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(54) Title: NOVEL IMMUNOCONJUGATES USEFUL FOR TREATMENT OF TUMOURS

(57) Abstract: The present invention relates to novel immunoconjugates that are devoid of light chains and comprise at least one variable domain of a heavy chain antibody. The immunoconjugates of the present invention can be used for the preparation of a medicament to treat tumours.

NOVEL IMMUNOCONJUGATES USEFUL FOR TREATMENT OF TUMOURS

Field of the invention

The present invention relates to novel immunoconjugates that are devoid of light chains and comprise at least one variable domain of a heavy chain antibody. The immunoconjugates of the present invention can be used for the preparation of a medicament to treat tumours.

Background of the invention

The selective delivery of cytotoxic agents to tumour cells is desirable because systemic administration of these agents often kills normal cells within the body as well as the tumour cells sought to be eliminated. Targeted drug delivery systems provide a mechanism for delivering cytotoxic agents directly to cancerous cells. Antitumour drug delivery systems currently in use typically utilize a cytotoxic agent conjugated to a tumour-specific antibody to form an immunoconjugate. This immunoconjugate binds to tumour cells and thereby "delivers" the cytotoxic agent to the site of the tumour. Basic research in the area of antibody-based tumour-targeted therapy has been driven for many years by the prospect of identifying surface antigens with sufficient restrictive tissue expression patterns to allow for the selective and specific accumulation of antibody in tumour tissue. The immunoconjugates utilized in these targeting systems include antibody-drug conjugates and antibody-toxin conjugates. Both polyclonal antibodies and monoclonal antibodies have been utilized in these immunoconjugates. Drugs used in these immunoconjugates include daunomycin, metotrexate, mitomycin C and vindesine. Toxins used in the antibody-toxin conjugates include bacterial toxins such as ricin and *Pseudomonas aeruginosa* exotoxin A. Despite the amount of research directed towards the use of immunoconjugates for therapeutic purposes, several limitations involved in these delivery approaches have become apparent. For example, the large amount of drug required to be delivered to the target tumour cell to effect killing of the cell is often unattainable because of limitations imposed by the number of tumour-associated antigens on the surface of the cells and the number of drug molecules that can be attached to any given antibody molecule. This limitation has led to the use of more potent cytotoxic agents such as plant toxins in these conjugates and to the development of polymer-bound antibody-drug conjugates having very high drug multiplicity ratios. However, even with the large drug loading ratios or

with the use of potent toxins, many immunoconjugates still display suboptimal cytotoxic activity and are unable to effect complete killing at doses where all available antigenic sites are saturated. It has also been recognized that the cytotoxic activity of an immunoconjugate is often dependent on its uptake, mediated by the antibody component of the conjugate into the tumour cell. This internalization is crucial when using an antibody-drug conjugate in which the drug has an intracellular site of action or when using antibody-toxin conjugates. However, the vast majority of tumour-associated antigens and thus the antibody-drug or antibody-toxin conjugates bound to those antigens, are not internalized. Those conjugates that are internalized are often transported to the lysosome of the cell where the drug or toxin is degraded. Accordingly, although an antibody-drug or antibody toxin conjugate may have excellent tumour-binding characteristics, the conjugate may nonetheless have a limited cytotoxic utility due to an inability to reach its site of action within the cell. Due to these drawbacks, the currently utilized antitumour drug or toxin delivery systems have had a limited amount of success, especially when used for *in vivo* treatment. Clinical trials have also demonstrated important limitations of mostly murine antibodies due to high immunogenicity, distribution to normal organs and poor penetration of solid tumours. Along with the recent progress in genetic engineering techniques, there have been major efforts to construct or engineer antibodies to obtain smaller binding units that retained the specificity and affinity of classical antibodies and/or to reduce the immunogenicity of the murine molecules ("humanisation") (Hudson, 1998). The variable fragment (Fv) composed of the paired variable domain of the immunoglobulin heavy chain (VH) and the variable domain of the immunoglobulin light chain (VL) is the smallest, intact antigen-binding fragment one can obtain from a conventional antibody. However, it is more convenient to produce Fv as recombinant single-chain Fv (scFv), i.e. an Fv where the VH and VL domains are tethered by a flexible oligopeptide linker (Bird *et al.*, 1988). To broaden the immunotherapeutic potential, more complex constructs have been engineered, e.g. by linking two different scFvs to bridge tumour cells with either T or NK cells (bispecific antibodies) or a scFv attached to a toxin or an enzyme to act on a prodrug (Hudson, 1999). However, several of these scFv-based constructs proved difficult to express and purify, and exhibited several serious shortcomings in functionality. Common hurdles were the tendency to form aggregates due to the presence of an oligopeptide linker, the susceptibility of the linker to

proteolytic cleavage and subsequent unfolding of the antibody constructs (Whitlow *et al.*, 1993).

The naturally occurring heavy-chain antibodies devoid of light chain and of CH1 domain that were discovered in camelids (Hamers-Casterman *et al.*, 1993) may constitute a promising alternative in this respect but they have never been evaluated as immunoconjugates. The observation that camelids possess large amounts of functional heavy-chain antibodies lacking light chains formed the basis for generating functional single-domain antibody fragments (referred to as cAb for camel single-domain antibody) (Ghahroudi *et al.*, 1997; Lauwereys *et al.*, 1998) from their variable domains (V_HH). These small-sized molecules are well expressed and were shown to overcome to a large extent the solubility, aggregation and degradation problems often encountered with scFvs. Furthermore, they show good specificity towards their corresponding antigens and can be obtained with affinities comparable to scFvs (Muyldermans and Lauwereys, 1999; Riechmann and Muyldermans, 1999). However, due to the number of complex parameters involved (efficiency of tumor targeting, efficiency of internalization, efficiency of killing tumors, immunogenicity, problems of expression) it cannot be predicted whether a particular class of immunoconjugate will be successful or not. We have constructed immunoconjugates which are fusions between camel variable heavy chain antibodies and an enzyme and have surprisingly found that these immunoconjugates have superior *in vivo* characteristics such as lower immunogenicity and a superior killing of tumour cells when compared to existing immunoconjugates.

Brief description of figures

Fig. 1: Structures of the cephalosporin mustard prodrug CCM and the parent drug phenylene-diamine mustard PDM.

Fig. 2: Cytotoxic effects of cAb-CEA5-βL + CCM combinations on LS 174T adenocarcinoma cells as determined by the incorporation of [^{3}H] thymidine into DNA. The LS 174T cells were incubated with the cAb-CEA5-βL conjugates, washed and treated with CCM for 1h. The effects were compared to cells treated with CCM or PDM for 1h without prior conjugate exposure and to cells that were treated with saturating amounts of unconjugated cAb-CEA5 prior to conjugate treatment.

