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(71) Applicant: **AVIDBIOLOGICS INC.** [CA/CA]; 101 College Street, Suite 218, Toronto, Ontario, M5G 1L7 (CA).

(72) Inventor: **TIKHOMIROV, Ilia Alexandre**; 954 The Queensway, Toronto, Ontario, M8Z 1P5 (CA).

(74) Agent: **PIASETZKI NENNIGER KVAS LLP**; 120 Adelaide Street West, Suite 2308, Toronto, Ontario, M5H 1T1 (CA).

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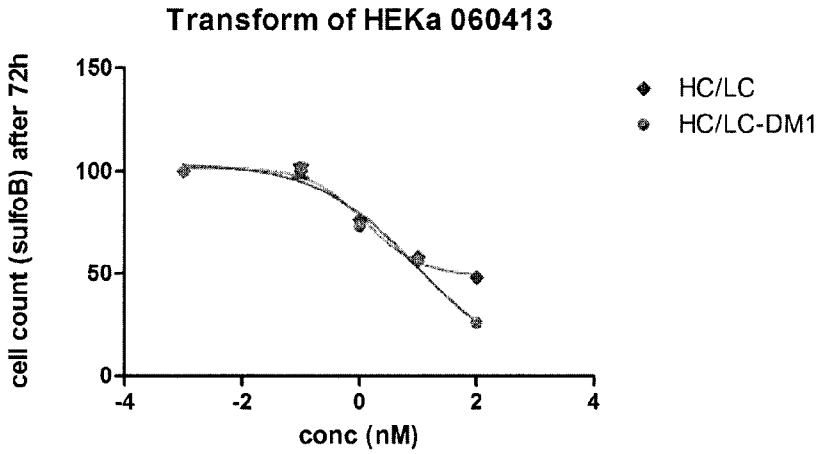


Figure 6

(57) Abstract: A maytansinoid is covalently linked through a non-cleavable linker to an EGFR antibody that is a full EGFR antagonist, such as cetuximab or panitumumab. The result is an anti-cancer agent having cytotoxicity that is potentiated in cancer cells but not normal cells. This benefit is not seen with EGFR antibodies that are partial antagonists, or with toxins that are not processed by lysosomes.

WO 2015/000062 A1

TITLE OF THE INVENTION

EGFR Antibody Conjugates

FIELD OF THE INVENTION

The present invention relates to an immunoconjugate that targets EGFR-expressing cancer cell populations and comprises an anti-EGFR antibody, such as panitumumab or cetuximab, conjugated to a microtubule damaging agent such as a maytansinoid.

BACKGROUND TO THE INVENTION

The conjugation of cell-binding proteins, such as antibodies, to potent cell-killing agents to enhance their anti-cancer activity, and provide the so-called "magic bullets" has had mixed clinical results.

Relative to naked antibodies, immunoconjugates often show enhanced cell-killing potency, and this increases their activity against cancer cells expressing the antibody-targeted antigen. The same increase in potency is also seen, however, in normal cells that express that same antigen. Of particular concern is the increased cytotoxicity against the rapidly proliferating tissues, such as skin. For example, a CD44v6-targeting immunoconjugate consisting of a maytansinoid and CD44v6 antibody was very active against cancer cells but was discontinued because of severe skin toxicities, such as toxic epidermal necrolysis, which occurred as a result of enhanced activity of the immunoconjugate against skin cells also expressing CD44v6 (Tijink *et al.*, Clin Cancer Res, 2006, 12:6064).

Another cell surface protein, epidermal growth factor receptor, or EGFR, is an attractive target for the development of anti-cancer immunoconjugates because of the antigen's expression by many tumors and its rapid internalization. However, because EGFR is also expressed by skin tissues, EGFR-targeting agents, such as the antibodies cetuximab and panitumumab, also show levels of skin toxicities that either demand dose reduction or in some cases are so severe as to warrant discontinuation of treatment.

With immunoconjugates, the toxicity of these antibodies is amplified through conjugation to a potent cell toxin. This exacerbates the problem for antibodies that are already inherently toxic to normal cells. For instance, conjugation with a toxic maytansinoid caused severe toxicity against skin cells when delivered via a CD44v6 antibody. As a result, development of anti-EGFR immunoconjugates based on approved anti-EGFR antibodies has not been pursued because of concerns over enhanced skin toxicity of such immunoconjugates. Alternative strategies are being pursued for the development of anti-EGFR immunoconjugates.

Anti-EGFR immunoconjugates are now being designed specifically to address these safety concerns. These conjugates are based on antibodies that target a mutated but naturally occurring version of EGFR, known as EGFRvIII, or on conformational forms of the EGFR, both of which predominate on tumour cells and not on skin cells (US 7628986, and US 7589180, respectively). For example, anti-EGFR antibody MAb806 is an antibody that targets an EGFR epitope found only on cancer cells, and potentially offers an advantage over the current EGFR antibodies, which all display significant binding to normal organs such as skin in humans. With this specificity, it is recognized that "the most important advantage of MAb 806 compared to current EGFR antibodies, is that MAb 806 can be directly conjugated to cytotoxic agents", an approach not feasible with other EGFR antibodies since the "cytotoxic conjugation would almost certainly induce severe toxicity" (US 7589180). An immunoconjugate comprising EGFR MAb 806 linked to an anti-microtubule payload is currently in phase I clinical testing in patients with advanced solid tumours.

Efforts continue through screening for naked anti-EGFR antibodies, and immunoconjugates thereof, to identify those with partial antagonistic activity against EGFR and reduced activity against keratinocytes (see US 2012/0156217), immunoconjugates based on "masked" anti-EGFR antibodies that are preferentially activated in the tumour microenvironment (WO 2009/025846), and immunoconjugates based on antibodies with medium affinity that preferentially accumulate in the tumor and

not normal tissues (WO 2012/100346). All of these strategies are aimed at reducing toxicities toward skin and other organs expressing EGFR, because currently approved anti-EGFR antibodies were deemed unsuitable for development as immunoconjugates.

An object of the present invention is to provide an immunoconjugate useful to treat EGFR+ disease cells including EGFR+ cancer cells and tumours comprising them. Another object of the present invention is to provide a method for potentiating the cytotoxicity of an EGFR antibody toward disease cells selectively. By this method, potentiation of toxicity toward normal cells is essentially avoided.

SUMMARY OF THE INVENTION

The present invention draws from the unexpected initial finding that conjugation of the anti-EGFR antibody cetuximab to a toxic maytansinoid through a non-cleavable linker yields an immunoconjugate that displays enhanced cytotoxicity against cancer cells without a corresponding increase in cytotoxicity against skin cells. The inventor demonstrates that the activity of some anti-EGFR antibodies against both cancer and keratinocytes is strongly potentiated by linking to maytansinoids and other cell-killing agents, whereas the activity of maytansinoid-conjugated cetuximab is potentiated only against cancer cells and not against keratinocytes. In these studies, conjugates of cetuximab with cell-killing agents other than anti-microtubule toxins, such as saporin, were found, as expected, to have an attendant and significantly enhanced toxicity toward keratinocytes.

It is further demonstrated that another antibody having full antagonist activity at EGFR, i.e. panitumumab, also demonstrates selective potentiation at EGFR+ cells when conjugated to an anti-microtubule payload such as a maytansinoid, in showing toxicity to EGFR+ cancer cells while sparing EGFR+ normal cells such as keratinocytes.

On the basis of these and other findings herein disclosed, the present invention enables the selection of components essential to yield an immunoconjugate that comprises an EGFR antibody and a toxic payload that potentiates antibody activity toward cancer cells

but not toward normal cells such as keratinocytes. Immunoconjugates having this property require the selection of an EGFR antibody that is a full antagonist, a toxin that is an anti-microtubule agent, and a linker that most desirably is not cleavable. By applying these criteria, there is provided an EGFR antibody-based immunoconjugate that has significant therapeutic activity against EGFR+ cancer cells without a corresponding increase in toxicity against EGFR+ normal cells including skin cells such as keratinocytes. Lack of additional potentiation of the toxicity against skin cells is critical because naked EGFR-targeting antibodies are already characterized by high prevalence dermatologic toxicities and it is beneficial not to potentiate these side effects.

Accordingly, in a general aspect, there is provided an immunoconjugate comprising an antibody having full antagonist activity at EGFR, and a toxin conjugated therewith through a non-cleavable linker, the immunoconjugate having a cytotoxic effect relative to a naked form of the antibody that is essentially not potentiated with respect to EGFR+ keratinocytes. The cytotoxic effect of the immunoconjugate with respect to EGFR+ cancer cells desirably is potentiated. The toxic payload desirably is an anti-microtubule toxin.

Also in a general aspect, there is provided a method useful to potentiate the anti-cancer activity of an EGFR antibody without potentiating the effect thereof on normal EGFR+ cells, the method comprising:

(i) selecting, for conjugation, an EGFR antibody that is a full EGFR antagonist and competes with cetuximab for binding to EGFR;

(ii) selecting, for delivery by the EGFR antibody, an anti-microtubule toxin;

(iii) selecting, for coupling the selected EGFR antibody and the anti-microtubule toxin, a linker; and

producing an immunoconjugate that incorporates the linker between the antibody and the toxin, thereby providing an immunoconjugate having a cytotoxicity that is potentiated against EGFR+ disease cells and essentially not potentiated against normal EGFR+ keratinocyte cells.

In one particular aspect, there is provided an immunoconjugate comprising cetuximab and a toxin conjugated therewith, the immunoconjugate having a cytotoxic effect relative to naked cetuximab that is (1) enhanced with respect to EGFR+ cancer cells, and (2) substantially not enhanced with respect to EGFR+ keratinocytes, wherein the immunoconjugate comprises cetuximab and an anti-microtubule toxin such as maytansinoid DM-1 conjugated by a non-cleavable linker. In alternative embodiments, the cetuximab is an equivalent of cetuximab, such as an EGFR-binding fragment of cetuximab, or an EGFR-binding variant of cetuximab that incorporates one or two or more benign substitutions in the antibody constant region or framework region without affecting the antibody binding to the receptor or antibody conjugate-mediated cell killing. For example, chimeric cetuximab can be further humanized using standard methods to create a more human-like version of the antibody. Alternatively, a fully human anti-EGFR antibody, such as necitumumab also known as IMC-11F8, which is considered to be functionally equivalent to cetuximab can be developed by screening of a human Fab library for an antibody that can bind and strongly inhibit EGFR and competes with cetuximab for receptor binding (Li S., Kussie P., Fergusson KM, Structural basis for EGF receptor inhibition by the therapeutic antibody IMC-11F8. Structure. 2008 Feb;16(2):216-27).

