

THERAPEUTIC ANTIBODIES AND USES THEREOF

Incorporation by Cross-Reference

The present application claims priority from Australian provisional patent application
5 number 2015901423 filed on 20 April 2015, the entire content of which is incorporated
herein by cross-reference.

Technical Field

The present invention relates generally to the field of prostate, pancreatic and/or
10 bladder cancers. More specifically, the present invention relates to the use of antibodies in
treatment of prostate, pancreatic and/or bladder cancers.

Background

Prostate cancer is the most commonly occurring tumour in males and is second only to
15 lung cancer in mortality. Treatment with surgery and/or radiotherapy is successful in many
patients if prostate cancer is diagnosed early. However, many patients with advanced disease
and a sizeable proportion of all prostate cancer patients eventually develop metastatic disease
following localised therapy.

Bladder cancer is estimated to affect approximately 77,000 adults in the US annually,
20 with an estimated 16,000 fatalities. A comparable number of persons in the US are predicted
to be diagnosed with pancreatic cancer in 2016 (53,000) with a very high mortality rate
(41,000). Common treatments for these cancers include surgical removal, radiation therapy,
and/or chemotherapy.

Despite their promise as effective agents in the treatment of cancer there are no
25 therapeutic antibodies with regulatory approval for the treatment of prostate, pancreatic or
bladder cancers. US patent no. 5,622,836 to Walker *et al.* discloses an antibody named
BLCA-38 (BLCA - "bladder cancer"). The document teaches that BLCA-38 is a monoclonal
antibody specific for an unknown antigen expressed by bladder carcinoma cells. BLCA-38 is
also taught to show specificity for human ovarian and colonic cancer cell lines, as well as
30 some melanoma cell lines, but not to lymphoid (T lymphoid or B lymphoid) and leukemic
cell lines.

Subsequently, Russell *et al.* (2004) (Russell *et al.*, "Cytotoxic properties of
immunoconjugates containing melittin-like peptide 101 against prostate cancer: in vitro & in

vivo studies". Cancer Immunol Immunother 2004; 53(5): 411-421) published a study in which BLCA-38 was used to target a cytotoxic peptide to prostate cancer cells. The authors indicate that BLCA-38 is a murine monoclonal antibody raised against the human bladder cancer cell line UCRU-BL-17CL.

5 A further publication by Russell *et al.* in 2004 (Russell et al., "Immunohistochemical characterization of the monoclonal antibody, BLCA38, for the detection of prostate cancer". Cancer Immunol Immunother 2004; 53(11) 995-1004) also teaches that BLCA-38 is a murine monoclonal antibody raised against a human bladder cell line which is capable of binding to bladder carcinoma cells, prostate cancer cells, and vulval epidermoid cells, but not to breast
10 cancer cells. The article indicates that BLCA-38 is specific for an antigen of approximately 30kDa in size that is difficult to characterise or identify.

Carter *et al.* (2004) (Carter et al., "Biodistributions of intact monoclonal antibodies and fragments of BLCA38, a new prostate cancer directed antibody". Cancer Immunol Immunother 2004; 53(6) 533-542) analysed timing and dosage for targeting therapeutic
15 agents to prostate cancer cells using BLCA-38, also indicating that it is a murine monoclonal antibody targeting an antigen of around 30kDa expressed on the cell surface and in the cytoplasm. The authors state that the nature of the antigen is elusive, and indicate that it is expressed on bladder and prostate cancer cells.

An article by Khatri *et al.* published in 2010 (Khatri et al. "Promise of BLCA38 as a
20 Targeting Antibody for Tissue-Specific Gene Delivery to Prostate Cancer". Austral-Asian J. Cancer 2010; 9(3): 195-203) reiterated that BLCA-38 is a murine monoclonal antibody specific for prostate cancer cells. The authors reveal that although BLCA-38 is not internalised upon binding to its antigen, conjugation with a virus facilitated internalisation of the antibody resulting in increased expression of the reporter gene.

25 Unlike many cancer treatments that may damage cancer and healthy cells alike, targeted cancer therapies are designed to selectively attack tumor cells while avoiding healthy cells and tissues in the patient under treatment. For example, targeted antibody-drug conjugates (ADCs) selectively deliver cytotoxic agents to cancer cells by virtue of specific binding to markers produced specifically by the cancer cells. The capacity of these agents to
30 deliver their toxic payload to cancerous cells and avoid damaging healthy tissue is obviously important. Additionally, internalisation of ADCs upon specifically binding to cancer cells can enhance their cytotoxic effects as opposed to ADCs that are not internalised. Internalisation can allow the tailoring of ADCs to enhance their effects upon uptake into cellular vesicles (e.g. endosomes and lysosomes). It is also noteworthy that internalisation of ADCs does not

necessarily induce effective cell killing, an observation that may at least in part arise from differential endosomal/lysosomal processing pathways in different types of cancer cells.

Therapeutic antibodies may require substantial quantities of product over multiple dosages to be clinically effective (e.g. 200–350mg/m² per dose). A therapeutic dose of ADC may often be marginally less than that of naked therapeutic monoclonal antibodies (TMAs) (e.g. at 160 mg per dose). However, ADC construction involves additional technologies such as chemical conjugation steps that complicate production streams and can raise the cost of manufacture significantly. It is therefore desirable to provide therapeutic dose ADCs at lower cost where possible.

Despite the promise that antibodies offer as therapeutic agents, a need continues to exist for effective therapeutic antibodies to treat prostate, bladder, and/or pancreatic cancer.

Summary of the Invention

The present inventors have surprisingly identified that the BLCA-38 antibody referred to and used in the aforementioned prior art is a combination of two distinct monoclonal antibodies in a mixed population. It has been determined that only one of these antibody species is capable of binding strongly to the relevant target antigen present on prostate cancer cells, whilst the second species cannot. Moreover, it has been unexpectedly discovered that the antibody species is capable of binding to the relevant target antigen present on prostate cancer cells is internalised upon doing so, despite the aforementioned prior art indicating to the contrary. Moreover, the present inventors have demonstrated effective internalisation in prostate, bladder and pancreatic cancer cells providing a means of targeting and killing these cancer cell types.

Accordingly, in a first aspect the present invention provides a use of an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof in the preparation of a medicament for treating prostate, bladder, and/or pancreatic cancer in a subject, wherein: the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject, and the antibody and/or fragment comprises: (a) a heavy chain variable region comprising: a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 3**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 3**; a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 3**; and (b) a light chain variable region comprising: a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 4**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 4**; a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by

positions 109-117 of **SEQ ID NO: 4**; and the medicament does not comprise an antibody with a light chain variable region comprising: a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 48-58 of **SEQ ID NO: 11**; a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 74-80 of **SEQ ID NO: 11**; a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 11**.. Also disclosed is a method for treating prostate, bladder, and/or pancreatic cancer in a subject, the method comprising administering to the subject a pharmaceutical preparation comprising an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof, wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is a method for killing a prostate, bladder, and/or pancreatic cancer cell in a subject, the method comprising administering to the subject a pharmaceutical preparation comprising an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof, wherein the antibody and/or fragment is

conjugated to at least one cytotoxic agent that is toxic to the prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is a method for treating a prostate, bladder, and/or pancreatic cancer tumour in a subject, the method comprising administering to the subject a pharmaceutical preparation comprising an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding
5 fragment thereof, wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the tumour.

Also disclosed is a use of an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof in the preparation of a medicament for treating prostate, bladder,
10 and/or pancreatic cancer in a subject, wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is a use of an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof in the preparation of a medicament for killing a prostate, bladder,
15 and/or pancreatic cancer cell in a subject, wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is a use of an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof in the preparation of a medicament treating a prostate, bladder,
20 and/or pancreatic cancer tumour in a subject, wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof for use in treating prostate, bladder, and/or pancreatic cancer in a subject,
25 wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof for use in killing a prostate, bladder, and/or pancreatic cancer cell in a subject, wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent
30 that is toxic to the prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof for use in treating a prostate, bladder, and/or pancreatic cancer tumour in a subject, wherein the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject.

Also disclosed is a method of radioimaging a prostate, bladder, and/or pancreas in a subject, the method comprising:

administering to the subject a pharmaceutical preparation comprising an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof, wherein the antibody and/or fragment is conjugated to at least one radioimaging agent,

detecting radiation emitted by the radioimaging agent, and

using the detected radiation to form a radioimage of the prostate, bladder, and/or pancreas.

In one example, the prostate of the subject is characterised by benign prostatic hyperplasia (BPH).

In one example, the prostate, bladder and/or pancreas of the subject is cancerous. In one embodiment of the tenth aspect, the subject suffers from prostate, bladder and/or pancreatic cancer.

In one example, the radiomaging agent is selected from the group consisting of: ^{18}F , ^{67}Ga , ^{68}Ga , ^{81}mKr , ^{82}Rb , ^{13}N , ^{99}mTc , ^{111}In , ^{123}I , ^{124}I , ^{133}Xe , ^{201}Tl , ^{177}Lu , ^{89}Zr , ^{64}Cu , ^{67}Cu , ^{32}P , and any combination thereof.

In one example, the radiomaging is single-photon emission computed tomography (SPECT) and the radioimaging agent is selected from the group consisting of: ^{99}mTc , ^{111}In , ^{67}Ga , ^{123}I , and any combination thereof.

In one example, the radiomaging is positron emission tomography (PET) and the radioimaging agent is selected from the group consisting of: ^{18}F , ^{68}Ga , ^{64}Cu , ^{86}Y , ^{124}I , ^{89}Zr , and any combination thereof.

In one example, the pharmaceutical preparation does not comprise an antibody with a light chain variable region comprising:

a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 48-58 of SEQ ID NO: 11;

a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 74-80 of SEQ ID NO: 11;

a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 11**.