Fig. 3 *In vitro* cytotoxicity of CCM (3μM) on LS 174T adenocarcinoma cells. The cells were treated with varying concentrations of the conjugates, washed and then exposed to CCM for 1 h. After 24h incubation and pulsing for 18h, cytotoxicity was quantified by measuring [^{3}H] thymidine incorporation relative to untreated control cells. Demonstration of the immunological specificity of prodrug activation was done by treating the cells with the non-binding control conjugate cAb-Lys3-βL prior to CCM exposure or by saturation with non-conjugated cAb-CEA5 (0.1mg/ml) prior to conjugate treatment.

Fig. 4 Pharmacokinetics of cAb-CEA5::βL and the nonbinding control cAb-Lys3:: βL in nude mice (three animals/group). βL conjugate levels in subcutaneous LS174T coloncarcinoma tumors and in normal tissues are shown at 6 h, 24 h and 48 h post administration. cAb-Lys3::βL served as nonbinding control.

Fig. 5 Therapeutic effect of cAb::βL/CCM combinations in nude mice with LS174T xenografts. Conjugates (1 mg/kg) were injected iv on days indicated by the arrows, and CCM was administered 24 h later. The therapeutic effects were compared to those of PDM at the MTD.

Aims and detailed description of the invention

The present invention relates to novel immunoconjugates comprising a fusion between at least one variable domain of a heavy chain antibody and an anti-tumour agent. It is understood that a particular immunoconjugate has a specificity for at least one tumour antigen. Various tumour antigens or tumour markers are known in the art and it has been proposed that therapy against tumours expressing these markers can be achieved by using specific immunoconjugates. The word 'tumour' is to be understood as referring to all forms of neoplastic cell growth including carcinomas, sarcomas, lymphomas and leukemias. Thus, an immunoconjugate of the present invention comprises a variable domain of a heavy chain antibody which has been linked to an anti-tumour agent. With the wording 'anti-tumour agent' it is understood that this is a cytotoxic agent (a toxin) or to an enzyme capable of converting a pro-drug into an active cytotoxic agent. In the present invention the immunoconjugate is devoid of any light chain but comprises at least one heavy chain antibody. Preferably, the variable domain of a heavy chain antibody is derived from camelids, but it can also be derived from other species (e.g. mouse, human). Said variable domain of a heavy chain antibody has an anti-tumour agent attached to it. It is desirable that the antibody has a

good affinity for its tumour marker (its target). This is required so that once the antibody has reached its target, it remains bound to that target for long enough to achieve the desired result, for example, cytotoxicity. In addition, the antibody should have good specificity for the target antigen so that binding to non-target antigens does not occur to any significant degree.

Thus in a first embodiment the invention provides an immunoconjugate, devoid of a light chain, specifically binding to a tumour antigen comprising at least one single domain variable domain of a heavy chain antibody having an anti-tumour agent attached thereto and further characterized by inhibiting the growth of tumour cells expressing said tumour antigen and leads to a reduction in tumour mass. The wording 'inhibiting the growth' comprises shrinking of the tumours, inducing necrotic lesions in the tumour, inducing tumour death and paralysing the growth of tumours. In a preferred embodiment the reduction in tumour mass is at least 50%, 60%, 70%, 80% and preferentially more than 90%.

The conjugation (or coupling) between the single domain variable heavy chain antibody and for example a prodrug converting enzyme or a toxin can be effected by chemical bonding or by splicing together nucleic acid sequences that code for both partners.

In a particular embodiment, the immunoconjugate is bivalent and formed by bonding, chemically or by recombinant DNA techniques, together two monovalent variable domain of heavy chains, The immunoconjugate can also be bispecific and formed by bonding together two variable domains of heavy chains, each one specific for a different tumour marker.

In another embodiment the invention provides an immunoconjugate, devoid of a light chain, specifically binding to carcinoembryonic antigen (CEA), but comprising at least one variable domain of a heavy chain antibody having an anti-tumour agent attached thereto and further characterized by inhibiting the growth of tumour cells expressing CEA.

Carcinoembryonic antigen (CEA) has been used as a marker antigen for cancer imaging and therapy. A large number of CEA antibodies with different specificities and affinities are known in the art. An optimal anti-CEA antibody is an antibody that has a higher proportion and amount of the antibody localized to tumour rather than to other body tissues and it is said that it is 'specifically binding to'. Preferably, no non-specific antibody localisation is observed. The specificity of an anti-CEA immunoconjugate is

preferably such that it binds to human colorectal carcinoma but does not bind to some or all of the following normal tissues: liver, kidney, large intestine, tonsil, lung, brain, testis, ovary, cervix, breast, blood films, placenta, spleen, thyroid, oesophagus, stomach, pancreas, lymph node and skeletal muscle.

The immunoconjugate according to the invention comprises at least one variable domain of a heavy chain antibody that is linked to an antitumour agent. This allows the antibody to target the antitumour agent to the tumour and hence results in inhibition of growth but preferably damage, destruction and/or killing of the tumour. Thus, the immunoconjugate is suitable for use in a method of treatment of the human or animal body. In particular, the immunoconjugate with a specificity for CEA is suitable for use in the manufacture of a medicament to treat a colorectal tumour. The antitumour agent linked to the antibody may be any agent that inhibits, destroys, damages or kills a tumour to which the antibody has bound or in the environment of the cell to which the antibody has bound. For example, the antitumour agent may be a toxic agent such as a chemotherapeutic agent, a radioisotope, an enzyme which activates a prodrug or a cytokine. Suitable chemotherapeutic agents are known to those skilled in the art and include anthracyclines (e.g. daunomycin and doxorubicin), methotrexate, vindesine, neocarzinostatin, cis-platinum, chlorambucil, cytosine arabinoside, 5-fluorouridine, melphalan, ricin and calicheamicin. The chemotherapeutic agents may be conjugated to the antibody using conventional methods known in the art. Suitable radioisotopes for use as antitumour agents are also known to those skilled in the art. For example ^{131}I or astatine such as ^{211}At may be used. These isotopes may be attached to the antibody using conventional techniques known in the art. The antitumour agent which is attached to the antibody may also be an enzyme which activates a prodrug. This allows activation of an inactive prodrug to its active, cytotoxic form at the tumour site as is undertaken in the so-called "antibody-directed enzyme prodrug therapy" (ADEPT). In clinical practice, the antibody-enzyme conjugate is administered to the patient and allowed to localise in the region of the tumour to be treated. The prodrug is then administered to the patient so that conversion to the cytotoxic drug is localised in the region of the tumour to be treated under the influence of the localised enzyme. One enzyme is bacterial carboxypeptidase G2 (CPG2) whose use is described in for example WO 88/07378. Another bacterial enzyme is beta-lactamase whose use is described in US 5773435. The antibody-enzyme conjugate may be modified in accordance with the teaching of WO 89/00427, in order to accelerate clearance from

areas of the body not in the vicinity of a tumour. The antibody-enzyme conjugate may also be used in accordance with WO 89/00427 by providing an additional component which inactivates the enzyme in areas of the body not in the vicinity of the tumour. The antitumour agent conjugated to the antibody may also be a cytokine such as interleukin-2 (IL-2), interleukin-12 (IL-12), granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumour necrosis factor alpha (TNF-alpha). The antibody targets the cytokine to the tumour so that the cytokine mediates damage to or destruction of the tumour without affecting other tissues. The cytokine may be fused to the antibody at the DNA level using conventional recombinant DNA techniques.

