ ID NO:8 (H8 HC P41C)

```
  1    QVQLVQSGAE VKKPGASVKV SCKASGYSFT GYYMHWVKQS CGQGLEWIGR
 51    INPNNGVTLY NQKFKDRVTM TRDTSISTAY MELSRLRSDD TAVYYCARST
101    MITNYVMDYW GQGTLVTVSS
```

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

```
  1    ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV
 51    HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP
101    KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS
151    HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
201    EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC
251    LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
301    QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
```

SEQ ID NO:10 (H8 HC P41C cDNA)

```
   1    ATGGCCTGTC CTGGATTTCT GTGGGCCCTC GTGATCAGCA CCTGTCTGGA ATTCAGCATG
  61    GCCCAGGTGC AGCTGGTGCA GTCTGGCGCC GAAGTGAAGA AACCAGGCGC CAGCGTGAAG
 121    GTGTCCTGCA AGGCCAGCGG CTACAGCTTC ACCGGCTACT ACATGCACTG GGTCAAGCAG
 181    AGCTGCGGCC AGGGCCTGGA ATGGATCGGC AGAATCAACC CCAACAACGG CGTGACCCTG
 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    GGCGGAACAG CCGCCCTGGG CTGCCTCGTG AAGGATTACT TCCCCGAGCC CGTGACCGTG
 541    TCCTGGAATA GCGGAGCCCT GACAAGCGGC GTGCACACCT TTCCAGCCGT GCTGCAGAGC
 601    AGCGGCCTGT ACTCTCTGAG CAGCGTCGTG ACTGTGCCCA GCAGCAGCCT GGGCACCCAG
 661    ACCTACATCT GCAACGTGAA CCACAAGCCC AGCAACACCA AGGTGGACAA GAAGGTGGAA
 721    CCCAAGAGCT GCGACAAGAC CCACACCTGT CCCCCTTGTC CTGCCCCTGA ACTGCTGGGC
 781    GGACCTTCCG TGTTCCTGTT CCCCCCAAAG CCCAAGGACA CCCTGATGAT CAGCCGGACC
 841    CCCGAAGTGA CCTGCGTGGT GGTGGATGTG TCCCACGAGG ACCCTGAAGT GAAGTTCAAT
 901    TGGTACGTGG ACGGCGTGGA AGTGCACAAC GCCAAGACCA AGCCCAGAGA GGAACAGTAC
 961    AACAGCACCT ACCGGGTGGT GTCCGTGCTG ACAGTGCTGC ACCAGGACTG GCTGAACGGC
1021    AAAGAGTACA AGTGCAAGGT GTCCAACAAA GCCCTGCCTG CCCCCATCGA GAAAACCATC
1081    AGCAAGGCCA AGGGCCAGCC CCGCGAACCC CAGGTGTACA CACTGCCTCC CAGCCGGGAA
1141    GAGATGACCA AGAACCAGGT GTCCCTGACA TGCCTCGTGA AAGGCTTCTA CCCCTCCGAT
1201    ATCGCCGTGG AATGGGAGAG CAACGGCCAG CCCGAGAACA ACTACAAGAC CACCCCCCCT
1261    GTGCTGGACA GCGACGGCTC ATTCTTCCTG TACAGCAAGC TGACCGTGGA CAAGTCCCGG
1321    TGGCAGCAGG GCAACGTGTT CAGCTGCAGC GTGATGCACG AGGCCCTGCA CAACCACTAC
1381    ACCCAGAAGT CCCTGAGCCT GAGCCCCGGC AAA
```

SEQ ID NO:11 (H8 LC)

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

SEQ ID NO:12 (H8 LC cDNA)

```
   1    ATGGCCTGTC CTGGATTTCT GTGGGCCCTC GTGATCAGCA CCTGTCTGGA ATTCAGCATG
  61    GCCGACATCG TGATGACCCA GAGCCCCGAT AGCCTGGCCG TGTCTCTGGG AGAGAGAGCC
 121    ACCATCAACT GCAAGGCCAG CCAGAGCGTG TCCAACGACG TGGCCTGGTA TCAGCAGAAG
 181    CCCGGCCAGA GCCCTAAGCT GCTGATCTCC TACACCAGCA GCAGATATGC CGGCGTGCCC
 241    GACAGATTTT CCGGCAGCGG CTCTGGCACC GACTTCACCC TGACAATCAG CTCCCTGCAG
 301    GCCGAGGACG TGGCCGTGTA CTTCTGTCAG CAAGACTACA ACAGCCCCCC CACCTTCGGC
 361    GGAGGCACCA AGCTGGAAAT CAAGCGGACA GTGGCCGCTC CCAGCGTGTT CATCTTCCCA
 421    CCTAGCGACG AGCAGCTGAA GTCCGGCACA GCCTCTGTCG TGTGCCTGCT GAACAACTTC
 481    TACCCCCGCG AGGCCAAGGT GCAGTGGAAG GTGGACAATG CCCTGCAGAG CGGCAACAGC
 541    CAGGAAAGCG TGACCGAGCA GGACAGCAAG GACTCCACCT ACAGCCTGAG CAGCACCCTG
 601    ACCCTGAGCA AGGCCGACTA CGAGAAGCAC AAGGTGTACG CCTGCGAAGT GACCCACCAG
 661    GGACTGAGCA GCCCTGTGAC CAAGAGCTTC AACCGGGGCG AGTGC
```