In another example, the cytotoxic agent is a prodrug designed for activation upon uptake into an endosome or lysosome of said prostate, bladder, and/or pancreatic cancer cell.

In another example, the prodrug is conjugated to the antibody and/or an antigen-binding fragment thereof by:

an acid sensitive-linker susceptible to cleavage at a pH that is less than: pH 7.2, pH 7.0, pH 6.5, pH 6.0, pH 5.5, pH 5.0, pH 4.5, or pH 4.

an enzyme-cleavable linker, wherein the enzyme capable of cleaving the linker exists within the endosome or lysosome.

In another example, the cytotoxic agent is linked to the antibody and/or antigen binding fragment thereof by binding to a chelating agent conjugated to the antibody and/or antigen binding fragment thereof.

In an additional example, the antibody comprises:

(a) a heavy chain variable region comprising:

a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 3**;

a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 3**;

a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 3**; and

(b) a light chain variable region comprising:

a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 4**;

a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 4**;

a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 109-117 of **SEQ ID NO: 4**.

In one example, the antibody is produced by a hybridoma cell as deposited at Cellbank Australia under accession number CBA20140026, or progeny thereof.

In a further example, the antibody is:

(i) a humanised anti-GPC1 antibody,

- (ii) a chimeric anti-GPC1 antibody,
- (i) a human anti-GPC1 antibody,
- (ii) a monoclonal anti-GPC1 antibody,
- (iii) a multimeric anti-GPC1 antibody, or
- (iv) a synthetic anti-GPC1 antibody.

In one example, the antibody is a chimeric antibody comprising:

(a) a heavy chain constant region comprising or consisting of an amino acid sequence as defined in residues 138-467 of **SEQ ID NO: 7**; and

(b) a light chain constant region comprising or consisting of an amino acid sequence as defined in residues of 128-234 **SEQ ID NO: 8**.

In another example, the antigen-binding fragment thereof is: a single chain variable fragment (scFv), a variable domain (Fv) fragment, a fragment antigen binding (Fab) fragment, a F(ab)₂ fragment, a peptide, or a proteolytic fragment containing an epitope binding region.

In yet another example, the antigen-binding fragment thereof is: a single chain variable fragment (scFv) comprising a sequence as defined in **SEQ ID NO: 9**.

In still another example, the cytotoxic agent is selected from the group consisting of: an adrenocortical suppressant, an alkylating agent, an alkyl sulfonate, an anthracycline, an anti-angiogenic agent, an antibiotic, an antimetabolite, an antimitotic, an auristatin, a calicheamycin, a camptothecin, a COX-2 inhibitor, an enzyme inhibitor, an epipodophyllotoxin, an ethylenimine derivative, a folic acid analog, an HDAC inhibitor, a heat shock protein (HSP90) inhibitor, a hormone antagonist, a maytansinoid, a methylhydrazine derivative, an mTOR inhibitor, a nitrogen mustard, a nitrosourea, a platinum coordination complex, a pro-apoptotic agent, a proteasome inhibitor, a purine analog, a pyrimidine analog, a radioisotope, a substituted urea, a taxane, triazene, a tubulin inhibitor, a tyrosine kinase inhibitor, and a vinca alkaloid.

In still another example, the cytotoxic agent is selected from the group consisting of: afatinib, aplidin, anastrozole, anthracyclines, AVL-101, AVL-291, axitinib, azaribine, bendamustine, bleomycin, bortezomib, bosutinib, bryostatin-1, busulfan, camptothecins, carboplatin, calicheamycin, camptothecin, carboplatin, carmustine, celecoxib, chlorambucil, cisplatin, cladribine, COX-2 inhibitors, crizotinib, cyano-morpholino doxorubicin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, dasatinib,

daunorubicin, dinaciclib, 3',5'-O-dioleoyl-FudR (FudR-d0), DM1, DM3, DM4, docetaxel, doxorubicin, doxorubicin glucuronide, duocarmycin, endostatin, entinostat, epidophyllotoxin, epirubicin glucuronide, erlotinib, estramustine, estrogen receptor binding agents, etoposide
5 glucuronide, etoposide phosphate, etoposide (VP16), exemestane, farnesyl-protein transferase inhibitors, fingolimod, flavopiridol, floxuridine (FudR), fludarabine, 5-fluorouracil, flutamide, fostamatinib, ganetespib, GDC-0834, gefitinib, gemcitabine, GS-1101, 10-hydroxycamptothecin, hydroxyurea, ibrutinib, idarubicin, idelalisib, ifosfamide, imatinib, irinotecan (CPT-11), lapatinib, lenolidamide, leucovorin, LFM-A13, lomustine,
10 mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mithramycin, mitomycin, mitotane, mitoxantrone, monomethylauristatin D (MMAD), monomethylauristatin E (MMAE), monomethylauristatin F (MMAF), navelbine, neratinib, nilotinib, nitrosurea, olaparib, paclitaxel, PCI-32765, pentostatin, plicomycin, a 2-PDox pro-drug (pro-2-PDox), procarbazine, PSI-341, 2-pyrrolinodoxorubicine (2-PDox), raloxifene,
15 semustine, SN-38, sorafenib, streptozocin, SU1 1248, sunitinib, tamoxifen, temazolomide, teniposide, thalidomide, thioguanine, thiotepa, topotecan, transplatinum, uracil mustard, vatalanib, vinblastine, vinca alkaloids, vincristine, vinorelbine, and ZD 1839.

In still another example, the cytotoxic agent is a radioisotope selected from the group consisting of: ^{90}Y , ^{188}Re , ^{166}Ho , ^{165}Dy , ^{109}Pd , ^{111}Ag , ^{186}Re , ^{198}Au , ^{153}Sm , ^{64}Cu , ^{177}Lu , ^{131}I , ^{125}I ,
20 ^{67}Cu , ^{175}Yb , ^{166}Dy , ^{169}Er , ^{212}Bi , ^{213}Bi , ^{225}Ac , ^{212}Pb , ^{66}Ga , ^{67}Ga , ^{68}Ga , ^{86}Y , $^{94\text{m}}\text{Tc}$, and ^{89}Zr .

In still another example, the antibody or antigen-binding fragment thereof is conjugated with a chelating agent selected from the group consisting of: DOTA, DTPA, NOTA, NODAGA, MeCOSAR, TETA, TRAP, TE2A, CBTE2A.

In still another example, the cytotoxic agent and the antibody or antigen-binding
25 fragment thereof are conjugated with a chelating agent selected from the group consisting of: DOTA, DTPA, NOTA, NODAGA, MeCOSAR, TETA, TRAP, TE2A, CBTE2A.

In a further example, the subject is a mammalian subject or a human subject.

In a second aspect, the present invention provides an isolated antibody population comprising:

30 first anti-glypican-1 (anti-GPC1) antibodies and/or antigen binding fragments thereof, that are conjugated to at least one cytotoxic agent which is toxic to a prostate, bladder, and/or pancreatic cancer cell, wherein the first anti-glypican-1 (anti-GPC1) antibodies comprise:

(a) a heavy chain variable region comprising:
a complementarity determining region 1 (CDR1) comprising or consisting of
an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 3**;
5 a complementarity determining region 2 (CDR2) comprising or consisting of
an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 3**;
a complementarity determining region 3 (CDR3) comprising or consisting of
an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 3**; and
(b) a light chain variable region comprising:
10 a complementarity determining region 1 (CDR1) comprising or consisting of
an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 4**;
a complementarity determining region 2 (CDR2) comprising or consisting of
an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 4**;
a complementarity determining region 3 (CDR3) comprising or consisting of
15 an amino acid sequence defined by positions 109-117 of **SEQ ID NO: 4**;
and wherein the antibody population does not contain second antibodies comprising a
light chain variable region comprising:
a complementarity determining region 1 (CDR1) comprising or consisting of
an amino acid sequence defined by positions 48-58 of **SEQ ID NO: 11**;
20 a complementarity determining region 2 (CDR2) comprising or consisting of
an amino acid sequence defined by positions 74-80 of **SEQ ID NO: 11**;
a complementarity determining region 3 (CDR3) comprising or consisting of
an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 11**.
In one example, the first antibodies and/or antigen binding fragments thereof are any
25 one or more of monoclonal antibodies, humanised antibodies, chimeric antibodies,
multimeric antibodies, and/or synthetic antibodies.
In another example, the antigen binding fragments are any one or more of single chain
variable fragments (scFv), variable domain (Fv) fragments, fragment antigen binding (Fab)
fragments, F(ab)2 fragments, peptides, or proteolytic fragments containing an epitope binding
30 region.
In an additional example, the first antibodies comprise or consist of a heavy chain
sequence as defined by positions 20-461 of **SEQ ID NO: 3** and a light chain sequence as
defined by positions 21-234 of **SEQ ID NO: 4**.
In a further example, the first antibodies and/or antigen binding fragments thereof are
35 chimeric.

In a further example, the first antibodies and/or antigen binding fragments thereof are chimeric antibodies comprising:

(a) a heavy chain constant region comprising or consisting of an amino acid sequence as defined in residues 138-467 of **SEQ ID NO: 7**; and

(b) a light chain constant region comprising or consisting of an amino acid sequence as defined in residues of 128-234 **SEQ ID NO: 8**.

In another example, the antigen binding fragments are single chain variable fragments (scFv) comprising a sequence as defined in **SEQ ID NO: 9**.