In another embodiment the invention provides an immuno-conjugate, devoid of a light chain, specifically binding to a tumour antigen, but comprising at least one variable domain of a heavy chain antibody derived from camelids having an anti-tumour agent attached thereto and further characterized by inhibiting the growth of tumour cells expressing said tumour antigen.

In the present invention a variable domain of a heavy chain antibody derived from a camelid is designated as V_HH.

In another embodiment the invention provides an immunoconjugate, devoid of a light chain, specifically binding to CEA, but comprising at least one variable domain of a heavy chain antibody, derived from camelids, having an anti-tumour agent attached thereto and further characterized by inhibiting the growth of tumour cells expressing CEA.

In the family of 'camelids' immunoglobulins devoid of light polypeptide chains are found. "Camelids" comprise old world camelids (*Camelus bactrianus* and *Camelus dromaderius*) and new world camelids (for example *Lama paccos*, *Lama glama* and *Lama vicugna*). EP0656946 describes the isolation and uses of camelid immunoglobulins and is incorporated herein by reference.

In another embodiment the invention provides an immunoconjugate, devoid of a light chain, specifically binding to a tumour antigen, but comprising at least one variable domain of a heavy chain antibody having an enzyme which activates a prodrug attached thereto and further characterized by inhibiting the growth of tumour cells expressing said tumour marker.

In a particular embodiment the enzyme is bacterial beta-lactamase.

In a more particular embodiment the immunoconjugate has the nucleotide sequence set forth in SEQ ID NO: 15 and the amino acid sequence set forth in SEQ ID NO:14.

In another embodiment the immunoconjugates described herein before can be used as a medicament.

In another embodiment the immunoconjugate provided by the invention can be used for the manufacture of a medicament to treat tumours expressing a tumour marker that is recognised by the immunoconjugate.

In yet another embodiment the invention provides a pharmaceutical composition comprising an immunoconjugate of the present invention.

The term 'medicament to treat' relates to a composition comprising immunoconjugates as described above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat or to prevent diseases as described herein. The administration of an immunoconjugate as described above or a pharmaceutically acceptable salt thereof may be by way of oral, inhaled or parenteral administration. The active compound may be administered alone or preferably formulated as a pharmaceutical composition. An amount effective to treat tumours that express the antigen recognized by the immunoconjugate depends on the usual factors such as the nature and severity of the disorders being treated and the weight of the mammal. However, a unit dose will normally be in the range of 0.01 to 50 mg, for example 0.01 to 10 mg, or 0.05 to 2 mg of immunoconjugate or a pharmaceutically acceptable salt thereof. Unit doses will normally be administered once or more than once a day, for example 2, 3, or 4 times a day, more usually 1 to 3 times a day, such that the total daily dose is normally in the range of 0.0001 to 1 mg/kg; thus a suitable total daily dose for a 70 kg adult is 0.01 to 50 mg, for example 0.01 to 10 mg or more usually 0.05 to 10 mg. It is greatly preferred that the compound or a pharmaceutically acceptable salt thereof is administered in the form of a unit-dose composition, such as a unit dose oral, parenteral, or inhaled composition. Such compositions are prepared by admixture and are suitably adapted for oral, inhaled or parenteral administration, and as such may be in the form of tablets, capsules, oral liquid preparations, powders, granules, lozenges, reconstitutable powders, injectable and infusable solutions or suspensions or suppositories or aerosols. Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tabletting agents, lubricants, disintegrants, colourants, flavourings, and wetting agents. The tablets may be coated according to well known methods in the art.

Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycollate. Suitable lubricants include, for example, magnesium stearate. Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulphate. These solid oral compositions may be prepared by conventional methods of blending, filling, tabletting or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavouring or colouring agents. Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating. Preferably, compositions for inhalation are presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. A favored inhaled dose will be in the range of 0.05 to 2 mg, for example 0.05 to 0.5 mg, 0.1 to 1 mg or 0.5 to 2 mg. For parenteral administration, fluid unit dose forms are prepared containing a compound of the present invention and a sterile vehicle. The active compound, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under

vacuum. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the active compound. Where appropriate, small amounts of bronchodilators for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included. As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

The present invention further provides a pharmaceutical composition for use in the treatment and/or prophylaxis of herein described disorders which comprises a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable solvate thereof, and, if required, a pharmaceutically acceptable carrier thereof.

It should be clear that the therapeutic method of the present invention against tumours can also be used in combination with any other tumour therapy known in the art such as irradiation, chemotherapy or surgery.

The following examples more fully illustrate preferred features of the invention, but are not intended to limit the invention in any way. All of the starting materials and reagents disclosed below are known to those skilled in the art, and are available commercially or can be prepared using well-known techniques.

Examples

1. Construction and Purification of the Camel Single-domain::β-lactamase Conjugates.

Several anti-CEA camel single domain VH and V_HH antibodies were retrieved from an immunized phage display library. FACS analysis was performed to analyze the ability of these antibodies to recognize CEA expressed on LS 174T cells (the human LS 174T adenocarcinoma cell line was obtained from ATCC (Manassas, VA). LS 174T is a trypsinized variant of the LS 180 colon-adenocarcinoma cell line and produces large amounts of carcinoembryonic antigen (CEA). Based on the FACS profiles, V_HHs cAb-CEA3 (SEQ ID NO: 1 for the amino acid sequence and SEQ ID NO: 2 for the nucleotide sequence), cAb-CEA5 (SEQ ID NO: 3 for the amino acid sequence and SEQ ID NO: 4 for the nucleotide sequence), cAb-CEA61 (SEQ ID NO: 5 for the amino

acid sequence and SEQ ID NO: 6 for the nucleotide sequence) and the VH cAbCEA72 (SEQ ID NO: 7 for the amino acid sequence and SEQ ID NO: 8 for the nucleotide sequence) were chosen for conjugate construction. cAb-CEA-β-lactamase conjugates were constructed in a stepwise fashion by insertion of the cAb-CEA sequence, the llama γ2c hinge (AHHSEDPSSKAPKAP) region sequence (SEQ ID NO: 9) and the β-lactamase (bL) gene followed by a 6xhis-tag into the pHEN6 expression vector. The particular bL was cloned from the *E. cloacae* P99 strain by PCR amplification. Primer-sequences used are 5'-CATGCCATGACTCGCGGCCCAGCCGGCCATGGC-3' (Fw primer) (SEQ ID NO: 10) and 5'-CATG***CCATGG***GAGCTTTGGGAGCTTTGGAGCTGGGGTCTTCGCTGTGGTGCGCT GAGGAGACGGTGACCTGGGT-3' (Rev primer: includes γ2c hinge coding sequence) for amplification and NcoI cloning of cAb-CEA/γ2c hinge (SEQ ID NO: 11).