In another particular aspect, there is provided an immunoconjugate comprising panitumumab and a toxin conjugated therewith, the immunoconjugate having a cytotoxic effect relative to naked panitumumab that is (1) enhanced with respect to EGFR+ cancer cells, and (2) substantially unaltered with respect to EGFR+ keratinocytes, wherein the immunoconjugate comprises panitumumab and an anti-microtubule toxin such as maytansinoid DM-1 conjugated by a non-cleavable linker. In alternative embodiments, the panitumumab is an EGFR-binding fragment of panitumumab, or is a variant of panitumumab that incorporates one, two, or more benign substitutions yet maintains EGFR binding and inhibitory characteristics of the panitumumab parent.

In preferred embodiments, conjugation of the antibody and toxin is achieved using a non-cleavable linker. With non-cleavable linkers, release of the cytotoxic payload occurs by

intracellular destruction of the drug conjugate by lysosomes. A non-cleavable linker is substantially resistant to acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, or disulfide bond cleavage, whereas cleavable linkers, which can be used optionally but less desirably, are linkers that can be cleaved by one or more of these recited cleaving agents. Examples of such non-cleavable linkers include those that are or can be derived from a haloacetyl-based moiety selected from the group consisting of N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl iodoacetate (SIA), N-succinimidyl bromoacetate (SBA), and N-succinimidyl 3-(bromoacetamido)propionate (SBAP). Alternatively, the non-cleavable linker is or is derived from a maleimido-based moiety selected from the group consisting of N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC), κ-maleimidoundecanoic acid N-succinimidyl ester (KMUA), γ-maleimidobutyric acid N-succinimidyl ester (GMBS), ε-maleimidcaproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-(α-maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-(β-maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), and N-(p-maleimidophenyl)isocyanate (PMPI); another non-cleavable linker is maleimidocaproyl. (See US 2005/0169933; , Yoshitake et al, 101 Eur. J. Biochem. 395-399 (1979); Hashida et al, J. Applied Biochem. 56-63 (1984); and Liu et al, 18 690-697 (1979), and Doronina et al,. Bioconjugate Chem., 2006 Jan-Feb;17(1):114-24 for additional details.)

In a preferred embodiment, conjugation is achieved using a non-cleavable cross-linking reagent as linker such as succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC). Other useful forms of non-cleavable linkers include N-Succinimidyl iodoacetate (SIA), sulfo-SMCC, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), sulfo-MBS and succinimidyl-iodoacetate, as described in the literature, which introduce 1-10 reactive groups. (see, Yoshitake et al, 101 Eur. J. Biochem. 395-399 (1979); Hashida et al, J. Applied Biochem. 56-63 (1984); and Liu et al, 18 690-697 (1979)). Particularly useful for linking auristatins as anti-microtubule

toxin are the non-cleavable maleimidocaproyl linkers described by Doronina et al, in Bioconjugate Chem., 2006 Jan-Feb;17(1):114-24).

In another aspect, there is provided a pharmaceutical composition comprising the present EGFR antibody-based immunoconjugate in an amount cytotoxic to EGFR+ cancer cells, and a pharmaceutically acceptable carrier.

In a further aspect, there is provided the use of the present pharmaceutical composition for the treatment of EGFR+ cancer cells. In a related aspect, there is provided a method for treating a subject presenting with an EGFR+ cancer cell, comprising administering the present immunoconjugate to the subject in an amount cytotoxic to the EGFR+ cancer cell.

These and other aspects of the present invention are now described in greater detail with reference to the accompanying drawings in which:

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing cytotoxic activity against keratinocytes of naked anti-EGFR antibodies and maytansinoid conjugates thereof. About 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 5 days at 37°C. Viability of the remaining cells was determined by WST-8 based colorimetric assay.

Figure 2 is a graph showing cytotoxic activity against cancer cell line of naked anti-EGFR antibodies and maytansinoid conjugates thereof. About 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 5 days at 37°C. Viability of the remaining cells was determined by WST-8 based colorimetric assay.

Figures 1 and 2 herein are reproductions of figures presented in US 2012/0156217.

Figure 3 is a graph showing cytotoxic activity against H226 cancer cell line of naked anti-EGFR antibody cetuximab and maytansinoid conjugates thereof. About 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 72 h at 37°C. Viability of the remaining cells was determined via alamar blue cell viability assay.

Figure 4 is a graph showing cytotoxic activity against A431 cancer cell line of naked anti-EGFR antibody cetuximab and maytansinoid conjugates thereof. About 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 72 h at 37°C. Viability of the remaining cells was determined via alamar blue cell viability assay.

Figure 5 is a graph showing the cytotoxic activity of naked anti-EGFR antibody cetuximab and maytansinoid conjugates thereof against normal keratinocyte cell line HaCaT. About 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 5 days at 37°C. Viability of the remaining cells was determined via alamar blue cell viability assay.

Figure 6 is a graph showing the cytotoxic activity of naked anti-EGFR antibody cetuximab and maytansinoid conjugates thereof against primary keratinocytes. 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 72 h or 5 days at 37°C. Viability of the remaining cells was determined via alamar blue cell viability assay.

Figure 7 is a graph showing the cytotoxic activity of naked anti-EGFR antibody panitumumab and maytansinoid conjugates thereof against primary keratinocytes. 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 72 h or 5 days at 37°C. Viability of the remaining cells was determined via alamar blue cell viability assay.

Figure 8 is a graph showing the cytotoxic activity of naked anti-EGFR antibody panitumumab and maytansinoid conjugates thereof against primary keratinocytes. 2-3,000 cells/well were seeded in a flat bottom 96-well tissue culture plate, and incubated with various concentrations of test article in culture media for 72 h or 5 days at 37°C. Viability of the remaining cells was determined via alamar blue cell viability assay.

Figures 9-13 show results with immunoconjugates that incorporate elements not selected according to the criteria prescribed herein, as discussed in Example 4.

Figure 9 shows that panitumumab and cetuximab are very strong EGFR activation blockers and thus will not be potentiated on normal keratinocytes by conjugation, whereas partially antagonistic antibodies such as J2898A is potentiated (See Figure 10, and 11).

Figure 10 shows that partially antagonistic anti-EGFR antibody by IMGN (J2989A) is potentiated by conjugation (IMGN289) on normal keratinocytes (see also Setiady et al, Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2013;73(8 Suppl):Abstract nr 5463).

Figure 11 shows the toxicity of cetuximab mutant antibody, 6-LC, (having reduced affinity to make it a partially antagonistic against EGFR) is potentiated by conjugation to payload via non-cleavable linker (6LC-DM1).

Figure 12 shows panitumumab, and panitumumab-DM1 effects on keratinocytes, and reveals that conjugation of panitumumab via non-cleavable linker to DM1 (Avid300-DM1) does not increase its activity against normal cells; 2C9-DM1 is cetuximab-DM1 conjugate used as a control.

Figure 13 shows that conjugation of cetuximab to MMAE anti-microtubule payload by a cleavable linker (valine-citruline) potentiates its toxicity against both normal cells and

MDA-MB-468 cancer cells (Cetux 2C9-MMAE), whereas conjugation via non-cleavable linker (SMCC) only (Cetux2C9-DM1) potentiates anti-cancer activity, thus demonstrating a favorable therapeutic window.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present immunoconjugates are based on antibodies that bind to the human epidermal growth factor receptor (hEGFR), a protein that is presented on the surface of many different cell types including particularly skin cells such as keratinocytes. As used herein, the term "hEGFR" refers to any protein that comprises the expressed and processed product of the human *her-1* gene, wherein the protein is designated as UniProtKB/Swiss-Prot P00533. The term EGFR is used generically herein, and refers to the wild type protein and all naturally occurring variants thereof. The term "wtEGFR" is used more specifically with reference only to the wild type form of human EGFR. The term "EGFRvIII" refers to the EGFR variant protein that comprises the expressed and processed product of a variant of the *her-1* gene lacking exons 2-7, and thus includes only the polypeptide sequence encoded by exons 1 and 8 of *her-1*. The term "domain III" is not related to EGFRvIII, and instead refers to a location within EGFR, and represents an extracellular site that is key for EGF ligand binding, and binding of highly antagonistic antibodies cetuximab and panitumumab (Voigt et al, 2012 November; 14(11): 1023–1031).

The present immunoconjugates comprise an EGFR antibody that is a full antagonist at the EGFR. An EGFR antibody that is a "full antagonist" is an antibody that blocks completely or nearly so the transmission of a signal that is stimulated, in the normal course, by the EGF ligand through wtEGFR to the wtEGFR-coupled tyrosine kinase. EGFR antibodies that are full antagonists are particularly EGFR antibodies that bind directly to EGFR domain III. EGFR antibodies having these properties and an EGFR binding affinity of 5 nanomolar (nM) or less are particularly preferred for inclusion in the present immunoconjugates. By this measure, and as shown in Figure 9, at least the following known antibodies are not suitable for use in the present immunoconjugates:

J2898A, intellimab 6-LC (a cetuximab variant taught in WO 2012/100346), nimotuzumab, and matuzumab.

It is found that when a full antagonist EGFR antibody is selected to deliver a toxic payload, the killing effect is potentiated only on EGFR+ cancer cells and not on EGFR+ normal cells such as keratinocytes. This is not the case when the EGFR antibody is a partial antagonist, i.e., an antibody that allows transmission of some EGF-mediated signal. When conjugated to a toxin, these partial antagonist antibodies show a potentiation of the killing effect at both normal cells that are EGFR+ and cancer cells that are EGFR+.