In yet another example, the cytotoxic agent is selected from the group consisting of: an adrenocortical suppressant, an alkylating agent, an alkyl sulfonate, an anthracycline, an anti-angiogenic agent, an antibiotic, an antimetabolite, an antimitotic, an auristatin, a calicheamycin, a camptothecin, a COX-2 inhibitor, an enzyme inhibitor, an epipodophyllotoxin, an ethylenimine derivative, a folic acid analog, an HDAC inhibitor, a heat shock protein (HSP90) inhibitor, a hormone antagonist, a maytansinoid, a methylhydrazine derivative, an mTOR inhibitor, a nitrogen mustard, a nitrosourea, a platinum coordination complex, a pro-apoptotic agent, a proteasome inhibitor, a purine analog, a pyrimidine analog, a radioisotope, a substituted urea, a taxane, triazene, a tubulin inhibitor, a tyrosine kinase inhibitor, and a vinca alkaloid.

In another example, the cytotoxic agent is selected from the group consisting of: afatinib, aplidin, anastrozole, anthracyclines, AVL-101, AVL-291, axitinib, azaribine, bendamustine, bleomycin, bortezomib, bosutinib, bryostatin-1, busulfan, camptothecins, carboplatin, calicheamycin, camptothecin, carboplatin, carmustine, celecoxib, chlorambucil, cisplatin, cladribine, COX-2 inhibitors, crizotinib, cyano-morpholino doxorubicin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, dasatinib, daunorubicin, dinaciclib, 3',5'-O-dioleoyl-FudR (FUDR-d0), DM1, DM3, DM4, docetaxel, doxorubicin, doxorubicin glucuronide, duocarmycin, endostatin, entinostat, epipodophyllotoxin, epirubicin glucuronide, erlotinib, estramustine, estrogen receptor binding agents, etoposide glucuronide, etoposide phosphate, etoposide (VP16), exemestane, farnesyl-protein transferase inhibitors, fingolimod, flavopiridol, floxuridine (FUDR), fludarabine, 5-fluorouracil, flutamide, fostamatinib, ganetespib, GDC-0834, gefitinib, gemcitabine, GS-1101, 10-hydroxycamptothecin, hydroxyurea, ibrutinib, idarubicin, idelalisib, ifosfamide, imatinib, irinotecan (CPT-11), lapatinib, lenolidamide, leucovorin, LFM-A13, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mithramycin, mitomycin, mitotane, mitoxantrone, monomethylauristatin D (MMAD),

monomethylauristatin E (MMAE), monomethylauristatin F (MMAF), navelbine, neratinib, nilotinib, nitrosurea, olaparib, paclitaxel, PCI-32765, pentostatin, plicomycin, a 2-PDox pro-drug (pro-2-PDox), procarbazine, PSI-341, 2-pyrrolinodoxorubicine (2-PDox), raloxifene, semustine, SN-38, sorafenib, streptozocin, SU1 1248, sunitinib, tamoxifen, temazolomide, 5 teniposide, thalidomide, thioguanine, thiotepa, topotecan, transplatinum, uracil mustard, vatalanib, vinblastine, vinca alkaloids, vincristine, vinorelbine, and ZD 1839.

In yet another example, the cytotoxic agent is a prodrug designed for activation upon uptake into an endosome or lysosome of said prostate, bladder, and/or pancreatic cancer cell.

In one example, the prodrug is conjugated to the antibody and/or an antigen-binding 10 fragment thereof by:

an acid sensitive-linker susceptible to cleavage at a pH that is less than: pH 7.2, pH 7.0, pH 6.5, pH 6.0, pH 5.5, pH 5.0, pH 4.5, or pH 4; or

an enzyme-cleavable linker, wherein the enzyme capable of cleaving the linker exists within the endosome or lysosome.

15 In one example, the cytotoxic agent is linked to the antibody and/or antigen binding fragment thereof by binding to a chelating agent conjugated to the antibody and/or antigen binding fragment thereof.

In still another example, the cytotoxic agent and the antibody or antigen-binding fragment thereof are conjugated with a chelating agent selected from the group consisting of: 20 DOTA, DTPA, NOTA, NODAGA, MeCOSAR, TETA, TRAP, TE2A, CBTE2A.

In still another example, the cytotoxic agent is a radioisotope selected from the group consisting of: ^{90}Y , ^{188}Re , ^{166}Ho , ^{165}Dy , ^{109}Pd , ^{111}Ag , ^{186}Re , ^{198}Au , ^{153}Sm , ^{64}Cu , ^{177}Lu , ^{131}I , ^{125}I , ^{67}Cu , ^{175}Yb , ^{166}Dy , ^{169}Er , ^{212}Bi , ^{213}Bi , ^{225}Ac , ^{212}Pb , ^{66}Ga , ^{67}Ga , ^{68}Ga , ^{86}Y , $^{94\text{m}}\text{Tc}$, and ^{89}Zr .

In still another example, the antibody or antigen-binding fragment thereof is conjugated 25 with a chelating agent selected from the group consisting of: DOTA, DTPA, NOTA, NODAGA, MeCOSAR, TETA, TRAP, TE2A, and CBTE2A.

Brief Description of the Drawings

Preferred embodiments of the present invention will now be described, by way of 30 example only, with reference to the accompanying figures wherein:

Figure 1 shows images from immunofluorescence assays using chimeric MIL-38 antibody and controls on DU-145 cells. **Figure 1A-D** show combined bright field and DAPI images of the stained cells. **Figure 1E-H** show binding to DU-145 cells with MIL-38 prep 33A (**1E**, positive control), chimeric MIL-38 (**1F**), Cetuximab (**1G**, positive control for human IgG1k), and negative control (**1H**, no 1° antibody);

Figure 2 shows western blot analysis of chimeric MIL-38 antibody. **Figure 2A** shows reactivity of murine MIL-38 with DU-145 MPEK extract, C3 MPEK extract and NS0-produced recombinant GPC-1 antigen. **Figure 2B** shows reactivity of chimeric MIL-38 with DU-145 MPEK extract, C3 MPEK extract and NS0-produced recombinant GPC-1 antigen.

Figure 2C shows reactivity of murine MIL-38 with DU-145 MPEK extract, C3 MPEK extract and NS0-produced recombinant GPC-1 antigen under equivalent conditions to **Figure 2B**;

Figure 3 shows FLOW cytometry of fluorescently-labelled MIL-38 and scFv MIL-38 antibodies binding to DU-145 cells. **Figure 3A** shows negative controls (cells alone and a negative control antibody). **Figure 3B** shows reactivity of murine MIL-38 with DU-145 cells and also a reduction in FLOW signal when cells are pre-incubated with un-labelled MIL-38 antibody. **Figure 3C** shows comparative binding of labelled MIL-38 and labelled MIL-38 scFv to DU-145 cells. MIL-38 scFv retains binding to DU-145 cells but at a slightly lower level than murine MIL-38;

Figure 4 shows binding of MIL-38 antibody to MDA-MB-231 breast cancer cells, T-24 bladder cancer cells, PANC-1 pancreatic cancer cells and DU-145 prostate cancer cells. **Figure 4A** shows binding of murine MIL-38 antibody to MDA-MB-231 cells. Binding of MIL-38 is considerably higher than isotype or secondary antibody only controls indicating MDA-MB-231 cells are positive for the MIL-38 antigen GPC-1. **Figure 4B** shows overlaid FLOW cytometry histograms for MIL-38 binding to MDA-MB-231, T-24 or DU-145 cells. MDA-MB-231 cells showed the lowest binding, while the highest binding was observed for DU-145 cells. **Figure 4C** shows overlaid FLOW cytometry histograms for MIL-38, anti-CD9 and anti-CD81 antibodies binding to PANC-1 cells.

Figure 5 shows internalisation of MIL-38 antibody by MDA-MB-231, T-24 and DU-145 cells. **Figure 5A** shows internalisation of the 1F5 murine MIL-38 parent antibody 30 min post binding to DU-145 cells. **Figure 5B** shows internalisation of the chimeric MIL-38 antibody 30 min post binding to DU-145 cells. **Figure 5C** shows lack of detectable antibody binding or internalisation of the chimeric MIL-38 antibody following 30 min of exposure to MDA-MB-231 cells. **Figure 5D** shows internalisation of the chimeric MIL-38 antibody

immediately post binding to T-24 cells; **Figure 5E** shows localisation of chimeric MIL-38 antibody (green) in PANC-1 cells after 15 minutes of a 60 minute timecourse. **Figure 5F** shows internalisation of anti-human antibody labelled with Alexa-Fluor 488 in PANC-1 cells after completion of the 60 minute timecourse.

5 **Figure 6** shows targeting of either CY5-labelled 1F5 murine MIL-38 or CY5-labelled chimeric MIL-38 to subcutaneous DU-145 xenograft tumours. **Figure 6A** shows targeting of 1F5 murine MIL-38 while **Figure 6B** shows targeting of the chimeric MIL-38. Tumours are located on the back of the mice and are indicated by arrows. There is also localisation to the sites of the ear puncture sites (indicated by diamonds).