5'-CATG***CCATGG***GCACGCCAGTGTCAGAAAAA-3' (Fw primer) (SEQ ID NO: 12) and 5'-CGC***GAATTC***TTAATGATGATGATGATGATGCTGTAGCGCCTGGAGG-3' (Rev primer: includes 6x his tag coding sequence) for amplification and directional NcoI-EcoRI cloning of β-lactamase (SEQ ID NO: 13). The resulting cAb-CEA-βL his-tagged conjugates were expressed in *E. coli* and purified on an IMAC column (Ni-NTA Superflow, QIAGEN) followed by gel filtration on a Superdex 75 HR 10/30 column (Pharmacia). The anti-lysozyme camel single-domain antibody cAb-Lys3 conjugated to β-lactamase was also engineered and used as non-binding control in further experiments. The isolation of the cAb-Lys3 antibody fragment was previously described (Ghahroudi *et al.*, 1997). The gene was recloned in an expression vector under control of the lac promoter, between the Pel B leader signal and a carboxyterminal hexahistidine tail (Lauwereys *et al.*, *EMBO J,* 17, 3512-3520 (1998).

Enzymatic activity assays of the bL portion of the conjugates were undertaken using nitrocefin as the substrate. Michaelis-Menten kinetic analyses confirmed that the fusion protein retained the full enzymatic activity from the enzyme from which it was derived.

2. *In vitro* cytotoxicity Assays using cAb-CEA5-βL conjugate.

A total of 10^4 LS 174T human adenocarcinoma cells/well (0.1ml of EMEM with 10% fetal bovine serum, 100units/ml penicillin, 0.1mg/ml streptomycin, 1mM sodium pyruvate and 0.1mM non-essential amino acids) were plated into 96-well microtiter plates and allowed to adhere overnight. For blocking experiments, the cells were incubated with unconjugated cAb-CEA5 at 0.1mg/ml for 30 minutes prior to treatment

with the cAb-CEA-βL conjugates. The cells were then exposed to the conjugates at 1, 5, and 10nM. After 30 minutes at 4°C, the plates were washed 3 times with antibiotic free RPMI 1640 medium with 10% fetal bovine serum, and then different amounts of the prodrug CCM (7-(4-carboxy-butanamido) cephalosporin mustard) or PDM (parental drug, phenylenediamine mustard) were added (see Fig. 1 for the structure). CCM and PDM were also added to cells that were not treated with the conjugates. We received the prodrug CCM and parental drug PDM for the *in vitro* cytotoxicity studies from Dr. Peter Senter (Director Chemistry, Seattle Genetics, Inc., Washington, U.S.A). After 1 hour at 37°C, the cells were washed with EMEM and incubated for 24 hours. The cells were then pulsed for 18 hours with [^{3}H] thymidine (1μCi/well) at 37°C, detached by freezing and thawing, and harvested onto glass fiber filter mats using a 96-well cell harvester. Radioactivity was counted using a β-plate counter. Another set of experiments was performed with varying concentrations of the anti-CEA-βL conjugates or cAb-Lys3-βL as a non-binding control. After conjugate exposure, cells were treated with a fixed amount of CCM. After 24 hours incubation, the cells were pulsed for 18 hours, harvested and radioactivity was counted with a β counter. The cytotoxic effects of a conjugated V_HH, cAb-CEA5-βL (SEQ ID NO: 14 for the amino acid sequence and SEQ ID NO: 15 for the nucleotide sequence) (Fig.2) in combination with CCM prodrug were determined on LS 174T human adenocarcinoma cells which express the CEA antigen. The cells were exposed to the conjugate, washed to remove unbound material, and treated with various amounts of two different batches of CCM (CCM1, CCM2). Cytotoxic activity was determined by measuring the incorporation of [^{3}H] thymidine into DNA relative to untreated cells. The prodrug CCM was approximately 40 fold less toxic to LS 174T cells than the parental drug PDM. cAb-CEA5-βL effectively activated the prodrug in a dose dependent manner, leading to a cytotoxicity equivalent in activity to PDM. Prodrug activation was immunologically specific since cAb-CEA5-βL activated CCM at marginal levels on cells that were saturated with unconjugated cAb-CEA5 prior exposure to the fusion protein. . In addition, to compare the relative abilities of the cAb-CEA-βL conjugate for prodrug activation, LS 174T cells were exposed to various amounts of conjugate. Unbound material was washed off, and CCM was added at a fixed concentration of 3μM, which has low cytotoxic activity in the absence of β-lactamase. cAb-CEA5-βL induced effectively the prodrug in a dose dependent manner and showed to be immunologically specific (Fig. 3 panel A and B).

Demonstration of the immunological specificity of prodrug activation was done by saturation with non-conjugated cAb-CEA or by treating the cells with non- binding control conjugate, cAb-Lys3-βL, prior to CCM. As expected, cAb-Lys3-βL did not activate the prodrug CCM.

3. Immunogenicity studies

To study the immune response to cAb-enzyme conjugates, BALB/c mice receive a single or multiple course of intravenous treatment with cAb-CEA5 antibody fragments conjugated to bacterial enzyme β-lactamase (1 mg of immunoconjugate/kg bodyweight). The development of mouse anti-camel antibodies and anti-β-lactamase antibodies is analyzed at day 7, 14 and 60 after the last treatment course by ELISA. Anti-β-lactamase antibodies present in serum of mice are tested for their capacity to inhibit β-lactamase activity *in vitro*.

4. *In vivo* therapy experiments in nude mice bearing LS 174T carcinoma tumour xenografts.