SEQ ID NO:13 (germline leader sequence)

```
   1    MDWTWRILFL VAAATGAHS
```

SEQ ID NO:14 (natalizumab HC)

```
   1    QVQLVQSGAE VKKPGASVKV SCKASGFNIK DTYIHWVRQA PGQRLEWMGR
  51    IDPANGYTKY DPKFQGRVTI TADTSASTAY MELSSLRSED TAVYYCAREG
 101    YYGNYGVYAM DYWGQGTLVT VSS
```

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

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

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

```
   1    ATGGACTGGA CCTGGCGCAT CCTGTTTCTG GTGGCCGCTG CTACCGGCGC TCACTCCCAG
  61    GTGCAGCTGG TGCAGTCTGG CGCCGAAGTG AAGAAACCTG GCGCCTCCGT GAAGGTGTCC
 121    TGCAAGGCCT CCGGCTTCAA CATCAAGGAC ACCTACATCC ACTGGGTCCG ACAGGCCCCT
 181    GGACAGCGGC TGGAATGGAT GGGCAGAATC GACCCCGCCA ACGGCTACAC TAAGTACGAC
 241    CCCAAGTTCC AGGGCAGAGT GACCATCACC GCCGACACCT CCGCCTCCAC AGCCTACATG
 301    GAACTGTCCT CCCTGCGGAG CGAGGACACC GCCGTGTACT ACTGCGCCAG AGAGGGCTAC
 361    TACGGCAACT ACGGCGTGTA CGCCATGGAC TACTGGGGCC AGGGCACCCT GGTCACCGTG
 421    TCCTCCGCTT CCACCAAGGG CCCCTCCGTG TTCCCTCTGG CCCCTTGCTC CCGGTCCACC
 481    TCCGAGTCTA CCGCCGCTCT GGGCTGCCTG GTCAAGGACT ACTTCCCCGA GCCCGTGACC
 541    GTGTCCTGGA ACTCTGGCGC CCTGACCTCT GGCGTGCACA CCTTCCCTGC TGTGCTGCAG
 601    TCCTCCGGCC TGTACTCCCT GTCCTCCGTC GTGACCGTGC CCTCCAGCTC CCTGGGCACC
 661    AAGACCTACA CCTGTAACGT GGACCACAAG CCCTCCAACA CCAAGGTGGA CAAGCGGGTG
 721    GAATCTAAGT ACGGCCCTCC CTGCCCCCCC TGCCCTGCCC CTGAATTTCT GGGCGGACCT
 781    TCCGTGTTCC TGTTCCCCCC AAAGCCCAAG GACACCCTGA TGATCTCCCG GACCCCCGAA
 841    GTGACCTGCG TGGTGGTGGA CGTGTCCCAG GAAGATCCCG AGGTCCAGTT CAATTGGTAC
 901    GTGGACGGCG TGGAAGTGCA 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 AACAACTACA AGACCACCCC CCCTGTGCTG
1261    GACTCCGACG GCTCCTTCTT CCTGTACTCT CGGCTGACCG TGGACAAGTC CCGGTGGCAG
1321    GAAGGCAACG TCTTCTCCTG CTCCGTGATG CACGAGGCCC TGCACAACCA CTACACCCAG
1381    AAGTCCCTGT CCCTGAGCCT GGGCAAG
```

SEQ ID NO:17 (germline leader sequence)

```
  1    MDMRVPAQLL GLLLLWLRGA RC
```

SEQ ID NO:18 (natalizumab LC)

```
  1    DIQMTQSPSS LSASVGDRVT ITCKTSQDIN KYMAWYQQTP GKAPRLLIHY
 51    TSALQPGIPS RFSGSGSGRD YTFTISSLQP EDIATYYCLQ YDNLWTFGQG
101    TKVEIK
```