10 **Figure 7** shows cell growth inhibition assays using a variety of targeting antibodies in DU-145 and MDA-MB-231 breast cancer cell lines. Briefly, cells were grown for 3 days in the presence of chimeric MIL-38 and protein G pre-labelled with the cytotoxic agents DM1, MMAE, MMAF and Duocarmycin after which cell viability was assessed. EC50s were determined and are shown to the right of the respective figures. Antibodies used were
15 chimeric MIL-38 (**Figure 7A**), BLCA-38 (bi-clonal population containing AM3 and AM4 populations, **Figure 7B**), AM3 (monoclonal derived from BLCA-38 population that has minimal GPC-1-binding, **Figure 7C**), 1F5 MIL-38 (AM4-like monoclonal derived from BLCA-38 population that has high GPC-1-binding, **Figure 7D**) and Erbitux (generic name cetuximab, a chimeric anti-EGFR monoclonal that serves as positive control, **Figure 7E**).
20 **Figure 7F** shows titration of protein G-duocarmycin in DU-145 cells (top panel) and cell growth inhibition assays using the optimized protein G-duocarmycin concentration in DU-145 cells;

Figure 8 shows cell growth and chimeric antibody expression characteristics from a pilot batch production using a stable cell pool. Viable cell density (**Figure 8A**), cell viability
25 (**Figure 8B**) and antibody production (**Figure 8C**) are shown, demonstrating scalable chimeric antibody expression using methodologies suitable for large scale GMP production;

Figure 9 shows DOTA conjugation of MIL-38 and labeling with ¹⁷⁷Lu. **Figure 9A** shows gel filtration standard markers run on HPLC. **Figure 9B** shows unconjugated MIL-38 run on HPLC. **Figure 9C** shows conjugated MIL-38 run on HPLC following PD-10 column
30 purification. **Figure 9D** shows radioactivity of MIL-38 labelled with ¹⁷⁷Lu run on HPLC. **Figure 9E** shows the corresponding A280 trace of the labelled MIL-38 run on HPLC;

Figure 10 shows FLOW cytometry of chimeric MIL-38, MIL-38 DOTA and mock radio-labelled MIL-38 DOTA binding to DU-145 cells. Essentially equivalent binding curves were obtained indicating that the DOTA conjugation did not cause a reduction in MIL-38

binding to DU-145 cells. Similarly, MIL-38 DOTA that had undergone a mock radiolabelling process had equivalent cell binding to the un-labelled conjugate, indicating retention of cell binding activity;

Figure 11 shows labeling of DOTA-conjugated MIL-38 with ^{67}Ga . **Figure 11A** shows a size exclusion chromatography HPLC chromatogram of the radioactivity of MIL-38 labelled with ^{67}Ga . The star indicates free ^{67}Ga remaining in the reaction mixture. **Figure 11B** shows a stability study of ^{67}Ga -labelled MIL-38 DOTA. The size exclusion chromatography HPLC chromatogram of the radioactivity of MIL-38 labelled with ^{67}Ga is shown together with the corresponding UV trace. The ^{67}Ga -MIL-38 preparation had free ^{67}Ga removed, then was allowed to remain at room temperature for two weeks prior to undergoing SEC HPLC chromatograph.

Figure 12 shows binding of chimeric MIL-38, MIL-38 DOTA prepared using three different DOTA conjugate ratios and mock radio-labelled MIL-38 DOTA to DU-145 and T-24 cells using FLOW cytometry, as well as binding in a direct antigen binding ELISA. **Figure 12A** shows binding of un-labelled chimeric MIL-38 or chimeric MIL-38 that had been conjugated to DOTA using either a 5, 10 or 20 fold molar excess of DOTA. Essentially equivalent binding curves were obtained indicating that the DOTA conjugation did not cause a reduction in MIL-38 binding to DU-145 cells. **Figure 12B** shows FLOW cytometry binding to T-24 cells of three separate batches of chMIL-38 DOTA (20 fold excess) that had each undergone a mock-labelling reaction. Essentially identical binding curves were observed indicating good inter-batch reproducibility. **Figure 12C** shows FLOW cytometry binding to T-24 cells of three separate FLOW reactions of chMIL-38 DOTA (20 fold excess) all prepared from one batch that had undergone a mock-labelling reaction. Essentially identical binding curves were observed indicating good intra-batch reproducibility. **Figure 12D** shows overlayed FLOW cytometry profiles of binding to T-24 cells for secondary antibody alone (red trace), chMIL-38 DOTA (green trace), one sample from the inter-batch reproducibility (Batch 1, blue trace) and one sample from the intra-batch reproducibility (Intra 1, orange trace). Essentially identical binding curves were observed indicating that the mock-labelling process did not affect binding to cells compared to the chMIL-38 DOTA control. **Figure 12E** shows binding of chMIL-38 and chMIL-38 that had been conjugated to DOTA using either a 5, 10 or 20 fold molar excess of DOTA to recombinant GPC-1 in a direct binding ELISA. **Figure 12F** shows direct binding to recombinant GPC-1 of chMIL-38 DOTA, the 3 different preparations of mock-labelled chMIL-38 DOTA prepared for the inter-batch comparison and 3 different reactions from the intra-batch preparation.

Figure 13 shows (A) fluorescence images observed from the back of Balb/c nude mice with DU-145 xenograft tumours after chimeric MIL-38 antibody injection; and (B) Multi-modal Animal Rotation System (MARS) image of a representative mouse from (A) after 144 hours.

Figure 14 shows a time course of biodistribution of ^{177}Lu -labelled MIL-38 DOTA when administered to normal rats. The graph shows the % injected dose per gram for blood, stomach, small intestine, large intestine, testes, muscle, bone thyroid, heart, lung, liver, kidneys, and bladder. Timepoints of 6hr, 1 day, 2 days, 1 week and 2 weeks post administration are shown.

Figure 15 shows a time course of biodistribution of ^{177}Lu -labelled MIL-38 DOTA when administered to normal rats. The graph shows the % injected dose per gram for blood, liver and kidneys. Timepoints of 6hr, 1 day, 2 days, 1 week and 2 weeks post administration are shown.

Figure 16 is a PET-CT image of ^{64}Cu -labelled MIL-38 NOTA taken 48 hours post injection in Balb/c nude mice with xenograft tumours. Arrow indicates site of DU-145 xenograft tumour.

Figure 17 is a graph representing *ex vivo* biodistribution of DOTA- and NOTA-labelled MIL-38 antibody in Balb/c nude mice with xenograft tumours.

Figure 18 shows (A) fluorescence images observed from the back for goat anti-GPC-1 antibody; (B) *ex vivo* fluorescence images of organs observed for goat anti-GPC-1 at 120 hours post injection. Tumours are indicated with red arrows; and (C) MARS image at 120 hrs.

Figure 19 shows comparative sandwich ELISAs performed using different MIL-38 antibody preparations as capture antibodies. **Figure 19A** shows comparative sandwich ELISAs using AM3 and AM4 as capture antibodies. **Figure 19B** shows comparative sandwich ELISAs using either a mixed preparation (34A) or a clonal population (AM4 1F5) as capture antibodies.

Definitions

As used in this application, the singular form “a”, “an” and “the” include plural references unless the context clearly dictates otherwise. For example, the phrase “an antibody” also includes multiple antibodies.

As used herein, the term “comprising” means “including.” Variations of the word “comprising”, such as “comprise” and “comprises,” have correspondingly varied meanings.

Thus, for example, a sample “comprising” antibody A may consist exclusively of antibody A or may include one or more additional components (e.g. antibody B).

As used herein the term “multiple” means more than one. In certain specific aspects or embodiments, multiple may mean 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, or more, and any integer derivable therein, and any range derivable therein.

As used herein, the terms “antibody” and “antibodies” include IgG (including IgG1, IgG2, IgG3, and IgG4), IgA (including IgA1 and IgA2), IgD, IgE, IgM, and IgY, whole antibodies, including single-chain whole antibodies, and antigen-binding fragments thereof. Antigen-binding antibody fragments include, but are not limited to, Fv, Fab, Fab' and F(ab')₂, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. The antibodies may be from any animal origin or appropriate production host. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region/s alone or in combination with the entire or partial of the following: hinge region, CH1, CH2, and CH3 domains. Also included are any combinations of variable region/s and hinge region, CH1, CH2, and CH3 domains. Antibodies may be monoclonal, polyclonal, chimeric, multispecific, humanised, and human monoclonal and polyclonal antibodies which specifically bind the biological molecule. The antibody may be a bi-specific antibody, avibody, diabody, tribody, tetrabody, nanobody, single domain antibody, VHH domain, human antibody, fully humanized antibody, partially humanized antibody, anticalin, adnectin, or affibody.

As used herein the term “monoclonal antibody” refers to an antibody that recognises a single antigenic epitope, and that is obtained from a population of substantially homogeneous antibodies which bind specifically to the same antigenic epitope, and are identical with the potential exception of naturally occurring mutation/s that may be present in minor amounts.

As used herein, the term “humanised antibody” refers to forms of antibodies that contain sequences from human antibodies as well as non-human antibodies (e.g. murine antibodies). For example, a humanised antibody can comprise substantially all of at least one and typically two variable domains, in which all/substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all/substantially all of the FR regions are from the human immunoglobulin sequence. The humanised antibody may optionally also comprise at least a portion of an immunoglobulin constant region (Fc) which may typically be that of a human immunoglobulin.

As used herein, the term “chimeric antibody” refers to an antibody which exhibits a desired biological activity, and in which a portion of the light chain and/or heavy chain is identical to or homologous with corresponding sequences in antibodies derived from a given/specific species, while the remaining chain/s is/are identical to or homologous with corresponding sequences in antibodies derived from another different species. For example, a chimeric antibody may comprise variable regions that are derived from a first species and comprise constant regions that are derived from a second species. Chimeric antibodies can be constructed for example by genetic engineering from immunoglobulin gene segments belonging to different species.

As used herein, the term “hybridoma” refers to a cell produced by the fusion of an immortal cell (e.g. a multiple myeloma cell) and an antibody-producing cell (e.g. a B lymphocyte), which is capable of producing monoclonal antibodies of a single binding specificity.