4.1 Conjugate localization

Studies were undertaken in nude mice to establish the extent of cAb-CEA5::β-lactamase conjugate localization in LS 174 T tumor xenografts. ^{125}I labeled cAb-CEA5::β-lactamase (4.728.481 cpm/μg conjugate) or cAb-Lys3::β-lactamase conjugate (2.691.621 cpm/μg conjugate) were injected i.v. (1 mg/kg) into mice (3 animals/group) that had subcutaneous LS 174T carcinoma tumors of about 0.5-1 cm diameter. The amount of radioactivity in the tumors, blood, and several other tissues was determined 6h, 24h and 48h later (Fig. 4). It was found that the concentration of cAb-CEA5::βL in tumors was much higher (>10-fold) than in any other of the tissues measured. This was most likely due to binding to the CEA antigen on tumor cells, since the irrelevant cAb-Lys3::βL showed no preferential intratumoral accumulation. We also noticed a rather high accumulation of both cAb-CEA5::βL and cAb-Lys3::βL conjugates in the kidneys (0.41-0.53 %ID/g tissue). In order to see whether the radioactivity measured originated from intact conjugate molecules or degraded material, we assessed the enzymatic activity of β-lactamase in targeted tumor, liver and kidney tissue using nitrocefin. The results showed that enzymatic activity was intact in the excised tumor tissue whereas no activity could be measured in liver nor kidney tissue, indicating that

the radioactivity measured in kidney and liver tissue was not derived from intact antibody-enzyme conjugate molecules (spiking these tissue suspensions with similar concentrations of cold cAb-CEA5::βL resulted in positive enzymatic activity, indicating that the tissue suspensions did not exhibit inhibitory activity on the enzymatic activity). Maximal tumor uptake of approximately 3% injected dose/g tumor was seen 6 h after dosing of the cAb-CEA::βL conjugate whereas no targeting was seen for the nonbinding control cAb-Lys3::βL conjugate. The blood and normal tissue levels were still high at this time-point and thus tumor/normal tissue ratios were low. After 24 h, although the amounts of cAb-CEA::βL conjugate in the tumors had fallen to approx. 1% injected dose/g tumor, the blood and normal tissue levels had fallen more rapidly, and consequently, tumor/normal tissue ratios were in the 10-50 fold range, except for the kidneys were a high amount of radioactivity could still be measured. After 48 h, a similar biodistribution was seen. Based on these data, an interval of 24 h between conjugate and prodrug administration was selected for antitumour studies.

4.2 Antitumour effect of the prodrug therapy

The antitumor effect of giving cAb-CEA::βL conjugate (1 mg/ kg body weight) followed 24 h later by escalating doses of CCM (100, 150, 200 mg/kg) are shown in Fig. 5 . The prodrug therapy combination gave a significant antitumor effect compared to non-treated tumor-bearing mice or mice receiving prodrug in combination with the nonbinding cAb-Lys3::βL conjugate. Therapeutic efficiency was dose-dependent. Significant antitumor activity including partial regression of the tumors was obtained in the animals that received 200 mg CCM/kg/injection. There were no apparent toxicities in any of the groups receiving CCM therapy. In contrast, systemic treatment of mice with the drug PDM at 4 mg/kg/injection had no beneficial effect on tumor growth since they grew out after the treatment was discontinued. Moreover, although the PDM dose given at about the maximal tolerated dose (4.5 mg/kg/injection), systemic administration led to toxicity and resulted in > 10% body weight loss.

Materials and Methods

Tumor localization studies

The cAb-CEA::βL conjugate was radioiodinated with carrier-free ^{125}I using the IODOGEN reagent, following the manufacturer's (Pierce, Rockford, Illinois, USA) recommended method. In vitro retention of immunoreactivity postradioiodination was confirmed by binding to LS174T cells. Approximately 1 mg of conjugate/ kg body

weight were injected intravenously into athymic nude mice bearing established tumor xenografts (2 x 10^6 LS174T tumor cells injected 10 days previously and tumors measured approximately 5-6 mm in diameter). Following injection of the conjugate, groups of three mice were killed 6, 24 and 48 h later. The tumor, a sample of blood, and a range of other tissues were removed, weighed, and counted in a gamma counter.

Antitumor studies

Groups of 5 female athymic nude mice were injected subcutaneously with 2 x 10^6 LS174T tumor cells. Ten days later when the tumors reached a size of about 100 mm^3, 1 mg/ kg bodyweight of βL conjugates was injected iv, followed 24 h later by the prodrug CCM. Treatment with cAb-βL + CCM was carried out on a weekly schedule for a total of 3 rounds. The animals were monitored twice a week for general health, weight and tumor growth and compared to control groups receiving no treatment. Tumor volumes were calculated using the formula (longest length x perpendicular $width^2$)/2.

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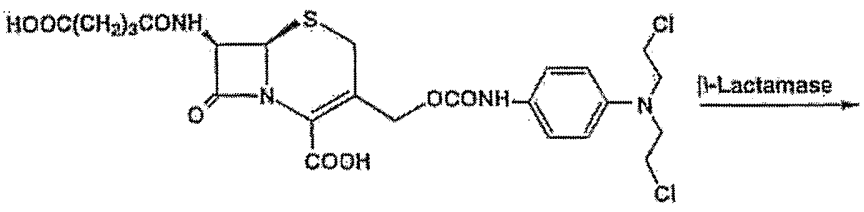
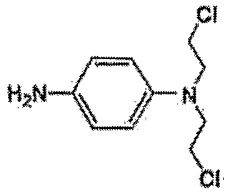
Claims

1. An immunoconjugate, devoid of a light chain, specifically binding to a tumour antigen comprising at least one variable domain of a heavy chain antibody having an anti-tumour agent attached thereto and further characterized by inhibiting the growth of tumour cells expressing said tumour antigen and leads to a reduction in tumour mass.
2. An immunoconjugate according to claim 1 wherein said reduction of tumour mass is at least 50%.
3. An immunoconjugate according to claims 1 and 2 which is specifically binding to carcinoembryonic antigen (CEA).
4. An immunoconjugate according to claims 1, 2 or 3 wherein said single domain heavy chain antibody is derived from camelids.
5. An immunoconjugate according to claim 1-4 wherein said anti-tumour agent is an enzyme which activates a prodrug.
6. An immunoconjugate according to claim 5 wherein said enzyme is bacterial beta-lactamase.
7. An immunoconjugate according to claims 3 and 6 and has the nucleotide sequence set forth in SEQ ID NO: 15 and the polypeptide sequence set forth in SEQ ID NO: 16.
8. Use of the immunoconjugate of claims 1-7 as a medicament.
9. Use of the immunoconjugate of claims 1-7 for the manufacture of a medicament to treat tumours expressing a tumour marker that is recognised by the immunoconjugate.
10. A pharmaceutical composition comprising an immunoconjugate according to claims 1-7.