The present immunoconjugates are based more particularly, and in one embodiment, on the hEGFR antibody known as cetuximab, now commercially available from Eli Lilly and Company under the trade name Erbitux®. Cetuximab is a recombinant, human/mouse chimeric IgG1 antibody that binds specifically to the extracellular domain of wtEGFR. The amino acid sequences of the CDRs for both the heavy chain of cetuximab (SEQ ID Nos.1-3) and the light chain of cetuximab (SEQ ID Nos. 4-6) are listed herein. Also listed are the amino acid sequences of the heavy chain variable region (SEQ ID No.7) and of the light chain variable region (SEQ ID No.8) of cetuximab, together with the amino acid sequences of the complete heavy chain (SEQ ID No. 9) and complete light chain (SEQ ID No.10) of cetuximab.

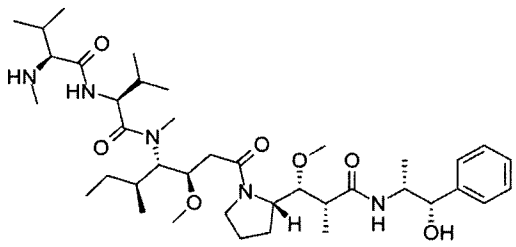
Cetuximab variants useful herein are highly antagonistic EGFR-binding agents that compete with cetuximab for binding to human EGFR. Useful cetuximab variants have been mentioned hereinabove, and include fragments of cetuximab comprising the EGFR binding sites of cetuximab, such as all of the light chain and heavy chain CDRs herein recited. Other cetuximab variants useful here in are cetuximab variants that incorporate one, two or more substitutions outside the antigen binding domains, such as in the framework region or in the constant region (Fc). Such substitutions are benign in the sense that they do not reduce cytotoxicity relative to cetuximab per se.

In another embodiment, the present immunoconjugates are based on the EGFR antibody known as panitumumab, now commercially available and sold under the trade name Vectibix®. Panitumumab is a recombinant, fully human IgG2 antibody that binds specifically to the extracellular domain of wtEGFR. The amino acid sequences of the heavy and light chains of panitumumab are listed in US 6,235,883 and US 7,807,798. A useful alternative to panitumumab is an EGFR binding variant thereof that competes with panitumumab for EGFR binding, such as a fragment of panitumumab that incorporates its antigen binding sites but has an otherwise lost or altered constant region.

The present immunoconjugates can also be based on still other EGFR antibodies provided they show full antagonist activity as defined above, such as EGFR antibodies that bind selectively to domain III of EGFR, and any other EGFR antibodies that compete with EGF and block fully or nearly so the transmission of EGF-stimulated downstream signalling.

In the present immunoconjugates, the full antagonist EGFR antibody, such as cetuximab or panitumumab, is conjugated to an anti-microtubule toxin such as maytansinoid toxin. By "anti-microtubule toxin" is meant an agent having cell toxicity mediated by interference with the microtubule structures important for cell mitosis, such as by inhibiting the formation of tubulin or by inhibiting the organization thereof.

Included within this toxin family are the maytansinoids and auristatins, and many other agents developed more recently and having the same mechanism of action. The auristatins in particular block cell replication by inhibiting polymerization of tubulin and are thus anti-mitotic. The structure of an auristatin useful herein and known as MMAE, or vedotin, is shown below:



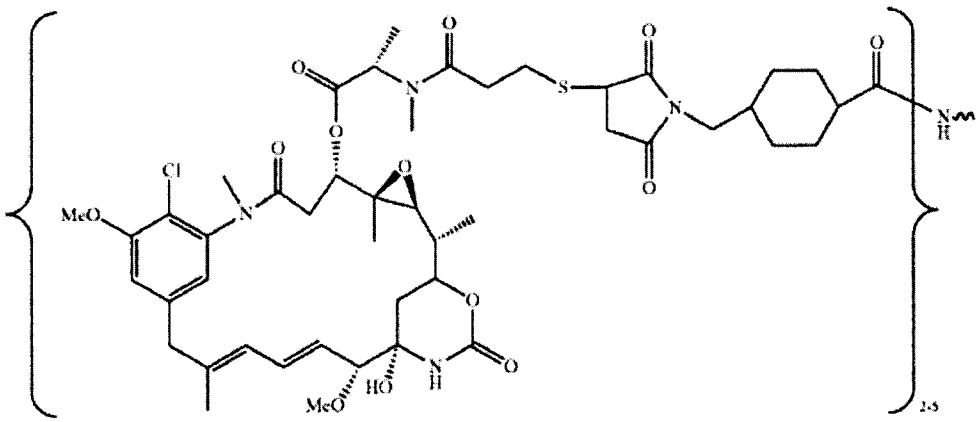
There are various forms of maytansinoids that are useful. These are all based on the complex structure of a natural molecule, maytansine.

Particularly useful are the maytansinoids including DM-1 and DM-4. In a specific embodiment, the toxin coupled to the EGFR MAb is DM-1 having the structure shown *infra*.

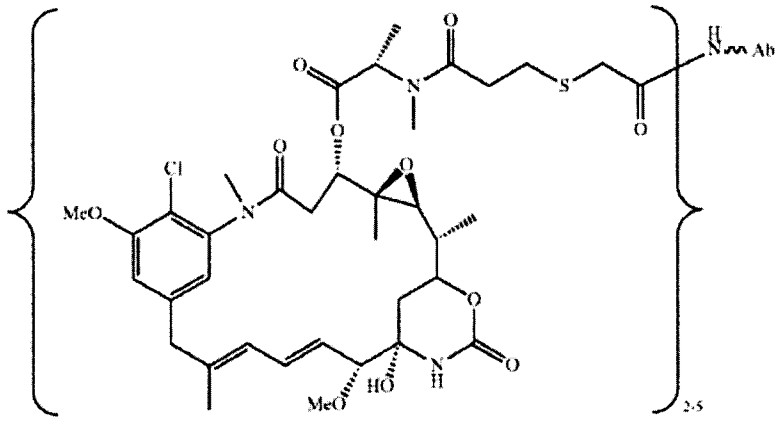
Also useful as anti-microtubule toxins are dolostatins, auristatins, tubulysins and cryptophycins. Specific examples of useful species within each genus include dolostatin 10, monomethyl dolostatin 10, auristatin E, monomethyl auristatin E (MMAE), auristatin F, monomethyl auristatin F, HTI-286, tubulysin M, as well as the tubulin binders such as tubulysin IM-1, tubulysin IM-2, tubulysin IM-3, colchicine DA, and maytansinoids AP-3, DM-1 and DM-4.

Conjugates of a full antagonist EGFR antibody such as cetuximab or panitumumab, and an anti-microtubule toxin such as a maytansinoid or auristatin can be formed using any technique presently known or later developed that couples a linker that is "non-cleavable". These linkers remain intact, and retain the antibody and toxin in covalent association, throughout conditions normally encountered following administration to a subject, including extracellular environments. More specifically, non-cleavable linkers result in ADC constructs for which release of the cytotoxic payload is achieved by destruction of the antibody by intracellular lysosomes.

Methods of linker integration are described for instance in US 5,208,020; US 8,088,387; and US 6,441,163. A preferred method is to modify the EGFR antibody, e.g., cetuximab, with succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) to introduce maleimido groups followed by reaction of the modified antibody with a thiol-containing maytansinoid to give a thioether-linked conjugate. The resulting chemical structures are shown below. Conjugates with 1 to 10 drug molecules per antibody molecule result.



Ab-SMCC-DM1
Ab = Antibody



Ab-SIA-DM1
Ab = Antibody

Other useful forms of non-cleavable linkers include N-Succinimidyl iodoacetate (SIA), sulfo-SMCC, m-maleimidobenzoyl-N-hydroxysuccinimide ester(MBS), sulfo-MBS and succinimidyl-iodoacetate, as described in the literature, to introduce 1-10 reactive groups. (see, Yoshitake et al, 101 Eur. J. Biochem. 395-399 (1979); Hashida et al, J. Applied Biochem. 56-63 (1984); and Liu et al, 18 690-697 (1979)). Particularly useful for linking auristatins as anti-microtubule toxin are the non-cleavable maleimidocaproyl linkers described by Doronina et al, in Bioconjugate Chem., 2006 Jan-Feb;17(1):114-24).

In a specific embodiment, the immunoconjugate comprises cetuximab linked to DM-1 by an SMCC linker.

In another specific embodiment, the immunoconjugate comprises panitumumab linked to DM-1 by an SMCC linker.

Thus, in a general aspect, the invention provides a method useful to potentiate the anti-cancer activity of an EGFR antibody without potentiating the effect thereof on normal EGFR+ cells, the method comprising:

(i) selecting, for conjugation, an EGFR antibody that is a full EGFR antagonist;

(ii) selecting, for delivery by the EGFR antibody, an anti-microtubule toxin;

(iii) selecting, for coupling the selected EGFR antibody to the anti-microtubule toxin, a linker that is non-cleavable; and

incorporating the linker between the antibody and the toxin to provide an immunoconjugate having a cytotoxic activity that is potentiated against EGFR+ disease cells but not against normal EGFR+ cells.

As will be appreciated from the preceding disclosure, the full antagonist EGFR antibody is preferably cetuximab or panitumumab. The anti-microtubule toxin is preferably an auristatin or a maytansinoid, and is most preferably DM-1. The non-cleavable linker is preferably SMCC.

In another specific embodiment, there is the proviso that the full antagonist EGFR antibody is not cetuximab. In a further specific embodiment, there is the proviso that the full antagonist EGFR antibody is not panitumumab.

Therapeutic formulations of the conjugate are prepared for therapeutic use directly or for storage by mixing the conjugate having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid

and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl, or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins such as serum, albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagines, histidine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN, PLURONICS or polyethylene glycol (PEG).

The active ingredients to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release include semipermeable matrices of solid hydrophobic polymers containing the conjugate, which matrices are in the form of shapes articles, e.g., films or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly (2-hydroxyethyl-methacrylate), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate, and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

The conjugates are useful to treat EGFR+ disease cells. Such treatment results in a reduction in the number, size or distribution of such disease cells in subjects presenting with them. In embodiments, the conjugates are used to treat EGFR+ disease cells that are EGFR+ cancer cells and tumours comprising them. Such treatment results preferably in a

reduction in the number, size, volume or distribution of such cancer cells and tumours comprising them, or at least in a reduction in the rate at which such disease cells increase in number, size, volume or distribution of such cells and tumours in subjects presenting with them.