As used herein, the terms “binding specifically” and “specifically binding” in reference to an antibody, antibody variant, antibody derivative, antigen binding fragment, and the like refers to its capacity to bind to a given target molecule preferentially over other non-target molecules. For example, if the antibody, antibody variant, antibody derivative, or antigen binding fragment (“molecule A”) is capable of “binding specifically” or “specifically binding” to a given target molecule (“molecule B”), molecule A has the capacity to discriminate between molecule B and any other number of potential alternative binding partners. Accordingly, when exposed to a plurality of different but equally accessible molecules as potential binding partners, molecule A will selectively bind to molecule B and other alternative potential binding partners will remain substantially unbound by molecule A. In general, molecule A will preferentially bind to molecule B at least 10-fold, preferably 50-fold, more preferably 100-fold, and most preferably greater than 100-fold more frequently than other potential binding partners. Molecule A may be capable of binding to molecules that are not molecule B at a weak, yet detectable level. This is commonly known as background binding and is readily discernible from molecule B-specific binding, for example, by use of an appropriate control.

As used herein, the term “cytotoxic agent” encompasses any compound that is toxic to a cell such as, for example by, inhibiting cell function, and including but not limited to, causing cell death directly and/or indirectly.

As used herein, the terms “conjugate”, “conjugated” and “conjugation” in the context of a “cytotoxic agent” as described herein and an antibody, antibody derivative, or antigen

binding fragment of an antibody described herein will be understood to mean that the cytotoxic agent is linked to the antibody, antibody derivative, or antigen binding fragment. The link may occur directly between the cytotoxic agent and the antibody, or alternatively the link between the cytotoxic agent and the antibody may occur indirect via one or more
5 intervening molecules (e.g. protein-G).

As used herein, the term “subject” includes any animal of economic, social or research importance including bovine, equine, ovine, primate, avian and rodent species. Hence, a “subject” may be a mammal such as, for example, a human or a non-human mammal.

Any description of prior art documents herein, or statements herein derived from or
10 based on those documents, is not an admission that the documents or derived statements are part of the common general knowledge of the relevant art. For the purposes of description all documents referred to herein are hereby incorporated by reference in their entirety unless otherwise stated.

15 Detailed Description

A need continues to exist for more effective methods and agents to treat prostate, bladder, and/or pancreatic cancer. Antibodies are useful diagnostic and therapeutic agents for cancer, having become a successful and important tool for diagnosing and treating patients with haematological malignancies and solid tumours. The identification of new relevant
20 antibodies targeting tumour-specific antigens offers one potential means of improving diagnostic and/or therapeutic outcomes for cancer patients. Another means by which these outcomes can be enhanced is through the improvement of existing antibody-based diagnostics and/or therapies.

The present inventors have surprisingly identified that the BLCA-38 antibody referred
25 to and used in the prior art is not a discrete monoclonal antibody as indicated, but rather a combination of two distinct monoclonal antibodies in a mixed population. The present inventors have determined that the hybridoma used to generate the BLCA-38 antibody, a representative sample of which was deposited at the American Tissue Type Culture Collection under accession number HB11785, is a mixed population of hybridoma cells,
30 which produces at least two discrete antibody species. Only one of these antibody species can bind strongly to the relevant target antigen present on prostate, bladder, and/or pancreatic cancer cells, whilst the second species cannot.

The unexpected determination that BLCA-38 as referred to in the prior art represents a mixed hybridoma/antibody population has facilitated the generation of a monoclonal

hybridoma capable of producing a single population of monoclonal antibodies with binding specificity for the target antigen on prostate, bladder, and/or pancreatic cancer cells. This circumvents the unnecessary production and application of an ineffectual therapeutic antibody. It is also well known that the cost of therapeutic antibody production is high, and in particular when post-production modifications are required such as ADC production or conjugation of chelating agents for radio-immunotherapy. It is clearly beneficial to avoid preparing ADC or chelated monoclonal antibodies from antibodies that do not bind the target prostate, bladder, and/or pancreatic cancer cell antigen. The actual target of the antibody species in the BLCA-38 mixed population that does not bind to the prostate, bladder, and/or pancreatic cancer antigen is unknown raising the risk of unwanted side-effects from potential binding to non-target cells.

The present inventors have also unexpectedly discovered that contrary to prior art teachings that the BLCA-38 antibody population is not internalised on binding to cancer cells, the present inventors have demonstrated that antibodies of the monoclonal antibody species are in fact internalised upon binding to the relevant target antigen present on prostate, bladder, and/or pancreatic cancer cells.

Accordingly, certain embodiments of the present invention relate to the provision of a monoclonal antibody population derived from clonal hybridoma cells, each member of the antibody population being capable of binding specifically to an antigen present on prostate, bladder, and/or pancreatic cancer cells. The present invention also provides antigen binding fragments of these antibodies, as well as derivatives and variants of the antibodies that maintain the same binding specificity. The antibodies and fragments are each conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject.

In view of providing antibody conjugates capable of specifically targeting prostate, bladder, and/or pancreatic cancer cells, other embodiments of the present invention relate to methods for treating prostate, bladder, and/or pancreatic cancer in subjects afflicted with the same, and methods for killing prostate, bladder, and/or pancreatic cells and tumours.

Further aspects of the present invention relate to medicaments and pharmaceutical preparations comprising the conjugated antibodies and/or fragments, and methods for their preparation.

Antibodies and antigen-binding fragments

The present invention provides antibodies, derivatives of such antibodies, and antigen binding fragments thereof, each conjugated to at least one cytotoxic agent.

In alternative embodiments, the antibodies, derivatives of such antibodies, and antigen binding fragments thereof, are provided without being conjugated to at least one cytotoxic agent (i.e. 'naked').

The antibodies, variants, derivatives, and antigen binding fragments are capable of binding specifically to an antigenic epitope present in glypican-1 heparan sulfate proteoglycan (GPC-1). The GPC-1 protein may be a human glypican-1 protein (e.g. as defined by a sequence set forth in any one of: NCBI reference sequence accession no. NP_002072.2, GenBank accession no. AAH51279.1, GenBank accession no. AAA98132.1, GenBank accession no. EAW71184.1, or UniProtKB/Swiss-Prot accession no. P35052.2). In some embodiments the GPC-1 protein may not include a signal peptide and/or a propeptide. Additionally or alternatively, the monoclonal antibodies, derivatives, and antigen binding fragments may be capable of binding specifically to an antigenic epitope present in a GPC-1 variant (e.g. a GPC-1 isoform, splice variant, or allotype).

By way of non-limiting example, the antibodies, variants, derivatives, and antigen binding fragments may comprise a heavy chain and/or a light chain, combinations thereof, or component/s thereof.

Non-limiting examples of suitable anti-GPC1 antibodies include those set out in **Table 1** below.

Table 1: non-limiting examples of anti-GPC1 antibodies

Company	Cat#	Reactivity	Use	Host	M/P	Binding specificity	Immunogen
R&D	BAF4519	Human	WB, FC	Goat	poly	aa24-530	mouse myeloma cell line NS0-derived recombinant human Glypican-1, Asp24-Ser530 (Accession# P35052)
Bioss	bs-2426R-Biotin	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly		Unknown immunogen. Biotin conjugated
Bioss	bs-2426-HRP	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly		Unknown immunogen. HRP conjugated
antibodies-online	ABIN740102	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly	C-term	Synthetic peptide derived from human glypican 1 C-terminus. Biotin conjugated.
antibodies-online	ABIN1174125	Human	IHC, WB, ELISA	Rabbit	poly		Unknown immunogen. Biotin conjugated
antibodies-online	ABIN740109	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F	Rabbit	poly	C-term	Synthetic peptide derived from human glypican 1 C-terminus. Enquire about sequence info. HRP conjugated
antibodies-online	ABIN653109	Human	WB, IHC, FACS, ELISA	Rabbit	poly	N-term	KLH conjugated sythetic peptide between 12-41aa from the N-terminal region of human Glypican-1
antibodies-online	ABIN952553	human	ELISA, IHC-p, WB, FACS	Rabbit	poly	N-term	KLH conjugated synthetic peptide between 12-41aa from the N-terminal region of human Glypican-1

antibodies-online	ABIN797896	human	IHC, WB	Rabbit	poly	N-term	synthetic peptide derived from N-terminal domain of human GPC1
antibodies-online	ABIN347483	mouse, rat, human	IHC, WB	Rabbit	poly	N-term	synthetic peptide derived from N-terminal domain of human GPC1
antibodies-online	ABIN347484	human	IHC, WB, ICC, ELISA	Rabbit	poly	N-term	synthetic peptide derived from N-terminal domain of human GPC1
antibodies-online	ABIN740100	Human, mouse, rat, dog, cow, horse	WB, ELISA, IHC-P&F, IF	Rabbit	poly	C-term	Synthetic peptide derived from human glypican 1 C-terminus. Enquire about sequence info.
antibodies-online	ABIN207433	human	WB, ELISA	Rabbit	poly	C-term	synthetic peptide corresponding to C-terminal residues of human GPC1 precursor
antibodies-online	ABIN964659	human, mouse, rat	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to an internal region of human GPC-1
antibodies-online	ABIN349638	human	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to human GPC1
antibodies-online	ABIN1101824	human	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to an internal region of human GPC-1
antibodies-online	ABIN595376	human	WB, ELISA	Rabbit	poly	internal region	synthetic peptide corresponding to an internal region of human GPC-1
antibodies-online	ABIN330371	human	WB, ELISA	goat	poly	aa24-530	NS0-derived rhGlypican 1 aa24-530
antibodies-online	ABIN1479675	human	FACS, IHC, WB, ELISA	Rabbit	poly	aa12-41	KLH conjugated synthetic peptide from N-terminal region of human GPC1

The heavy chain or component/s thereof may comprise a heavy chain variable region comprising one, two, or three complementarity determining regions (CDR1, CDR2, and/or CDR3), also known in the art as heavy chain hypervariable (HV) regions. The heavy chain CDR1 may comprise or consist of an amino acid sequence as defined by
5 residues 50-54 of **SEQ ID NO: 3**. The heavy chain CDR2 may comprise or consist of an amino acid sequence as defined by residues 69-85 of **SEQ ID NO: 3**. The heavy chain CDR3 may comprise or consist of an amino acid sequence as defined by residues 118-126 of **SEQ ID NO: 3**.