SEQ ID NO:19 (natalizumab LC cDNA)

```
  1    ATGGACATGA GAGTGCCCGC CCAGCTGCTG GGACTGCTGC TGCTGTGGCT GAGAGGCGCC
 61    AGATGCGACA TCCAGATGAC CCAGTCCCCC TCCAGCCTGT CCGCCTCCGT GGGCGACAGA
121    GTGACCATCA CATGCAAGAC CTCCCAGGAC ATCAACAAGT ACATGGCCTG GTATCAGCAG
181    ACCCCCGGCA AGGCCCCTCG GCTGCTGATC CACTACACCT CCGCTCTGCA GCCTGGCATC
241    CCCTCCAGAT TCTCCGGCTC CGGCTCTGGC CGGGACTATA CCTTCACCAT CTCCAGTCTG
301    CAGCCCGAGG ATATCGCCAC CTACTACTGC CTGCAGTACG ACAACCTGTG GACCTTCGGC
361    CAGGGCACCA AGGTGGAAAT CAAGCGGACC GTGGCCGCTC CCTCCGTGTT CATCTTCCCA
421    CCCTCCGACG AGCAGCTGAA GTCCGGCACC GCCTCCGTCG TGTGCCTGCT GAACAACTTC
481    TACCCCCGCG AGGCCAAGGT GCAGTGGAAG GTGGACAACG CCCTGCAGTC CGGCAACTCC
541    CAGGAATCCG TCACCGAGCA GGACTCCAAG GACAGCACCT ACTCCCTGTC TCCACCCTG
601    ACCCTGTCCA AGGCCGACTA CGAGAAGCAC AAGGTGTACG CCTGCGAAGT GACCCACCAG
661    GGCCTGTCCA GCCCCGTGAC CAAGTCCTTC AACCGGGGCG AGTGC
```

CLAIMS

1. An antibody-drug conjugate 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 and 89 (according to Kabat numbering); and

   light chain 40 and 41 (according to Kabat numbering).

2. The compound according to claim 1, wherein said engineered cysteine is at one or more positions of said antibody selected from heavy chain 40 and 41 and light chain 40 and 41 in the Fab part of said antibody.

3. The compound according to claim 1 or 2, further comprising an engineered cysteine at position 375 in the Fc part of said antibody (according to Eu numbering).

4. The compound according to any one of claims 1-3, wherein said linker drug comprises a duocarmycin derivative.

5. An antibody-drug conjugate compound wherein a linker drug is site-specifically conjugated to an antibody or antigen binding fragment thereof through an engineered cysteine at one or more positions of said antibody selected from

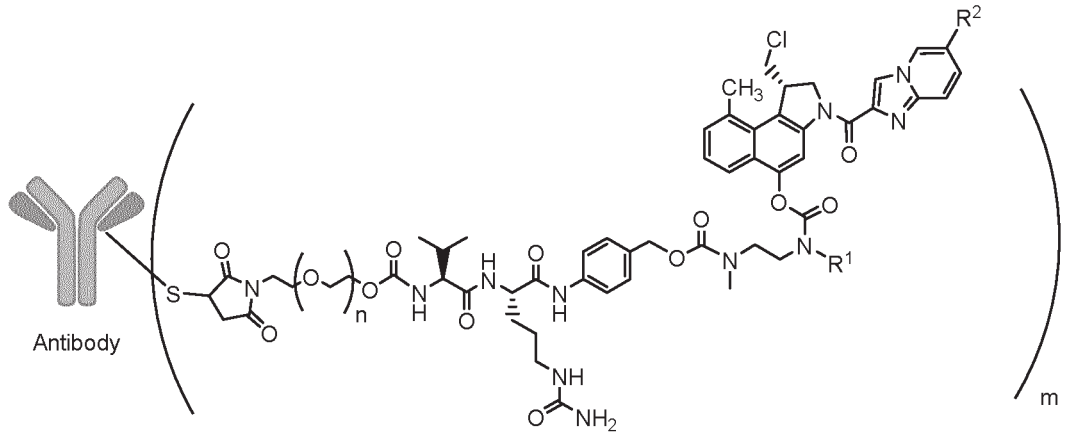
   heavy chain 40, 41 and 89 (according to Kabat numbering);

   heavy chain 152, 153, 155, 171, 247, 297, 339, 375 and 376 (according to Eu numbering); and

   light chain 40, 41, 165 and 168 (according to Kabat numbering),

   wherein said linker drug comprises a duocarmycin derivative.

6. The compound according to any one of claims 1-5 of the formula (I)



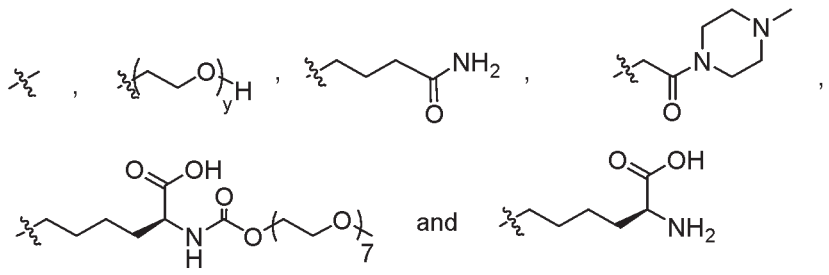
(I)

wherein

n is 0-3,

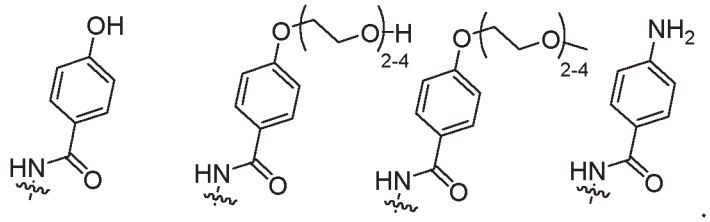
m represents an average DAR of from 1 to 6,