Subjects presenting with EGFR+ cancer cells can be identified with the aid of assays that detect the receptor, as protein or as nucleic acid precursor (DNA or RNA) in physiological samples such as biopsied tissue. A suitable test for EGFR protein is the commercially available and FDA approved Dako EGFR pharmDx® test kit.

For the treatment of subjects presenting with EGFR+ cancer cells, the appropriate dosage of the conjugate will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the agent is administered for preventative or therapeutic purposes, previous therapy, the patients clinical history and response to the agent, and the discretion of the attending physician. The agent is suitably administered to the patient at one time or over a series of treatments.

For example, depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g., 0.1-20 mg/kg) of conjugate is a candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 500 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.
It will thus be appreciated that an effective amount of the immunoconjugate is an amount effective alone or as part of a treatment regimen that retards or inhibits the rate of growth or proliferation of EGFR+ disease cells.

An EGFR+ disease cell is a disease cell that presents EGFR on its surface as detectable for instance by EGFR antibody binding, or by detection of intracellular mRNA encoding

her-1. Particular EGFR+ disease cells include those having on their surface an abnormally high density and/or activity of EGFR molecules, or the presence of the EGFRvIII variant of EGFR.

It may be useful to administer to administer the present conjugates by intravenous infusion first as loading dose, followed by maintenance dose, such as at an initial dose of 4mg/kg over 90 minutes, then 2 mg/kg over 30 minutes, once weekly for as many as 52 weeks, with follow up as required. In the specific case of the panitumumab conjugate, dosing might be based on that utilized for panitumumab per se, which comprises 6mg/kg given once every two weeks as a one hour infusion.

The conjugates are useful in the treatment of a variety of cancers, to inhibit the growth or proliferation of EGFR+ cancer cells and tumours comprising them, including hematopoietic cell cancers and solid tumours. Conditions or disorders to be treated include benign or malignant tumors (e.g., renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulva, and thyroid); hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors; leukemias and lymphoid malignancies. In particular embodiments, the antibody or bivalent fragment are used in the treatment of such cancer cells that express EGFRvIII, as determined by the screening assays herein described. In particular embodiments, the cancer cells are EGFR+-presenting cancer cells that include head and neck cancers and especially squamous cell carcinoma of the head and neck, colorectal cancers, gastrointestinal cancers, brain tumours including glioblastomas, and tumours of the lung including non-small-cell lung carcinoma, and of the breast, pancreas, esophagus, kidney, ovary, cervix and prostate. In specific embodiments, the EGFR+ cancer is one for which cetuximab has received FDA marketing approval, such as squamous cell carcinoma of the head and neck and colorectal cancers.

It will be appreciated that subjects who could benefit from the present method include mammals including humans as well as livestock, and pets.

Other therapeutic regimens may be combined with the administration of the conjugates of the instant invention. For example, the patient to be treated may also receive radiation therapy, such as external beam radiation. Alternatively, or in addition, a chemotherapeutic agent may be administered to the patient. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration or the conjugate or may be given simultaneously therewith. The conjugate may be combined with any anti-cancer toxins, or any other suitable drug particularly including irinotecan (CPT-11), cisplatin, cyclophosphamide, melphalan, dacarbazine, doxorubicin, daunorubicin, and topotecan, as well as tyrosine kinase inhibitors, including particularly EGFR kinase inhibitors such as AG1478 ((4-(3-chloroanilino-6,7-dimethoxyquinazoline), gefitinib (Iressa®), erlotinib (Tarceva®), lapatinib (Tykerb®), canertinib (PD183805, Pfizer), PKI-166 (Novartis), PD158780 and pelitinib.

It may also be desirable to administer antibodies or conjugates against other tumor associated antigens or their ligands, such as antibodies which bind to the ErbB2 (including trastuzumab marketed as Herceptin®, and pertuzumab marketed as Omnitarg®), ErbB3, ErbB4, or vascular endothelial factor (VEGF), and/or antibodies that bind to EGF or TGFα.

In another embodiment of the invention, an article of manufacture containing conjugate in an amount useful for the treatment of the disorders described herein is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle). The label on or associated with the container indicates that

the composition is used for treating a cancer condition. The article of manufacture may further compromise a second container compromising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other matters desirable from a commercial and use standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

An anti-cancer immunoconjugate according to the invention may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Unit doses of the conjugate are suitably 50mgs, 100mgs, 150mgs, 200mgs, 250mgs, 300mgs and 400mgs. The drug can be formulated in single use vials at a concentration such as 20mg/mL, for instance 100mg in 5mL vehicle such as 0.9% saline, 200mg in 10mL or 400mg in 20mL.

Any appropriate route of administration can be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, pulmonary, or oral administration.

Example 1 – Preparation of Cetuximab-SMCC-DM1 conjugate (A-H)

A. PREPARATION AND MEASUREMENT OF CETUXIMAB ANTIBODY

Cetuximab is obtained from the open market, or is produced as described in WO 2012/100346, for conjugation to DM1 using the non-cleavable heterobifunctional cross-linking reagent SMCC.

Cetuximab antibody was then buffer exchanged into 50 mM potassium phosphate, 50 mM sodium chloride, 2 mM EDTA; pH 6.5 buffer (Buffer A). All buffers in this experiment were tested to be free of endotoxin using a chromogenic Limulus amoebocyte lysate (LAL) method (Cambrex). The concentration of antibody was measured using an extinction coefficient of 1.45 mL/mg/cm at 280 nm and a molecular weight of 145,781g.

B. PREPARATION AND MEASUREMENT OF SMCC STOCK SOLUTION

A 20 mM solution of SMCC (6.69 mg/mL) (Concortis Biosystems Corp.) was prepared in DMSO. The solution was diluted 1/40 in Assay Buffer and the absorbance of the samples was measured at 302 nm. The concentration of the stock solution was calculated using a molar extinction coefficient of 602/M/cm.

C. PREPARATION AND MEASUREMENT OF DM1 STOCK SOLUTION

A 10 mM solution of DM1 (free thiol form; Concortis Biosystems Corp.) was prepared in DMA (7.37 mg/mL). The absorbance of dilutions of the stock solution in ethanol was measured at 280 nm. The concentration of stock DM1 was calculated by using a molar extinction coefficient of 5700/M/cm at 280 nm. The concentration of free —SH in the stock DM1 preparation was measured using Elman's reagent (DTNB). Dilutions of the stock solution were prepared in Assay buffer made to 3% (v/v) DMA, and then 100 mM DTNB in DMSO (1/100th volume) was added. The increase in absorbance at 412 nm was measured against a reagent blank and the concentration was calculated using an extinction coefficient of 14150/M/cm. The concentration of —SH resulting from the Elman's assay was used to represent the DM1 stock concentration in calculations for conjugation conditions.

D. MODIFICATION OF CETUXIMAB WITH SMCC CROSSLINKER

The antibody was modified using a 7.5-fold molar excess of SMCC at 20 mg/mL antibody. The reaction was carried out in Buffer A (95% v/v) with DMSO (5% v/v) for 2 hours at room temperature with stirring.

E. G25 CHROMATOGRAPHY TO REMOVE EXCESS SMCC

The cetuximab-SMCC reaction mixture was gel-filtered through a 1.5×4.9 cm pre-packed column of Sephadex G25 resin equilibrated in Buffer A. The load and elution volumes were according to manufacturer's instructions (Amersham Biosciences). The concentration of the modified antibody solution was assayed spectrophotometrically using the extinction co-efficient described above.

F. CONJUGATION OF CETUXIMAB-SMCC WITH DM1

The modified antibody was reacted with a 1.7-fold excess of DM1 over linker (assuming 5 linkers per antibody). The reaction was carried out at 10 mg/mL antibody concentration in Buffer A (94% v/v) with DMA (6% v/v). After addition of DM1, the reaction was incubated at room temperature for 16.5 hours with stirring.

G. CONJUGATION PURIFICATION BY G25 CHROMATOGRAPHY

The conjugation reaction mixture was gel-filtered through a 1.5×4.9 cm pre-packed column of Sephadex G25 resin equilibrated in 1× phosphate buffered saline (PBS), pH 6.5 (Buffer B). The load and elution volumes were according to manufacturer's instructions (Amersham Biosciences). The number of DM1 molecules linked per mole of cetuximab was determined by measuring absorbance at both 252 nm and 280 nm of the eluted material. The DM1/antibody ratio was found to be 2 and 4. The resulting conjugate was analyzed for binding and cytotoxicity.

H. TESTING OF CETUXIMAB-SMCC-DM1

The cell lines used in these studies have the following characteristics:

A431: human epithelial carcinoma of vulva cell line; available from ATCC; plated at 4000 cells/well in DMEM-10%FBS, 100µl/well in 96 well plate.

H226: lung squamous cell carcinoma cell line; available at ATCC; plated at 4000 cells/well in RPMI-10%FBS, 100µl/well in 96-well culture plate.

MDA-MB-468: mammary gland/breast; derived from metastatic site: pleural effusion; available from ATCC; plated at 4000 cells/well in ATCC-formulated Leibovitz's L-15 Medium (Catalog No. 30-2008) with added fetal bovine serum to a final concentration of 10%; 100µl/well in 96 well plate.

HaCaT: in vitro spontaneously transformed keratinocytes from histologically normal skin; available from Chinese Center for Type Culture Collection of Wuhan University; plated at 2000 cells/well in DMEM-10%FBS, 100µl/well in 96-well culture plate.

HEKa: Normal Human Primary Epidermal Keratinocytes, adult; available from PromoCell GmbH; plated at 4000cells/well in EpiLife media 100µl/well, EpiLife medium #MEP1500CA, Invitrogen +HKGS human keratinocyte growth supplement (#S-001-5)

Binding studies showed that the conjugation of antibody to DM1 did not affect the K_D; both naked cetuximab antibody and cetuximab-SMCC-DM1 conjugate had the same binding affinity to EGFR by surface plasmon resonance (~0.3 nM, Table 1). Evaluation of the *in vitro* cytotoxicity of the sample showed that the cetuximab-SMCC-DM1 conjugate is significantly more cytotoxic compared to cetuximab against cancer cell lines (Figs 3&4), but that surprisingly cetuximab-SMCC-DM1 and naked cetuximab have the same cytotoxic activity against keratinocytes (Figs 5&6).