Additionally or alternatively, the heavy chain variable region may comprise one,
10 two, three, or four framework regions (FR1, FR2, FR3, and/or FR4). The heavy chain FR1 may comprise or consist of an amino acid sequence as defined by residues 20-49 of **SEQ ID NO: 3**. The heavy chain FR2 may comprise or consist of an amino acid sequence as defined by residues 55-68 of **SEQ ID NO: 3**. The heavy chain FR3 may comprise or consist of an amino acid sequence as defined by residues 86-117 of **SEQ ID NO: 3**. The
15 heavy chain FR4 may comprise or consist of an amino acid sequence as defined by residues 127-137 of **SEQ ID NO: 3**.

Additionally or alternatively, the heavy chain variable region may comprise a leader sequence. The heavy chain leader sequence may comprise or consist of an amino acid sequence as defined by residues 1-19 of **SEQ ID NO: 3**. The skilled person will
20 recognise that the leader sequence is a signal sequence which facilitates the transport of a newly synthesised heavy chain into the endoplasmic reticulum, and is generally not present in the heavy chain of the final assembled form of the monoclonal antibody.

Additionally or alternatively, the light chain or component/s thereof may comprise a light chain variable region comprising one, two, or three complementarity determining
25 regions (CDR1, CDR2, CDR3) also known in the art as light chain hypervariable (HV) regions. The light chain CDR1 may comprise or consist of an amino acid sequence as defined by residues 44-54 of **SEQ ID NO: 4**. The light chain CDR2 may comprise or consist of an amino acid sequence as defined by residues 70-76 of **SEQ ID NO: 4**. The light chain CDR3 may comprise or consist of an amino acid sequence as defined by
30 residues 109-117 of **SEQ ID NO: 4**.

Additionally or alternatively, the light chain variable region may comprise one, two, three, or four framework regions (FR1, FR2, FR3, FR4). The light chain FR1 may comprise or consist of an amino acid sequence as defined by residues 21-43 of **SEQ ID NO: 4**. The light chain FR2 may comprise or consist of an amino acid sequence as
35 defined by residues 55-69 of **SEQ ID NO: 4**. The light chain FR3 may comprise or

consist of an amino acid sequence as defined by residues 77-108 of **SEQ ID NO: 4**. The light chain FR4 may comprise or consist of an amino acid sequence as defined by residues 118-127 of **SEQ ID NO: 4**.

5 Additionally or alternatively, the light chain variable region may comprise a leader sequence. The light chain leader sequence may comprise or consist of an amino acid sequence as defined by residues 1-20 of **SEQ ID NO: 4**. The skilled person will recognise that the leader sequence is a signal sequence which facilitates transport of a newly synthesised light chain into the endoplasmic reticulum, and is generally not present in the light chain of the final assembled form of the monoclonal antibody.

10 Additionally or alternatively, the heavy chain may comprise one, two, or three heavy chain constant regions. The heavy chain constant region may comprise or consist of an amino acid sequence as defined by residues 138-461 of **SEQ ID NO: 3**.

Additionally or alternatively, the light chain may comprise a light chain constant region. The light chain constant region may comprise or consist of an amino acid
15 sequence as defined by residues 128-234 of **SEQ ID NO: 4**.

In some embodiments, monoclonal antibodies, variants, derivatives, and antigen binding fragments according to the present invention may comprise a heavy chain variable region which comprises or consists of an amino acid sequence as defined by residues 20-137 of **SEQ ID NO: 3**. The monoclonal antibodies, variants, derivatives, and
20 antigen binding fragments may comprise one or two of the heavy chain variable regions.

In some embodiments, monoclonal antibodies, variants, derivatives, and antigen binding fragments according to the present invention may comprise a light chain variable region which comprises or consists of an amino acid sequence as defined by residues 21-127 of **SEQ ID NO: 4**. The monoclonal antibodies, variants, derivatives, and antigen
25 binding fragments may comprise one or two of the light chain variable regions.

In some embodiments, monoclonal antibodies, variants, derivatives, and antigen binding fragments according to the present invention may comprise a heavy chain variable region which comprises or consists of residues 20-137 of **SEQ ID NO: 3**, and a light chain variable region which comprises or consists of residues 21-127 of **SEQ ID**
30 **NO: 4**. The monoclonal antibodies, variants, derivatives, and antigen binding fragments may comprise a combination of two of the heavy chain variable regions and two of the light chain variable regions.

In some embodiments, monoclonal antibodies, variants, derivatives, and antigen binding fragments according to the present invention may comprise a heavy chain
35 comprising or consisting of an amino acid sequence as defined by residues 20-461 of

SEQ ID NO: 3. The monoclonal antibodies, variants, derivatives, and antigen binding fragments may comprise one or two of the heavy chains.

In some embodiments, monoclonal antibodies, variants, derivatives, and antigen binding fragments according to the present invention may comprise a light chain comprising or consisting of an amino acid sequence as defined by residues 21-234 of **SEQ ID NO: 4.** The monoclonal antibodies, variants, derivatives, and antigen binding fragments may comprise one or two of the light chains.

In some embodiments, monoclonal antibodies, variants, derivatives, and antigen binding fragments according to the present invention may comprise a heavy chain comprising or consisting of an amino acid sequence as defined by residues 20-461 of **SEQ ID NO: 3,** and a light chain comprising or consisting of an amino acid sequence as defined by residues 21-234 of **SEQ ID NO: 4.** The monoclonal antibodies, variants, derivatives, and antigen binding fragments may comprise or consist of a combination of two of the heavy chains and two of the light chains.

Monoclonal antibodies, variants and derivatives of such antibodies, and antigen binding fragments thereof according to the present invention are not restricted to any particular isotype, and thus may be IgA (IgA1 or IgA2), IgD, IgE, IgG (IgG1, IgG2, IgG3, IgG4), or IgM isotype. In some embodiments, they are IgG1 isotype.

Included within the scope of the present invention are monoclonal antibodies produced by hybridoma cells submitted under the terms of the Budapest Treaty at Cellbank Australia at 214 Hawkesbury Road, Westmead NSW 2145, Australia on 22nd August 2014 under accession number CBA20140026. The hybridoma is a clonal population that produces a single antibody species having binding specific for an epitope existing in glypican-1 heparan sulfate proteoglycan (GPC-1).

Included within the scope of the present invention are “fragments” of the antibodies described herein. In general, the fragments are “antigen binding fragments” in the sense that they are capable of specifically binding to same antigen/epitope (e.g. GPC-1) as the parent antibody from which they are derived or upon which they are based. Typically, an antigen binding fragment retains at least 10% of the antigen/epitope binding capacity of the parent antibody, or, at least 25%, 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% (or more) of the antigen/epitope binding capacity of the parent antibody. It is also contemplated that an antigen binding fragment of an antibody described herein may include conservative amino acid substitutions that do not substantially alter its antigen/epitope binding specificity/capacity (e.g. at least 70%, 80%, 90%, 95%, 99% or 100% (or more) of its antigen/epitope binding specificity/capacity may be retained).

Non-limiting examples of antigen binding fragments include portions of a full length antibody, peptides and derivatives thereof including, for example, Fab, Fab', F(ab)₂, F(ab')₂, F(ab)₃, Fv, single-chain Fv (scFv), dsFv, Fd fragments, dAB fragments Fse, VH, VL, VhH, and V-NAR domains, paratopes, CDR regions, single-chain antibody
5 molecules (e.g. sc-Fv), minibodies, diabodies, triabodies, tetrabodies, kappa bodies, linear antibodies, multispecific antibodies, domain antibodies formed from antibody fragments, multispecific antibody fragments formed from antibody fragments, and any portion or peptide sequence of the antibody that is capable of specifically binding to the relevant antigen/epitope (e.g. GPC-1).

10 Also included within the scope of the present invention are “derivatives” of the antibodies described herein. A “derivative” of an antibody of the present invention refers to an antibody described herein that is modified to incorporate additional components or have existing component/s altered, but is still capable of specifically binding to the same antigen/epitope (e.g. GPC-1) as the parent antibody from which it is derived. Typically,
15 an antibody derivative as contemplated herein retains at least 10% of the antigen/epitope binding capacity of the parent antibody, or, at least 25%, 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% (or more) of the antigen/epitope binding capacity of the parent antibody.

Non-limiting examples of modifications suitable to form antibody derivatives
20 include amidation, glycosylation, phosphorylation, pegylation, linkage to a cellular ligand or other protein, derivatisation by known protecting/blocking groups, acetylation, and the like. Additionally or alternatively, the derivative may contain one or more non-classical amino acids. Modifications may also include conjugation with a chelating agent such as DOTA, DTPA, NOTA, NODAGA, MeCOSAR, TETA, TRAP, TE2A, and CBTE2A.

25 The antibody derivatives may include labelled antibodies such as, for example, monoclonal antibodies labelled with radioactive iodine, indium, sulphur, carbon, tritium or the like; monoclonal antibodies conjugated with avidin or biotin, monoclonal antibodies conjugated with enzymes (e.g. horseradish, glucose 6-phosphate dehydrogenase, glucose oxidase, beta-D-galactosidase, alkaline phosphatase,
30 glucoamylase, acetylcholine esterase, carboxylic acid anhydrase, malate dehydrogenase, lysozyme, or peroxidase), and monoclonal antibodies conjugated with chemoluminescent agents (e.g. acridine esters), bioluminescent agents (e.g. luciferase), or fluorescent agents (e.g. phycobiliproteins). Further examples of antibody derivatives include bifunctional antibodies, such as bispecific antibodies generated by combining parts of two separate

antibodies that recognize two different antigenic groups (e.g. by recombinant techniques or crosslinking).