Fig. 1:



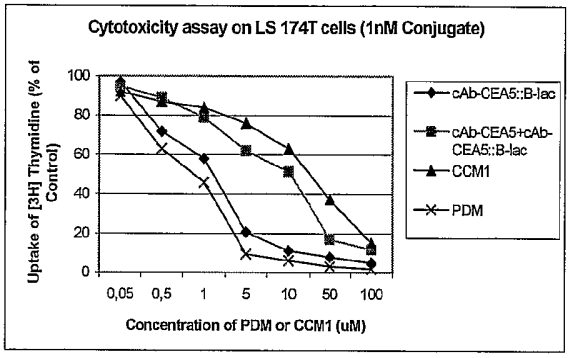
CCM



PDM

Fig. 2:

A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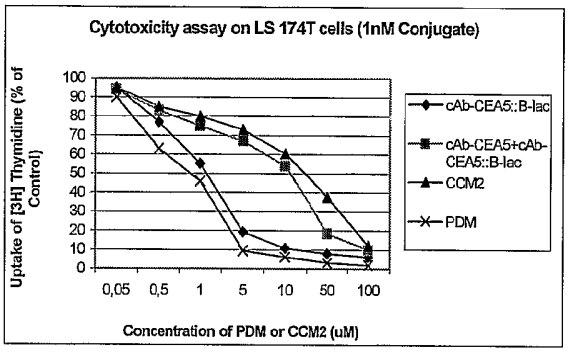
cAb-CEA5+cAb-CEA5::B-lac
CCM1
PDM

B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80
70
60
50
40
30
20
10
0
0,05 0,5 1 5 10 50 100
Concentration of PDM or CCM2 (uM)
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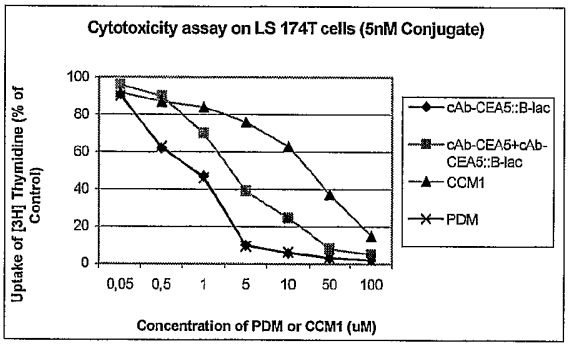
cAb-CEA5+cAb-CEA5::B-lac
CCM2
PDM

C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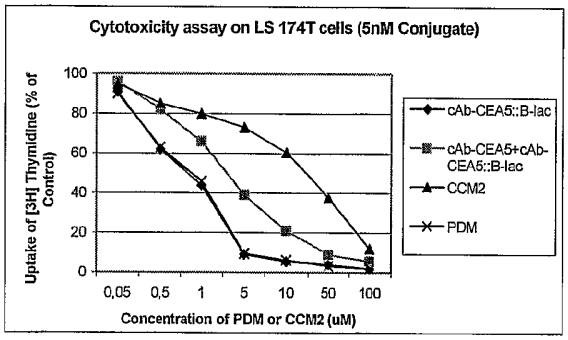
CCM1
PDM

D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CCM2
PDM

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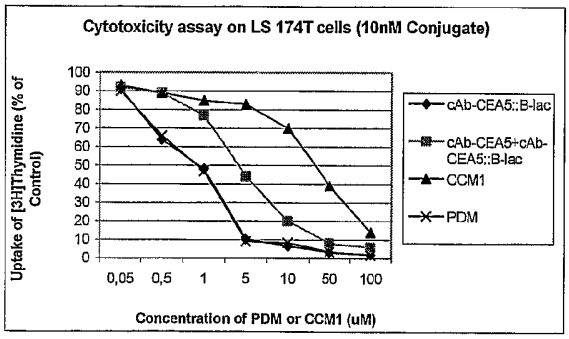
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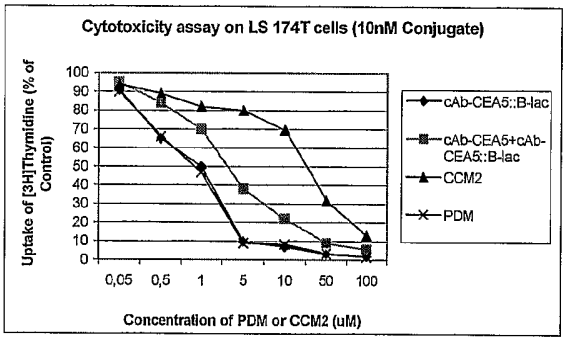
Concentration of PDM or CCM1 (uM)
cAb-CEA5::B-lac
cAb-CEA5+cAb-CEA5::B-lac
CCM1
PDM

F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0
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Concentration of PDM or CCM2 (uM)
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cAb-CEA5+cAb-CEA5::B-lac
CCM2
PDM

Fig. 3:

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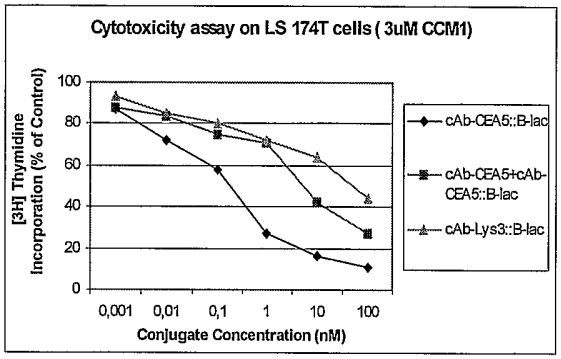
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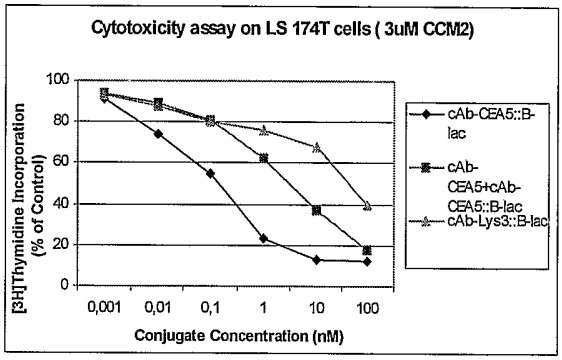
cAb-Lys3::B-lac

B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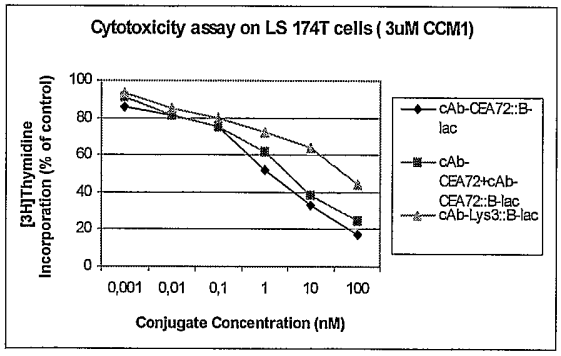
cAb-Lys3::B-lac