Table 1: effects of conjugation to DM1 on cetuximab KD

Sample	# of experiments	EGFR ecd KD (nM)
Commercial cetuximab	3	0.27 +/- 0.02
Cetuximab-SMCC-DM1	3	0.28 +/- 0.02

The effect of DM1 conjugation on different EGFR MAbs (huML66 and huEGFR-7R) is demonstrated in ImmunoGen's published US patent application 2012/0156217, and Figures 1 and 2 herein are reproductions of figures appearing in that application. More particularly, as shown in Figure 1, conjugation of anti-EGFR antibodies huML66-SMCC-DM1 and huEGFR-7R to maytansinoids to create huML66-SMCC-DM1 and huEGFR-7R-SMCC-DM1 immunoconjugates increases cytotoxic activity of against normal keratinocytes. Similarly, and as expected, conjugation of anti-EGFR antibodies huML66 and huEGFR-7R to maytansinoids to create huML66-SMCC-DM1 and huEGFR-7R-SMCC-DM1 immunoconjugates significantly potentiates cytotoxic activity of the antibodies against H226 cancer cell line (Figure 2).

As shown in Figures 3 and 4, DM1 conjugation of anti-EGFR antibody cetuximab (HC-LC) to create cetuximab-SMCC-DM1 immunoconjugate (HC-LC/DM1) significantly potentiates cytotoxic activity of the antibody against cancer cells, as expected and shown in Figure 3. As shown in Figure 4, and also as expected, conjugation of anti-EGFR antibody cetuximab (HC-LC) to maytansinoids to create cetuximab-SMCC-DM1 immunoconjugate (HC-LC/DM1) significantly potentiates cytotoxic activity of the antibody against cancer cells.

However, and quite surprisingly, as shown in Figures 5 and 6, conjugation of anti-EGFR antibody cetuximab (HC-LC) to maytansinoids to create cetuximab-SMCC-DM1 immunoconjugate (HC-LC/DM1) did not increase cytotoxicity against the keratinocytes as is reflected in substantially the same IC_{50} values for these agents (IC_{50} of 0.8269 for conjugate vs. IC_{50} of 0.4324 for naked antibody; Fig 5, HaCaT - normal human keratinocytes cell line & IC_{50} of 2.210 for conjugate vs. IC_{50} of 0.9348 for naked antibody, Fig 6; HEKa- primary human keratinocyte cells). As used herein, IC_{50} refers to the concentration of drug required to kill 50% of cells.

The measure of whether cytotoxicity is increased or not is determined above by comparing IC_{50} values produced using the alamar blue cell viability assay (each experiment performed in triplicates in a 96-well plate). A difference within one log order is deemed not significant and is considered to reveal no change in cytotoxicity, whereas a difference greater than one log order reveals a significant change in cytotoxicity.

Example 2 - Evaluation in Primates

Cynomolgus monkey species has been previously demonstrated to be a valuable and highly predictive model for evaluating anti-EGFR antibodies toxicities, including dermatologic side-effects. The high level of correlation between Cynomolgus monkey and human toxicity data for EGFR-targeting antibodies is in part due high homology between the monkey and human EGFR receptors that results in very similar K_D values for the antibodies:

Table 1: Binding affinity of cetuximab and panitumumab to human and cynomologus EGFR is consistent across the species. Apparent antibody affinity defined as the antibody concentration (picomolar, pM) required to achieve half-maximal binding in ELISA with recombinant extracellular domains of human EGFR (huEGFR), and Cynomolgus monkey EGFR (cyEGFR).Adapted from Koefoed et al, MABs. 2011 Nov-Dec; 3(6):584-595.

Apparent affinity, ELISA assay		
	Domain IIIB Binding	
Antigens	cetuximab	panitumumab
human sEGFR (pM)	12	32
cynomolgus sEGFR (pM)	14	32

The cetuximab-SMCC-DM1 immunoconjugate was tested in primates particularly to identify any significant potentiation of antibody side-effects by the conjugated toxin on normal cells. In particular, close attention was paid to dermatologic side-effects that are qualitatively different and/or significantly exacerbated relative to what is expected with the naked fully antagonistic anti-EGFR antibody.

The study initially made use of two Cynomolgus macaques, all males that were sedated with ketamine and anesthetized with isoflurane to facilitate IV administration of the antibody-drug conjugate administered at 10mg/kg. Animals were then observed for 5 days prior to sacrifice for general signs of acute toxicity, and examined further.

The detailed histopathology analysis revealed no severe dermatologic toxicities or other severe and qualitatively new dermatologic side-effects other than those that are expected as a result of naked cetuximab treatment. Also, no other severe on-target toxicities were observed in these animals demonstrating safety of ADCs of present invention and absence of significant exacerbation of the naked antibody toxicities.

Subsequent to successful completion of the acute primate study, repeat-dose safety study has been initiated in additional animals. The ADC was administered once every three weeks at 10mg/kg dose over 4 cycles. This dose and schedule was selected based on dosing schedules commonly used with other ADCs in primates and humans (Poon KA et al, Preclinical safety profile of trastuzumab emtansine (T-DM1): mechanism of action of its cytotoxic component retained with improved tolerability. Toxicol Appl Pharmacol. 2013 Dec 1;273(2):298-313; FDA Package Insert for Kadcyla™, accessed February 24, 2014: http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/125427lbl.pdf).

With the exception of reversible liver enzyme elevations which is an expected class side-effect of ADCs, no other severe and/or qualitatively new dermatologic or other on-target side-effects other than those that are already expected as a result of naked cetuximab treatment (cetuximab cynomolgus monkey data available at: http://www.accessdata.fda.gov/drugsatfda_docs/bla/2004/125084_ERBITUX_PHARMR_P3.PDF) have been observed in the ongoing study to date. Severe skin toxicity and toxic epidermal necrolysis previously reported with anti-CD44v6 ADC (which also uses SMCC-DM1 payload technology and targets an antigen expressed by normal keratinocytes) has not been observed in this study (Tijink *et al.*, Clin Cancer Res, 2006, 12:6064). These safety data further validates *in vitro* results that on-target toxicities of fully antagonist naked anti-EGFR antibodies is not significantly potentiated as result of conjugation to the cytotoxic payloads.

Example 3 – Preparation of Panitumumab-SMCC-DM1 conjugate (A-H)

A DM-1 conjugated panitumumab was prepared essentially as described above for the cetuximab counterpart. More particularly,

A. PREPARATION AND MEASUREMENT OF PANITUMUMAB ANTIBODY

Panitumumab is obtained from the open market, or is produced as described in US 6,235,883 or US 7,807,798 for conjugation to DM1 using the non-cleavable heterobifunctional cross-linking reagent SMCC.

Panitumumab antibody was then buffer exchanged into 50 mM potassium phosphate, 50 mM sodium chloride, 2 mM EDTA; pH 6.5 buffer (Buffer A). All buffers in this experiment were tested to be free of endotoxin using a chromogenic Limulus amoebocyte lysate (LAL) method (Cambrex). The concentration of antibody was measured using an extinction coefficient of 1.45 mL/mg/cm at 280 nm and a molecular weight of 145,781g.

B. PREPARATION AND MEASUREMENT OF SMCC STOCK SOLUTION

A 20 mM solution of SMCC (6.69 mg/mL) (Concortis Biosystems Corp.) was prepared in DMSO. The solution was diluted 1/40 in Assay Buffer and the absorbance of the samples was measured at 302 nm. The concentration of the stock solution was calculated using a molar extinction coefficient of 602/M/cm.

C. PREPARATION AND MEASUREMENT OF DM1 STOCK SOLUTION

A 10 mM solution of DM1 (free thiol form; Concortis Biosystems Corp.) was prepared in DMA (7.37 mg/mL). The absorbance of dilutions of the stock solution in ethanol was measured at 280 nm. The concentration of stock DM1 was calculated by using a molar extinction coefficient of 5700/M/cm at 280 nm. The concentration of free —SH in the stock DM1 preparation was measured using Elman's reagent (DTNB). Dilutions of the stock solution were prepared in Assay buffer made to 3% (v/v) DMA, and then 100 mM DTNB in DMSO (1/100th volume) was added. The increase in absorbance at 412 nm was measured against a reagent blank and the concentration was calculated using an extinction coefficient of 14150/M/cm. The concentration of —SH resulting from the Elman's assay was used to represent the DM1 stock concentration in calculations for conjugation conditions.

D. MODIFICATION OF PANITUMUMAB WITH SMCC CROSSLINKER

The antibody was modified using a 7.5-fold molar excess of SMCC at 20 mg/mL antibody. The reaction was carried out in Buffer A (95% v/v) with DMSO (5% v/v) for 2 hours at room temperature with stirring.

E. G25 CHROMATOGRAPHY TO REMOVE EXCESS SMCC

The panitumumab-SMCC reaction mixture was gel-filtered through a 1.5×4.9 cm pre-packed column of Sephadex G25 resin equilibrated in Buffer A. The load and elution volumes were according to manufacturer's instructions (Amersham Biosciences). The concentration of the modified antibody solution was assayed spectrophotometrically using the extinction co-efficient described above.

F. CONJUGATION OF PANITUMUMAB-SMCC WITH DM1

The modified antibody was reacted with a 1.7-fold excess of DM1 over linker (assuming 5 linkers per antibody). The reaction was carried out at 10 mg/mL antibody concentration in Buffer A (94% v/v) with DMA (6% v/v). After addition of DM1, the reaction was incubated at room temperature for 16.5 hours with stirring.

G. CONJUGATION PURIFICATION BY G25 CHROMATOGRAPHY

The conjugation reaction mixture was gel-filtered through a 1.5×4.9 cm pre-packed column of Sephadex G25 resin equilibrated in 1× phosphate buffered saline (PBS), pH 6.5 (Buffer B). The load and elution volumes were according to manufacturer's instructions (Amersham Biosciences). The number of DM1 molecules linked per mole of panitumumab was determined by measuring absorbance at both 252 nm and 280 nm of the eluted material. The DM1/antibody ratio was found to be 2 and 4. The resulting conjugate was analyzed for binding and cytotoxicity.