The antibody derivatives may be formed from covalent modification of the antibodies described herein, for example, by reacting targeted amino acid residues of the antibody with an agent capable of reacting with selected side chains or terminal residues. For example, derivatisation with bifunctional agents is a useful means for cross-linking an antibody or fragment thereof to macromolecular carriers such as water-insoluble support matrices. Antibody derivatives as contemplated herein may have an agent attached to a base antibody or a fragment thereof capable of increasing its half-life *in vivo* (e.g. extending the length of time before clearance from the blood stream). A non-limiting example of such a technique includes addition of PEG moieties.

In certain embodiments, the antibody derivative may be a multimer, such as, for example, a dimer, comprising one or more monomers, where each monomer includes (i) an antigen-binding region of an anti-GPC-1 antibody as described herein, or a polypeptide region derived therefrom (such as, for example, by conservative substitution of one or more amino acid/s), and (ii) a multimerising (e.g. dimerising) polypeptide region, such that the antibody derivative forms multimers (e.g. homodimers) that specifically bind to GPC-1. For example, an antigen binding region of an anti-GPC-1 antibody as described herein, or a polypeptide region derived therefrom, may be recombinantly or chemically fused with a heterologous protein, wherein the heterologous protein comprises a dimerisation or multimerisation domain. The derivative may be subjected to conditions allowing formation of a homodimer or heterodimer. The heterodimer may comprise identical dimerisation domains but different anti-GPC-1 antigen-binding regions, identical anti-GPC-1 antigen-binding regions but different dimerisation domains, or different anti-GPC-1 antigen-binding regions and different dimerisation domains. Suitable dimerisation domains include those that originate from transcription factors (e.g. a basic region leucine zipper), a basic-region helix-loop-helix protein, and an immunoglobulin constant region (e.g. a heavy chain constant region or a domain thereof such as a CH1 domain, a CH2 domain, or a CH3 domain).

In other embodiments, the antibody derivative may be an anti-GPC1 antibody as described herein conjugated to a second antibody (an “antibody heteroconjugate”).

Also contemplated herein are humanised derivatives of the antibodies described herein. A “humanised” antibody as contemplated herein is a human/non-human chimeric antibody that contains a minimal sequence derived from non-human immunoglobulin. For example, a humanised antibody may be a human immunoglobulin (recipient antibody) in

which residues from CDR region/s of the recipient are replaced by residues from a CDR region of a non-human species (donor antibody) (e.g. a mouse, rat, rabbit, or non-human primate having the desired specificity and affinity for a GPC-1 antigen/epitope). Framework region (FR) residues of the human immunoglobulin may also (optionally) be replaced by corresponding non-human residues, and in some cases humanised antibodies may comprise residues not present in the recipient antibody or in the donor antibody to enhance antibody performance.

Further contemplated herein are “chimeric” antibody derivatives in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences of an antibody described herein derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain/s is/are identical with or homologous to corresponding sequences in antibodies derived from another different species or belonging to another different antibody class or subclass. For example, a chimeric antibody as contemplated herein may comprise variable regions derived from an anti-GPC-1 monoclonal antibody as described herein, and constant regions derived from a second species. Chimeric antibodies may be generated, for example, by genetic engineering of immunoglobulin gene segments belonging to different species. The chimeric antibodies and antigen-binding fragments thereof may or may not be conjugated to a cytotoxic agent as described herein.

By way of non-limiting example only, a chimeric antibody according to the present invention may comprise a chimeric Mouse Human CH1-CH3 Chain Sequence Mouse VH-Human CH1-CH3 Chain (heavy chain) and/or a Mouse Human Kappa Chain Sequence Mouse VK-Human CK sequence MIL-38 Mouse VK (light chain). The heavy chain of the chimeric antibody may comprise or consist of an amino acid sequence as set out in residues 20-467 of **SEQ ID NO: 7**. The light chain of the chimeric antibody may comprise or consist of an amino acid sequence as set out in residues 21-234 of **SEQ ID NO: 8**. The heavy chain variable region may comprise: a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 7**; and/or a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 7**; and/or a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 7**. Additionally or alternatively, the light chain variable region may comprise: a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 8**; and/or a complementarity

determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 8**; and/or a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 109-117 of **SEQ ID NO: 8**. A chimeric antibody according to the present invention may be a
5 “variant” of this chimeric antibody.

Included within the scope of the present invention are “variants” of the antibodies described herein. A “variant” antibody refers to an antibody which differs in amino acid sequence from a “parent” anti-GPC-1 antibody amino acid sequence by virtue of addition, deletion, and/or substitution of one or more amino acid residue/s in the parent antibody
10 sequence. For example, the variant antibody may comprise one or more amino acid substitution/s in one or more CDR and/or framework region/s of the parent antibody (e.g. between 1 and 10, between 2 and 5, or 1, 2, 3, 4, or 5 substitutions in one or more heavy and/or light chain CDR and/or framework regions of the parent antibody). The antibody variant may comprise a heavy chain variable domain sequence and/or a light chain
15 variable domain sequence amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% amino acid sequence homology (i.e. sequence identity) with the corresponding variable domain of the parent antibody.

Sequence homology or identity between two sequences is defined herein as the
20 percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. If the two sequences which are to be compared with each other differ in length, sequence identity relates to the percentage of amino acid residues of the shorter sequence which are identical with the amino acid
25 residues of the longer sequence. Sequence identity can be determined conventionally with the use of computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive Madison, Wis. 53711) and/or the program “fasta20u66” (version 2.0u66, September 1998 by William R. Pearson and the University of Virginia; see also W. R.
30 Pearson (1990), Methods in Enzymology 183, 63-98).

In some embodiments, a variant antibody as described herein may differ from a parent antibody by way of conservative amino acid change/s in the sequence of the variable antibody. A “conservative change” refers to an alteration that is substantially antigenically or conformationally neutral, producing minimal changes in the tertiary
35 structure of the variant antibody, or producing minimal changes in the antigenic

determinants of the variant antibody, as compared to the parent antibody, and one which does not render the derivative incapable of binding to the same epitope in GPC-1 as the parent antibody. Non-limiting examples of conservative amino acid changes include substitution of hydrophobic amino acids and substitution of physiochemically similar amino acids. Persons of ordinary skill in the art can routinely and without difficulty assess whether a given amino acid substitution can be made while maintaining conformational and antigenic neutrality (see, for example, Berzofsky, (1985) *Science* 229:932-940; Bowie *et al.* (1990) *Science* 247:1306-1310). Alterations in protein conformation may be achieved using well-known assays including, but not limited to, microcomplement fixation methods (see Wasserman *et al.* (1961) *J. Immunol.* 87:290-295; Levine *et al.* (1967) *Meth. Enzymol.* 11:928-936) and through binding studies using conformation-dependent monoclonal antibodies (see Lewis *et al.* (1983) *Biochem.* 22:948-954). The conservative amino acid change/s may occur in one or more CDR and/or framework region/s of the parent antibody (e.g. between 1 and 10, between 2 and 5, or 1, 2, 3, 4, or 5 conservative substitutions in one or more CDR and/or framework regions of the parent antibody).

In general, humanised, chimeric, derivative, fragment and variant antibodies as contemplated herein are still capable of specifically binding to same antigen/epitope (e.g. GPC-1) as the parent antibody from which they derive or which they contain component/s of. Typically, they may retain at least 10% of the antigen/epitope binding capacity of the parent antibody, or, at least 25%, 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% (or more) of the antigen/epitope binding capacity of the parent antibody. For example, they may have a stronger binding affinity and/or binding specificity compared to the parent antibody.

The capacity of an antibody fragment, derivative, or variant to bind specifically to an antigen/epitope that is targeted by the parent antibody (i.e. a GPC-1 antigen/epitope) can be tested using known methods in the art including, for example, competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, enzyme linked immunosorbent assay (ELISA), immunoprecipitation assays, "sandwich" immunoassays, immunodiffusion assays, precipitin reactions, protein A immunoassays, fluorescent immunoassays, gel diffusion precipitin reactions, complement-fixation assays, immunoradiometric assays, agglutination assays, and the like (see, for example, Ausubel *et al.*, eds., *Short Protocols in Molecular Biology* (John Wiley & Sons, Inc., New York, 4th ed. 1999); Harlow & Lane, *Using Antibodies: A*

Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999)).

Specifically included within the scope of the present invention are variants of any antibody or antigen binding fragment thereof described herein including, but not limited to, antibodies (including chimeric antibodies) and antigen binding fragments defined by specific sequences herein, and antibodies produced by hybridomas described herein including the hybridoma submitted under the terms of the Budapest Treaty at Cellbank Australia at 214 Hawkesbury Road, Westmead NSW 2145, Australia on 22nd August 2014 under accession number CBA20140026.

Cytotoxic agents

Antibodies and antibody-binding fragments thereof according to the present invention may be conjugated to at least one cytotoxic agent. The cytotoxic agent may be any compound that is toxic to a cell (e.g. a prostate, bladder, and/or pancreatic cancer cell) such as, for example by, inhibiting cell function, and including but not limited to, causing cell death directly and/or indirectly.

Cytotoxic agents may cause direct toxicity to the prostate, bladder, and/or pancreatic cancer cell, or may induce apoptosis. Alternatively, in the case of a radiolabel, the radiation may cause DNA damage resulting in cell growth arrest, apoptosis induction or cell death.