C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cAb-Lys3::B-lac

D

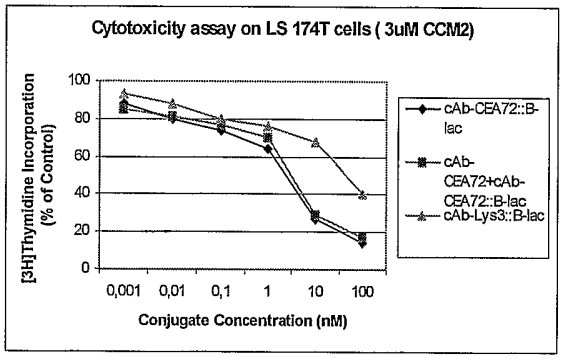
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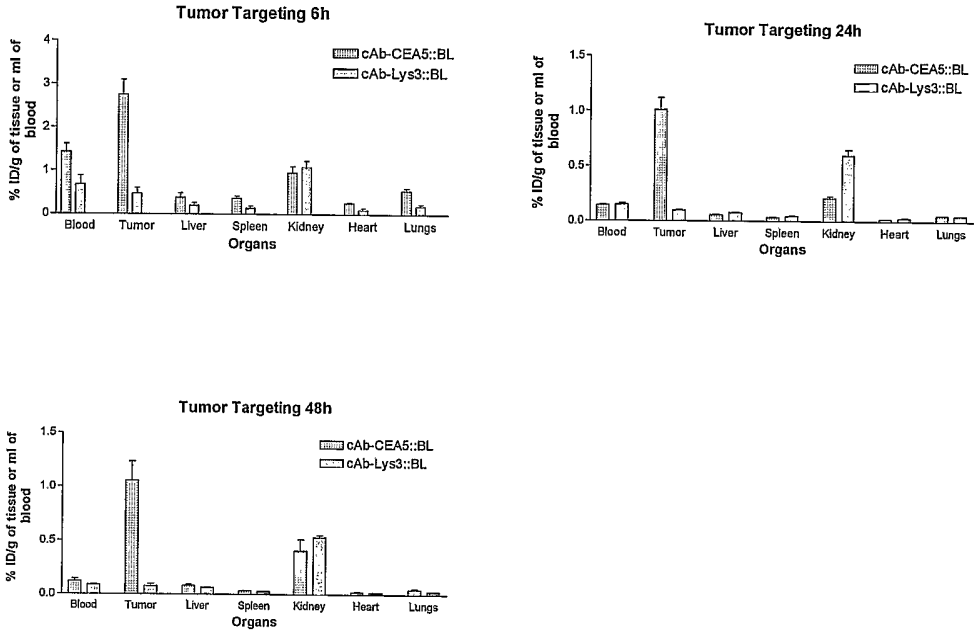
Fig. 4: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Liver
Spleen
Kidney
Heart
Lungs
Organs
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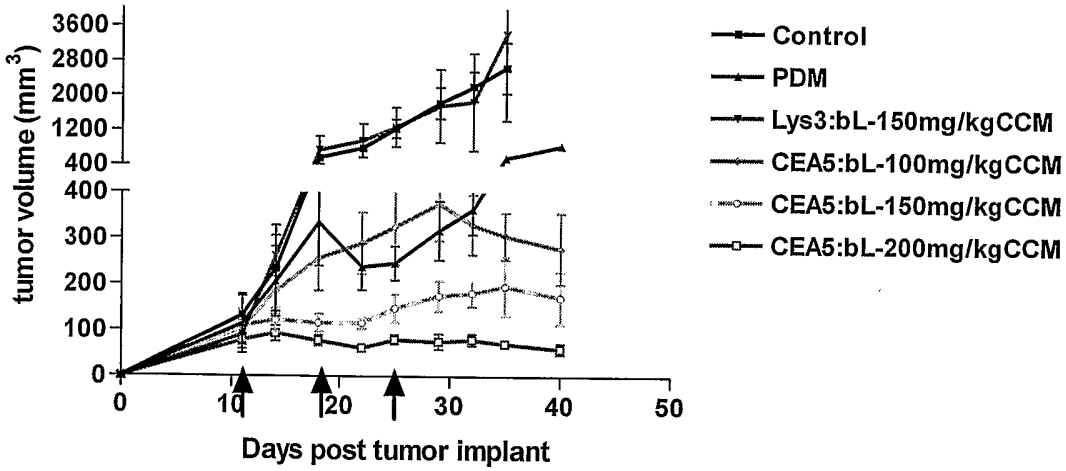
Tumor Targeting 48h

Fig. 5: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Days post tumor implant
Control
PDM
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<400> 5

Asp Val Gln Leu Val Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Pro Ser Gly Tyr Asn Ile Trp Thr Asn
20 25 30

Ser Cys Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly
35 40 45

Val Ala Leu Ile Tyr Ser Gly Gly Gly Thr Thr Tyr Tyr Ala Asp Ser

50 55 60

Val Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Arg Asn Thr Val
65 70 75 80

Tyr Leu Gln Met Asp Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr
85 90 95

Cys Ala Ala Arg Arg Cys Gly Thr Tyr Ser Asn Asp Leu Asp Val Arg
100 105 110

Thr Trp Asn Arg Tyr Gly Phe Trp Gly Gln Gly Thr Gln Val Thr Val
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Ser Ser
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<211> 390

<212> DNA

<213> Artificial Sequence

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<223> cAb-CEA61

<400> 6

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gctccaggga aggagcgcga gggggtcgcg ttgatttatt ctggtggtgg tacgacatac 180

tatgccgact ccgtgaaggg ccgattcacc atctcccaag acaacgccag gaacacggtg 240

tatctacaaa tggacagcct gaaacctgag gacactgcca tgtactactg tgccgcaagg 300

aggtgtggga cctactcgaa cgaccttgac gtccgaactt ggaatcggta tggcttctgg 360

ggccagggga cccaggtcac cgtctcctca 390

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<212> PRT

<213> Artificial Sequence

<220>

<223> cAb-CEA72

<400> 7

Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Glu Phe Thr Phe Ser Ser Ser
20 25 30

Tyr Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ser Gly Ile Asn Thr Asp Gly Ser Phe Thr Arg Tyr Ala Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Lys Ser Glu Asp Thr Ala Leu Tyr Tyr Cys
85 90 95

Ala Val Gly Gly Gly Leu Gly Tyr Gly Pro Arg Gly Gln Gly Thr Gln
100 105 110

Val Thr Val Ser Ser
115

<210> 8

<211> 351

<212> DNA

<213> Artificial Sequence

<220>

<223> cAb-CEA72

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ccagggaagg ggctggagtg ggtgtccggc attaataccg atggaagttt cacgcgctat 180

gcagactccg tgaagggccg attcaccatc tccagagaca acgccaagaa cacgctgtat 240

ctgcaaatga acagcctgaa atctgaggac acggccctgt attactgtgc cgtaggcggc 300

gggttaggct atggccccag gggccagggg acccaggtca ccgtctcctc a 351

<210> 9

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> the llama gamma2c hinge region