H. TESTING OF PANITUMUMAB-SMCC-DM1

The cell lines used in these studies have the following characteristics:

MDA-MB-468: mammary gland/breast; derived from metastatic site: pleural effusion; available from ATCC; plated at 4000 cells/well in ATCC-formulated Leibovitz's L-15 Medium (Catalog No. 30-2008) with added fetal bovine serum to a final concentration of 10%; 100µl/well in 96 well plate.

HaCaT: in vitro spontaneously transformed keratinocytes from histologically normal skin; available from Chinese Center for Type Culture Collection of Wuhan University; plated at 2000 cells/well in DMEM-10%FBS, 100µl/well in 96-well culture plate.

In vitro studies with keratinocytes demonstrate that conjugation of panitumumab to an anti-microtubule toxin via non-cleavable linker does not potentiate the toxicity of the antibody. The resulting ADC has a similar safety profile on keratinocytes as the naked panitumumab, as well as another ADC based on a fully antagonistic antibody, cetuximab. On MDA-MB-468 cancer cells, significant potentiation of anticancer activity of panitumumab is observed as a result of conjugating the antibody to anti-microtubule toxin via non-cleavable linker. Similar to toxicity observations, panitumumab-based ADC was similarly active as cetuximab-based ADC.

Example 4 – Results with partial antagonist EGFR MAb conjugates

The effect of selecting immunoconjugate components different from those recommended herein is shown in accompanying drawings. Figure 9 shows that a partial antagonist EGFR antibody, designated J2989A has an activity that is potentiated on normal keratinocytes when conjugated to DM-1. Figure 10 shows, similarly, that another partial antagonist antibody, designated 6-LC (a substitution variant of cetuximab having lower relative affinity for EGFR) has an activity that is also potentiated at normal keratinocytes when conjugated to DM-1. Figure 11 shows that the effect of panitumumab of keratinocytes is not potentiated on keratinocytes when conjugated to DM1, while the anti-cancer effect of the conjugate is potentiated dramatically (Figure 12). And, Figure 13 shows that conjugation of cetuximab to MMAE by a cleavable linker (valine-citrulline) potentiates its toxicity against normal cells and MDA-MB-468 cancer cells, whereas conjugation via non-cleavable linker (SMCC) potentiates anti-cancer activity.

Thus, in these studies, only fully antagonistic anti-EGFR antibodies were not potentiated on normal cells by payload conjugation via non-cleavable linkers, whereas toxicity against normal cells of partial antagonist EGFR antibodies was potentiated by the conjugation. Moreover, we subsequently tested the effect on this activity profile of the

cleavable vs. non-cleavable linkers, while maintaining payload mechanism of action the same (i.e. anti-microtubule agent). The antibodies were conjugated via cleavable linker to anti-microtubule payload, MMAE as described by Doronina et al, Nature Biotechnology Nov 7, 2003, Vol 21: pp 778-784. Cleavable linker data demonstrate that when the fully antagonistic antibodies are conjugated to their payloads via cleavable linkers, their toxicity against normal cells is potentiated. Thus, a safe anti-EGFR ADC should incorporate a strongly antagonistic anti-EGFR antibody linked to an anti-microtubule payload by a non-cleavable linker.

The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

Referenced Sequences

SEQ ID No. Description

1 Cetuximab heavy chain CDR1

NYGVH

2 Cetuximab heavy chain CDR2

VIWSGGNTDYNTPFTS

3 Cetuximab heavy chain CDR3

ALTYYDYEFAY

4 Cetuximab light chain CDR1

RASQSIGTNIH

5 Cetuximab light chain CDR2

ASESIS

6 Cetuximab light chain CDR3

QQNNNWPTT

7 Cetuximab heavy chain variable region (V_H)

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTS
RLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSA

8 Cetuximab light chain variable region (V_L)

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGS
GSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELK

9 Cetuximab complete heavy chain

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTS
RLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAASTKGPSVFPL
APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE
VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

10 Cetuximab complete light chain

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGS
GSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTA
SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE
VTHQGLSSPVTKSFNRGE

WE CLAIM:

1. A method useful to potentiate the cytotoxicity of an EGFR antibody or an EGFR-binding fragment thereof on EGFR+ disease cells without potentiating the cytotoxicity thereof on normal EGFR+ cells, the method comprising:

(i) selecting, for conjugation, an EGFR antibody that is a full EGFR antagonist and competes with cetuximab for binding to EGFR, or an EGFR-binding fragment of said antibody;

(ii) selecting, for delivery by the EGFR antibody or fragment thereof, an anti-microtubule toxin;

(iii) selecting, for coupling the selected EGFR antibody and the anti-microtubule toxin, a linker; and

producing an immunoconjugate that incorporates the linker between the antibody and the toxin, thereby providing an immunoconjugate having a cytotoxicity that is potentiated against EGFR+ disease cells and essentially not potentiated against EGFR+ keratinocyte cells.

2. The method according to claim 1, wherein the selected full antagonist EGFR antibody is cetuximab or panitumumab.

3. The method according to claim 1 or claim 2, wherein the selected anti-microtubule toxin is a maytansinoid.

4. The method according to claims 1, 2 or 3, wherein the selected linker is a non-cleavable linker, preferably SMCC.

5. An immunoconjugate comprising (i) a full antagonist EGFR antibody that binds to an EGFR epitope to which cetuximab binds, or an EGFR binding fragment of said antibody or single chain polypeptide based on said antibody, and (ii) an anti-microtubule toxin conjugated therewith, the immunoconjugate having a cytotoxic effect relative to a naked form of said antibody that is (1) potentiated with respect to EGFR+ cancer cells,

and (2) substantially unaltered with respect to EGFR+ keratinocytes, wherein the antibody and toxin are conjugated by a linker.

6. The immunoconjugate according to claim 5, wherein the antibody is cetuximab or cetuximab variant comprising one, two or more benign substitutions in the constant region.

7. The immunoconjugate according to claim 5, wherein the antibody is panitumumab.

8. The immunoconjugate according to claims 5, 6 or 7 wherein the anti-microtubule toxin is a maytansinoid.

9. The immunoconjugate according to claim 8, wherein the anti-microtubule toxin is DM-1.

10. The immunoconjugate according to claims 5-9, wherein the linker is a non-cleavable linker, preferably succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC).

11. The immunoconjugate according to claim 5, which is cetuximab-SMCC-DM1.

12. The immunoconjugate according to claim 5, which is panitumumab-SMCC-DM1.

13. A pharmaceutical composition comprising the immunoconjugate of claims 5-12 in an amount cytotoxic to EGFR+ disease cells, and a pharmaceutically acceptable carrier.

14. A method for producing an anti-cancer composition, comprising the step of combining a pharmaceutically acceptable carrier, and an EGFR antibody that is conjugated to an anti-microtubule toxin to form an immunoconjugate, the immunoconjugate having an effect on cancer cells that is potentiated and an effect on

keratinocytes that is essentially not potentiated, relative to the effects of the naked antibody on such cells.

15. The use of a pharmaceutical composition according to claim 13, for the treatment of EGFR+ disease cells.

16. A method for treating a subject presenting with an EGFR+ disease cell, comprising administering to the subject an amount an immunoconjugate according to claims 5-12 that is cytotoxic to the EGFR+ disease cell.

17. The method according to claim 16, wherein the EGFR+ disease cell is an EGFR+ cancer cell, such as a head and neck or colorectal cancer cell.

18. A method for potentiating the effect of a full antagonist EGFR antibody on EGFR+ disease cells without potentiating the effect thereof on normal EGFR+ cells, comprising linking the antibody to an anti-microtubule toxin by a non-cleavable linker.

19. In a method for treating a subject presenting with a tumour that responds to treatment with a full antagonist EGFR antibody, wherein treatment therewith elicits an EGFR antibody-mediated adverse response by keratinocytes, the improvement comprising treating said subject with the full antagonist EGFR antibody in a form conjugated with an anti-microtubule toxin, whereby the tumour response to treatment with conjugated antibody is enhanced essentially without enhancing the adverse keratinocyte response to treatment, relative to treatment with naked antibody alone.