R^1 is selected from



y is 1-16, and

R^2 is selected from

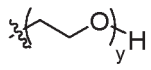


7. The compound according to claim 6, wherein

n is 0-1,

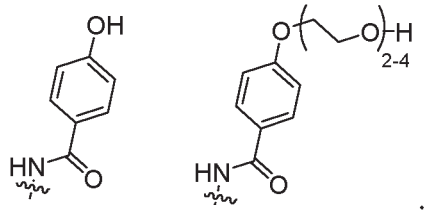
m represents an average DAR of from 1.5 to 2,

R^1 is

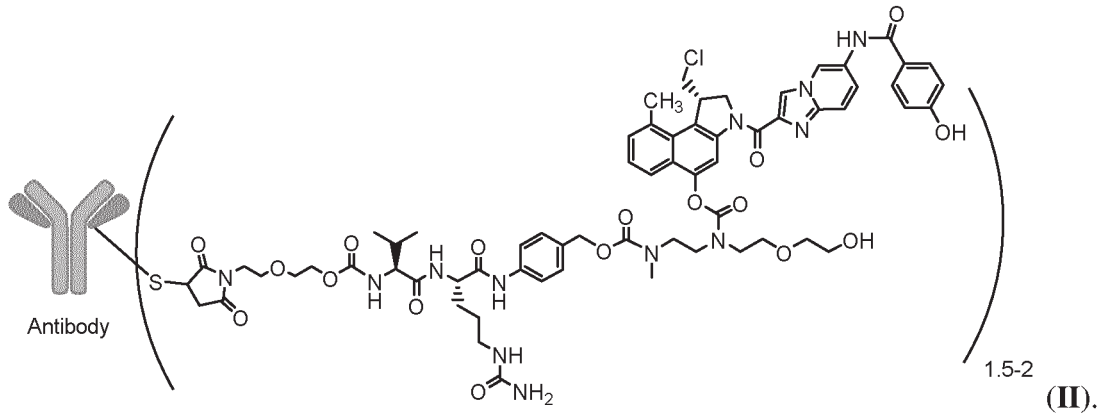


y is 1-4, and

R^2 is selected from



8. The compound according to any one of claims 1-7 of the formula (II)



(II).

9. The compound according to any one of claims 1-8, wherein said antibody binds to an antigen target that is expressed in or on the cell membrane of a tumour cell and wherein said antibody is internalised by the cell after binding to said target.

10. The compound according to any one of claims 1-9, wherein said antibody is an anti-annexin A1 antibody, an anti-CD115 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.

11. The compound according to any one of claims 1 to 10, wherein said antibody is an anti-PSMA monoclonal antibody or an anti-5T4 monoclonal antibody.

12. The compound according to claim 11, wherein said linker drug is conjugated at heavy chain variable region position 41 of said anti-PSMA antibody, preferably wherein the heavy chain of said anti-PSMA antibody comprises the amino acid sequence of SEQ ID NO:2 and the light chain of said anti-PSMA antibody comprises the amino acid sequence of SEQ ID NO:5.

13. The compound according to claim 11, wherein said linker drug is conjugated at heavy chain variable region position 41 of said anti-5T4 antibody, preferably wherein the heavy chain of said anti-5T4 antibody comprises the amino acid sequence of SEQ ID NO:8 and the light chain of said anti-5T4 antibody comprises the amino acid sequence of SEQ ID NO:11.

14. A pharmaceutical composition comprising a compound according to any one of claims 1-13 and one or more pharmaceutically acceptable excipients, preferably in the form of a lyophilized powder.

15. The compound according to any one of claims 1-13 or the pharmaceutical composition according to claim 14 for use as a medicament.

16. The compound according to any one of claims 1-13 or the pharmaceutical composition according to claim 14 for use in the treatment of human solid tumours and haematological malignancies.

17. The compound or the pharmaceutical composition according to claim 16, wherein the human solid tumours are selected from the group consisting of breast cancer, gastric

cancer, colorectal cancer, urothelial cancer, ovarian cancer, uterine cancer, lung cancer, mesothelioma, liver cancer, pancreatic cancer, and prostate cancer.