<400> 9

Ala His His Ser Glu Asp Pro Ser Ser Lys Ala Pro Lys Ala Pro
1 5 10 15

<210> 10

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Fw primer

<400> 10
catgccatga ctcgcggccc agccggccat ggc 33

<210> 11

<211> 74

<212> DNA

<213> Artificial Sequence

<220>

<223> Rev primer: includes gamma2c hinge coding sequence

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acggtgacct gggt 74

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<212> DNA

<213> Artificial Sequence

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<223> Fw primer

<400> 12
catgccatgg gcacgccagt gtcagaaaaa 30

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<211> 46

<212> DNA

<213> Artificial Sequence

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<223> Rev primer: includes 6x his tag coding sequence

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<210> 14

<211> 507

<212> PRT

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<223> cAb-CEA5

<220>

<221> MISC_FEATURE

<222> (124)..(138)

<223> llama gamma2c hinge

<220>

<221> MISC_FEATURE

<222> (140)..(491)

<223> beta-lactamase

<220>

<221> MISC_FEATURE

<222> (492)..(497)

<223> 6xhis tag

<400> 14

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Asp Thr Tyr Gly Ser Tyr
            20                  25                  30

Trp Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
        35                  40                  45

Ala Ala Ile Asn Arg Gly Gly Gly Tyr Thr Val Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Thr Ala Lys Asn Thr Val Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Pro Asp Asp Thr Ala Asp Tyr Tyr Cys
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85 90 95

Ala Ala Ser Gly Val Leu Gly Gly Leu His Glu Asp Trp Phe Asn Tyr
100 105 110

Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser Ala His His Ser Glu
115 120 125

Asp Pro Ser Ser Lys Ala Pro Lys Ala Pro Met Gly Thr Pro Val Ser
130 135 140

Glu Lys Gln Leu Ala Glu Val Val Ala Asn Thr Ile Thr Pro Leu Met
145 150 155 160

Lys Ala Gln Ser Val Pro Gly Met Ala Val Ala Val Ile Tyr Gln Gly
165 170 175

Lys Pro His Tyr Tyr Thr Phe Gly Lys Ala Asp Ile Ala Ala Asn Lys
180 185 190

Pro Val Thr Pro Gln Thr Leu Phe Glu Leu Gly Ser Ile Ser Lys Thr
195 200 205

Phe Thr Gly Val Leu Gly Gly Asp Ala Ile Ala Arg Gly Glu Ile Ser
210 215 220

Leu Asp Asp Ala Val Thr Arg Tyr Trp Pro Gln Leu Thr Gly Lys Gln
225 230 235 240

Trp Gln Gly Ile Arg Met Leu Asp Leu Ala Thr Tyr Thr Ala Gly Gly
245 250 255

Leu Pro Leu Gln Val Pro Asp Glu Val Thr Asp Asn Ala Ser Leu Leu
260 265 270

Arg Phe Tyr Gln Asn Trp Gln Pro Gln Trp Lys Pro Gly Thr Thr Arg
275 280 285

Leu Tyr Ala Asn Ala Ser Ile Gly Leu Phe Gly Ala Leu Ala Val Lys
290 295 300

Pro Ser Gly Met Pro Tyr Glu Gln Ala Met Thr Thr Arg Val Leu Lys
305 310 315 320

Pro Leu Lys Leu Asp His Thr Trp Ile Asn Val Pro Lys Ala Glu Glu
325 330 335

Ala His Tyr Ala Trp Gly Tyr Arg Asp Gly Lys Ala Val Arg Val Ser
340 345 350

Pro Gly Met Leu Asp Ala Gln Ala Tyr Gly Val Lys Thr Asn Val Gln
355 360 365

Asp Met Ala Asn Trp Val Met Ala Asn Met Ala Pro Glu Asn Val Ala
370 375 380

HRE-Ant-V105.ST25

Asp Ala Ser Leu Lys Gln Gly Ile Ala Leu Ala Gln Ser Arg Tyr Trp
385 390 395 400

Arg Ile Gly Ser Met Tyr Gln Gly Leu Gly Trp Glu Met Leu Asn Trp
405 410 415

Pro Val Glu Ala Asn Thr Val Val Glu Gly Ser Asp Ser Lys Val Ala
420 425 430

Leu Ala Pro Leu Pro Val Ala Glu Val Asn Pro Pro Ala Pro Pro Val
435 440 445

Lys Ala Ser Trp Val His Lys Thr Gly Ser Thr Gly Gly Phe Gly Ser
450 455 460

Tyr Val Ala Phe Ile Pro Glu Lys Gln Ile Gly Ile Val Met Leu Ala
465 470 475 480

Asn Thr Ser Tyr Pro Asn Pro Ala Arg Val Glu Ala Ala Tyr His Ile
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Leu Glu Ala Leu Gln His His His His His His
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<210> 15

<211> 1521

<212> DNA

<213> Artificial Sequence

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<222> (1)..(369)

<223> cAb-CEA5

<220>

<221> misc_feature

<222> (370)..(415)

<223> llama gamma2c hinge

<220>

<221> misc_feature

<222> (422)..(1503)

<223> beta-lactamase

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<221> misc_feature

<222> (1504)..(1521)

<223> 6xhis tag

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ccagggaagg agcgtgaggg ggtcgcagct attaataggg gtggtggcta tacagtctac 180

gccgactccg tgaagggccg attcaccatc tcccgagaca ccgccaagaa cacggtgtat 240

ctgcaaatga acagcctgag acctgacgac acggccgact attactgtgc ggctagcggg 300

gtactaggtg gtttacatga ggactggttt aactactggg gccaggggac ccaggtcacc 360

gtctcctcag cgcaccacag cgaagacccc agctccaaag ctcccaaagc tccaatgggc 420

acgccagtgt cagaaaaaca gctggcggag gtggtcgcga atacgattac cccgctgatg 480

aaagcccagt ctgttccagg catggcggtg gccgttattt atcagggaaa accgcactat 540

tacacatttg gcaaggccga tatcgcggcg aataaacccg ttacgcctca gaccctgttc 600

gagctgggtt ctataagtaa aaccttcacc ggcgttttag gtggggatgc cattgctcgc 660

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gtaccggatg aggtcacgga taacgcctcc ctgctgcgct tttatcaaaa ctggcagccg 840

cagtggaagc ctggcacaac gcgtctttac gccaacgcca gcatcggtct ttttggtgcg 900

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