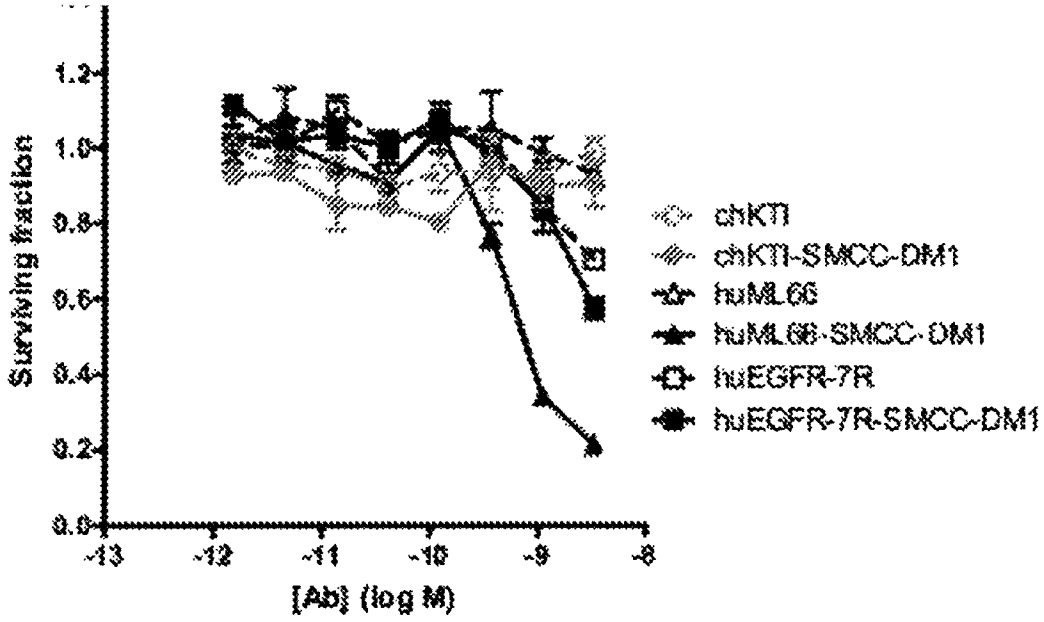


Figure 1

totox assay in cell lines independent of EGFR pathwa

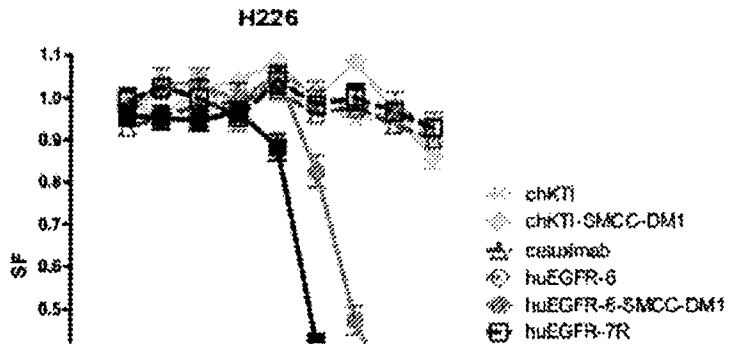


Figure 2

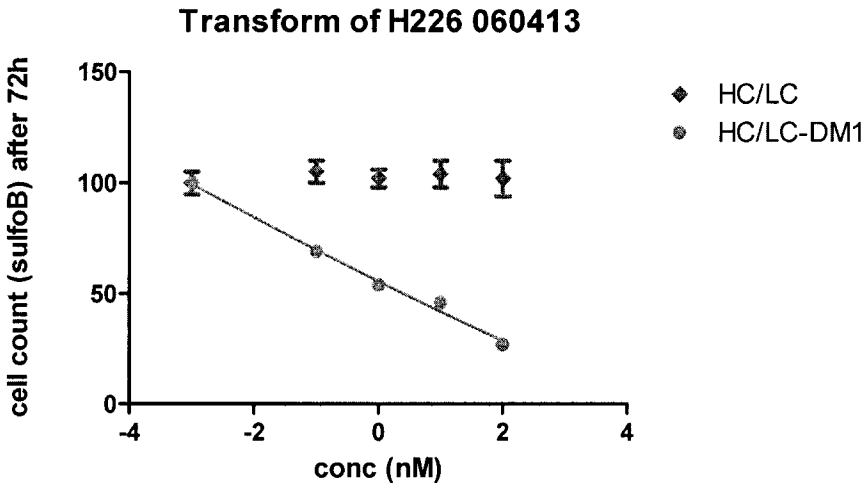


Figure 3

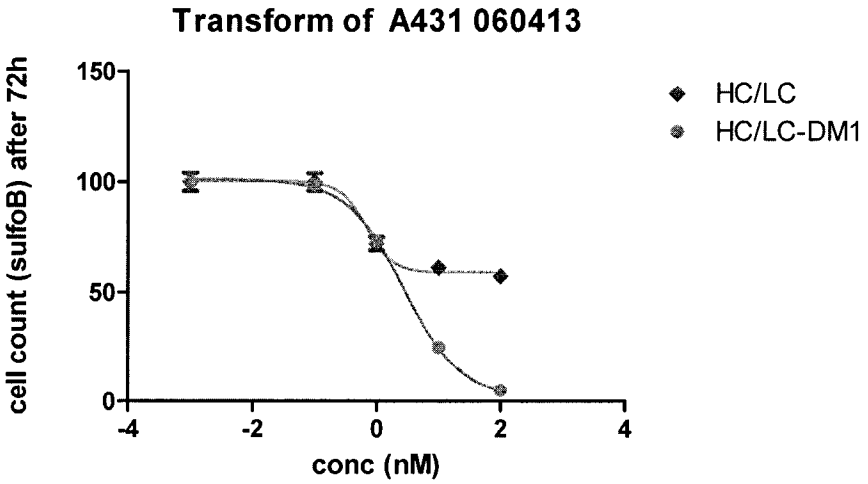


Figure 4

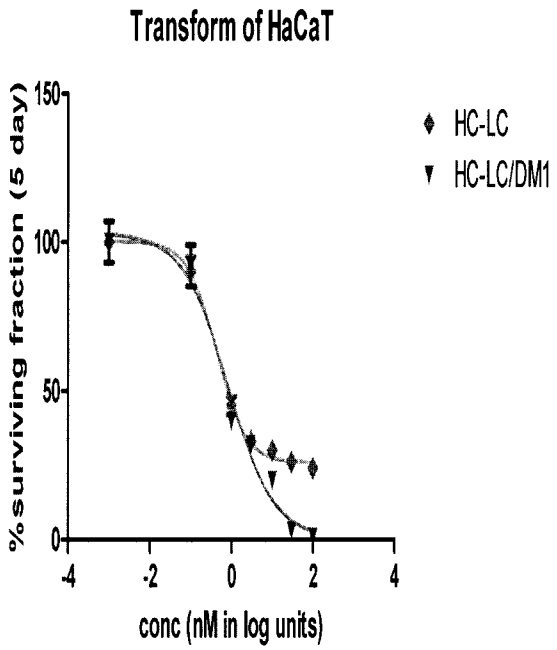


Figure 5

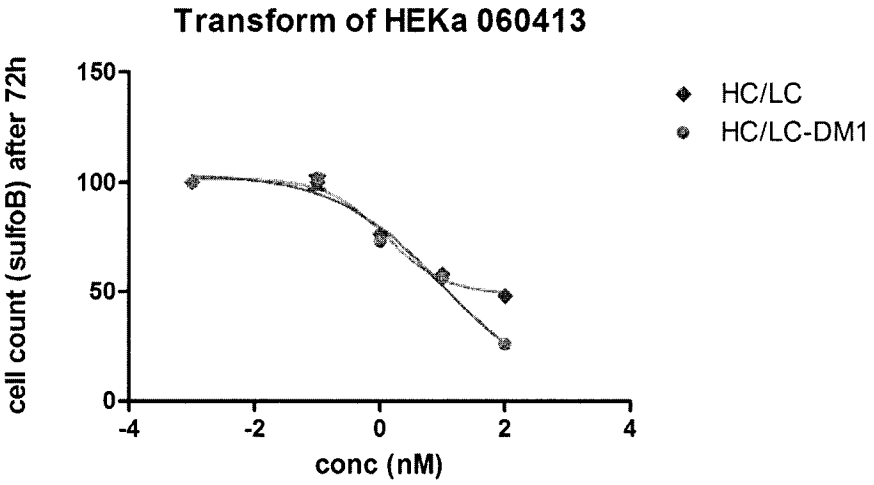


Figure 6

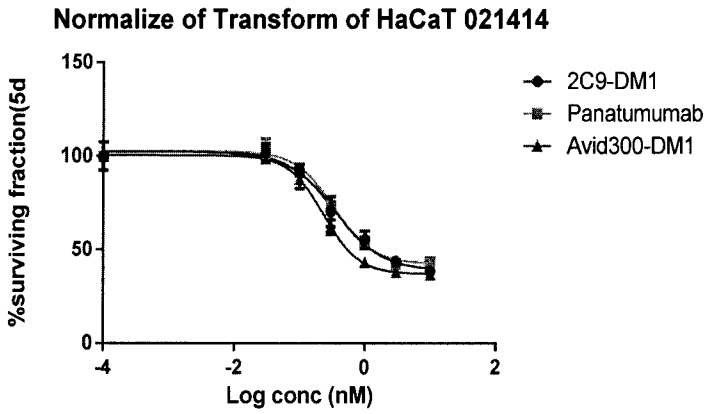


Figure 7

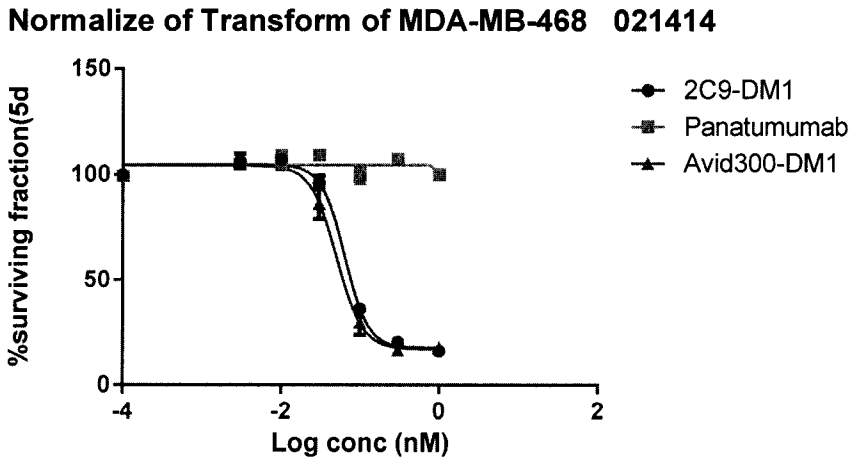


Figure 8

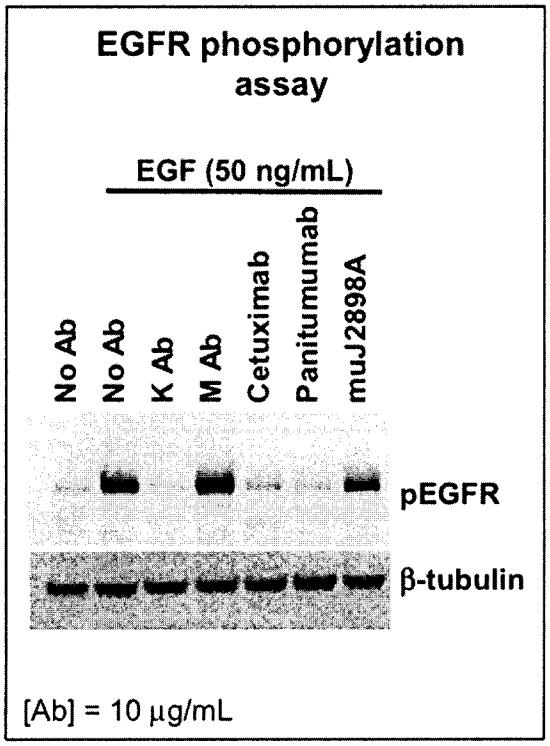


Figure 9

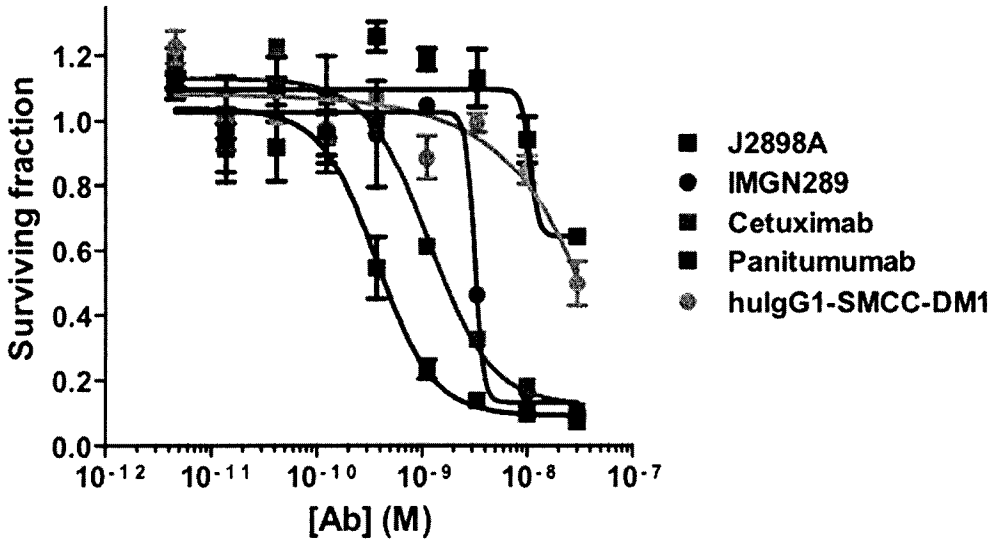


Figure 10

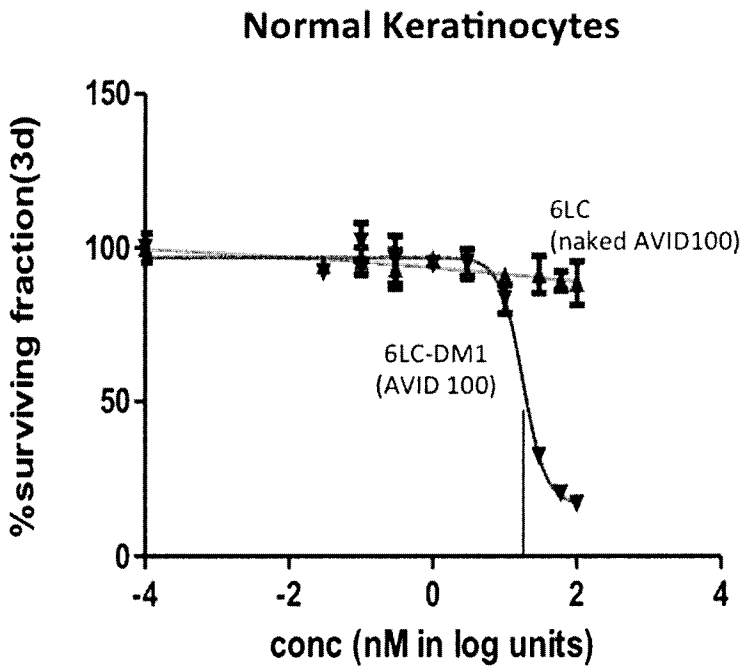


Figure 11

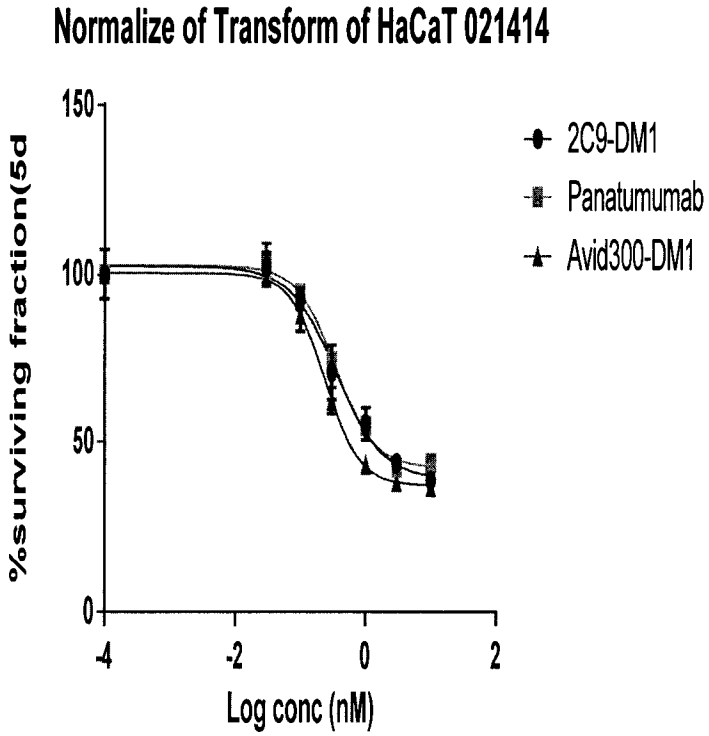
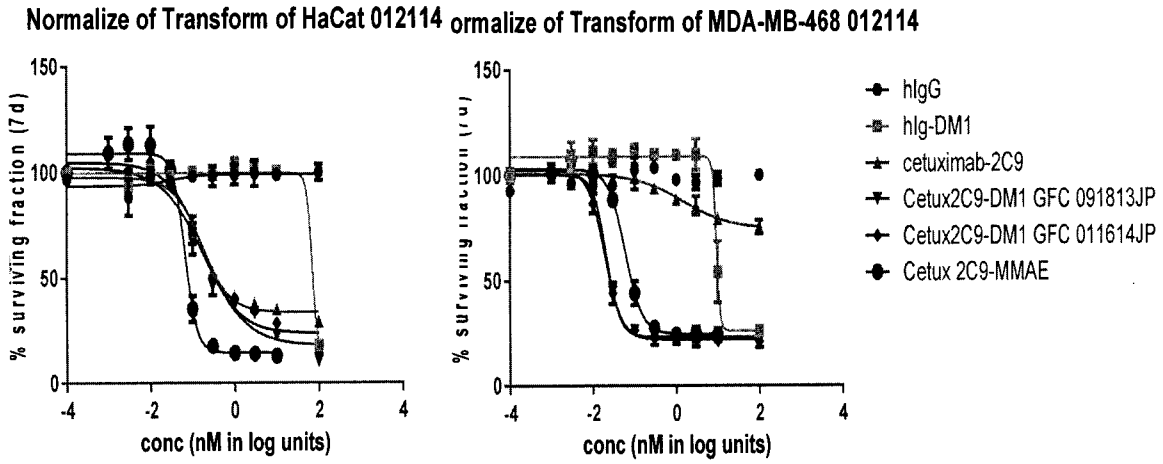


Figure 12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conc (nM in log units)
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hIg-DM1
cetuximab-2C9
Cetux2C9-DM1 GFC 091813JP
Cetux2C9-DM1 GFC 011614JP
Cetux 2C9-MMAE

Figure 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2014/000543

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 16/28*** (2006.01), ***A61K 47/48*** (2006.01), ***A61P 35/00*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2006.01): ***C07K, A61K, A61P***

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
TotalPatent, Scopus

Keywords: EGFR, antibody, cetuximab, maytansinoid, DM1, immunoconjugate, conjugate, antagonist, ErbB1, Her1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US2013/0156796 A1 (SETIADY, J. et al.) 20 June 2013 (20-06-2013) *paragraphs 0012-0016*	5-17 and 19 2
X Y	US2012/0156217 A1 (SETIADY, J. et al.) 21 June 2012 (21-06-2012) *Figures 2, 3 and 23, Examples 15-17, claims*	1, 3-5, 8-10 and 13-19 2, 6, 7, 11, 12

☐ Further documents are listed in the continuation of Box C.

☑ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier application or patent but published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	"&" document member of the same patent family

Date of the actual completion of the international search 30 July 2014 (30-07-2014)	Date of mailing of the international search report 11 August 2014 (11-08-2014)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Kerry Ferguson (819) 994-9451

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

- [x] on paper
- [ ] in electronic form

b. (time)

- [x] in the international application as filed
- [ ] together with the international application in electronic form
- [ ] subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claim Nos.: 16, 17 and 19
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 16, 17 and 19 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the use of the an immunoconjugate comprising a full antagonist EGFR antibody that binds to an EGFR epitope to which cetuximab binds, or an EGFR binding fragment of said antibody or single chain polypeptide based on said antibody, conjugated to an anti-microtubule toxin by a linker for treating a subject presenting with an EGFR+ disease cell or a tumor that responds to treatment with a full antagonist EGFR antibody.

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant=s protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2014/000543

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US2013/0156796 A1	20 June 2013 (20-06-2013)	WO2013078271A1	30 May 2013 (30-05-2013)
		AU2012340686A1	19 June 2014 (19-06-2014)
US2012/0156217 A1	21 June 2012 (21-06-2012)	WO2012158588A2	03 May 2012 (03-05-2012)
		WO2012158588A3	18 October 2012 (18-10-2012)
		WO2012158588A4	06 December 2012 (06-12-2012)
		AU2011320314A1	11 April 2013 (11-04-2013)
		CA2815277A1	03 May 2012 (03-05-2012)
		CN103298489A	11 September 2013 (11-09-2013)
		EA201390472A1	28 February 2014 (28-02-2014)
		JP2014500711A	16 January 2014 (16-01-2014)
		KR20140008308A	21 January 2014 (21-01-2014)
		MX2013004761A	27 August 2013 (27-08-2013)
		SG189929A1	28 June 2013 (28-06-2013)
		US2014099308A1	10 April 2014 (10-04-2014)
		US8790649B2	29 July 2014 (29-07-2014)