Enhanced prostate, bladder, and/or pancreatic cancer death may result from immune recognition of proteins from dead or dying prostate, bladder, and/or pancreatic cancer cells thereby resulting in an “immunisation” effect.

Non-limiting examples of suitable cytotoxic agents include: afatinib, aplidin, anastrozole, anthracyclines, AVL-101, AVL-291, axitinib, azaribine, bendamustine, bleomycin, bortezomib, bosutinib, bryostatin-1, busulfan, camptothecans, carboplatin, calicheamycin, camptothecin, carboplatin, carmustine, celecoxib, chlorambucil, cisplatin, cladribine, COX-2 inhibitors, crizotinib, cyano-morpholino doxorubicin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, dasatinib, daunorubicin, dinaciclib, 3',5'-O-dioleoyl-FudR (FudR-d0), DM1, DM3, DM4, docetaxel, doxorubicin, doxorubicin glucuronide, duocarmycin, endostatin, entinostat, epidophyllotoxin, epirubicin glucuronide, erlotinib, estramustine, estrogen receptor binding agents, etoposide glucuronide, etoposide phosphate, etoposide (VP16), exemestane, farnesyl-protein transferase inhibitors, fingolimod, flavopiridol, floxuridine (FudR), fludarabine, 5-fluorouracil, flutamide, fostamatinib, ganetespib, GDC-0834, gefitinib, gemcitabine,

GS-1101, 10-hydroxycamptothecin, hydroxyurea, ibrutinib, idarubicin, idelalisib, ifosfamide, imatinib, irinotecan (CPT-11), lapatinib, lenolidamide, leucovorin, LFM-A13, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mithramycin, mitomycin, mitotane, mitoxantrone, monomethylauristatin D (MMAD), monomethylauristatin E (MMAE), monomethylauristatin F (MMAF),
 5 navelbine, neratinib, nilotinib, nitrosurea, olaparib, paclitaxel, PCI-32765, pentostatin, plicomycin, a 2-PDox pro-drug (pro-2-PDox), procarbazine, PSI-341, 2-pyrrolinodoxorubicine (2-PDox), raloxifene, semustine, SN-38, sorafenib, streptozocin, SU1 1248, sunitinib, tamoxifen, temazolomide, teniposide, thalidomide, thioguanine,
 10 thiotepa, topotecan, transplatinum, uracil mustard, vatalanib, vinblastine, vinca alkaloids, vincristine, vinorelbine, and ZD 1839.

Additionally or alternatively, the cytotoxic agent may be a radioisotope. Non-limiting examples of suitable radioisotopes include: ^{90}Y , ^{188}Re , ^{166}Ho , ^{165}Dy , ^{109}Pd , ^{111}Ag , ^{186}Re , ^{198}Au , ^{153}Sm , ^{64}Cu , ^{177}Lu , ^{131}I , ^{125}I , ^{67}Cu , ^{175}Yb , ^{166}Dy , ^{169}Er , ^{212}Bi , ^{213}Bi , ^{225}Ac ,
 15 ^{212}Pb , ^{66}Ga , ^{67}Ga , ^{68}Ga , ^{86}Y , $^{94\text{m}}\text{Tc}$, and ^{89}Zr

In some embodiments, the antibody or antigen-binding fragment thereof is conjugated to the cytotoxic agent or agent(s) directly.

In other embodiments the antibody or antigen-binding fragment thereof is conjugated to the cytotoxic agent or agent(s) indirectly (e.g. via a chelating agent selected
 20 from the group consisting of: DOTA, DTPA, NOTA, NODAGA, MeCOSAR, TETA, TRAP, TE2A, CBTE2A).

Conjugated antibody production

Processes for the preparation of the monoclonal antibodies, derivatives and variants thereof, and antigen binding fragments thereof are readily available and capable of being
 25 performed without difficulty by persons of ordinary skill in the art.

Apart from the hybridoma method of Kohler *et al.* (1975) and described above in the section entitled “*Hybridomas*”, another non-limiting process that may be utilised is recombinant DNA technology (see, for example, US Patent No. 4816567). For example,
 30 the monoclonal antibodies, derivatives and variants thereof, and antigen binding fragments thereof, may be recombinantly produced in any well-established expression system including, but not limited to, baculovirus, yeast (e.g. *Pichia* sp., *Saccharomyces* sp.) *E. coli*, mammalian cells, plants, or transgenic animals (see Breitling and Dubel, 1999, Recombinant Antibodies, John Wiley & Sons, Inc., NY, pp. 119-132).

In some embodiments, nucleic acid sequences encoding monoclonal antibodies, derivatives and variants thereof, and antigen binding fragments thereof in accordance with the present invention may be used in production processes based on recombinant DNA technologies. Non-limiting examples include a heavy chain polynucleotide
5 sequence as defined in **SEQ ID NO: 1** or a variant or fragment thereof, and/or a light chain polynucleotide sequence as defined in **SEQ ID NO: 2** or a variant or fragment thereof.

A “variant” polynucleotide refers herein to a polynucleotide which differs in sequence from a parent or reference polynucleotide. Polynucleotide sequence divergence
10 may result from mutational changes such as deletions, substitutions, or additions of one or more nucleotides. Each of these changes may occur alone or in combination, one or more times in a given sequence. A “variant” polynucleotide refers to a polynucleotide that has a substantially similar sequence to a parent or reference polynucleotide. In general, two sequences are “substantially similar” if the two sequences have a specified percentage of
15 nucleotides that are the same (percentage of sequence “homology” or sequence “identity”). Sequence homology or identity between two polynucleotide sequences is defined herein as the percentage of nucleotides in the candidate (“variant”) sequence that are identical with those of the parent/reference polynucleotide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence
20 identity. If the two sequences which are to be compared with each other differ in length, sequence identity relates to the percentage of the nucleotides of the shorter sequence which are identical with the nucleotides of the longer sequence. Sequence identity can be determined conventionally with the use of computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group,
25 University Research Park, 575 Science Drive Madison, Wis. 53711) and/or the program “fasta20u66” (version 2.0u66, September 1998 by William R. Pearson and the University of Virginia; see also W. R. Pearson (1990), Methods in Enzymology 183, 63-98). The degree of sequence homology/identity between the variant polynucleotide and the reference/parent polynucleotide may, for example, be at least 75%, 80%, 83% 85%, 88%,
30 90%, 93%, 95%, 96%, 97%, 98% or 99%.

A polynucleotide “fragment” is a polynucleotide molecule that encodes a constituent or is a constituent of a large parent/reference polynucleotide. In general, the fragment will encode a fragment of an antibody of the present invention, the fragment being capable of specifically binding to GPC-1.

Monoclonal antibodies, derivatives and variants thereof, and antigen binding fragments thereof produced in accordance with the present invention may be isolated from various sources using appropriate methods including, but not limited to, immunoglobulin-binding molecules (for example, proteins A, L, G or H), tags operatively
5 linked to the antibody or antibody fragment (for example, His-tag, c-myc tag), affinity chromatography, and the like.

Monoclonal antibodies, derivatives and variants thereof, and antigen binding fragments thereof as described herein may be produced by hybridomas and/or cell cultures comprising single or mixed populations of hybridomas including, for example,
10 those described in the section above entitled “*Hybridomas*”, then isolated using known techniques. In some embodiments, the monoclonal antibodies can be produced by culturing a single (monoclonal) species of hybridoma cells deposited under the terms of the Budapest Treaty at Cellbank Australia under accession number CBA20140026, and isolated from the culture.

Processes for the preparation and cultivation of the hybridoma cell lines and isolation of the antibody produced are well known to those of ordinary skill in the art and are standard procedures.
15

Antibodies and antibody-binding fragments thereof according to the present invention may be conjugated to at least one cytotoxic agent. Methods for conjugating
20 cytotoxic agents to antibodies and antigen binding fragments thereof are well known to those skilled in the art (see, for example, Zhang *et al.* PLOS One December 2011, Volume 6 Issue 12).

Medicaments and pharmaceutical formulations

Medicaments and pharmaceutical formulations according to the present invention comprise antibodies and antibody-binding fragments thereof, which may be conjugated to at least one cytotoxic agent, as described herein, or alternatively be provided without such agent(s) (i.e. naked). The medicaments and pharmaceutical formulations may be prepared using methods known to those of ordinary skill in the art. Non-limiting examples of
30 suitable methods are described in Gennaro *et al.* (Eds), (1990), “*Remington’s Pharmaceutical Sciences*”, Mack Publishing Co., Easton, Pennsylvania, USA.

The medicaments and pharmaceutical formulations may comprise a pharmaceutically acceptable carrier, excipient, diluent and/or adjuvant. “Pharmaceutically acceptable” carriers, excipients, diluents and/or adjuvants as contemplated herein are
35 substances which do not produce adverse reaction(s) when administered to a particular

recipient such as a human or non-human animal. Pharmaceutically acceptable carriers, excipients, diluents and adjuvants are generally also compatible with other ingredients of the medicaments and pharmaceutical formulations. Non-limiting examples of suitable excipients, diluents, and carriers can be found in the “*Handbook of Pharmaceutical*
5 *Excipients*” 4th Edition, (2003) Rowe *et al.* (Eds), The Pharmaceutical Press, London, American Pharmaceutical Association, Washington.

Non-limiting examples of pharmaceutically acceptable carriers, excipients or diluents include demineralised or distilled water; saline solution; vegetable based oils such as peanut oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oils, arachis
10 oil or coconut oil; silicone oils, including polysiloxanes, such as methyl polysiloxane, phenyl polysiloxane and methylphenyl polysiloxane; volatile silicones; mineral oils such as liquid paraffin, soft paraffin or squalane; cellulose derivatives such as methyl cellulose, ethyl cellulose, carboxymethylcellulose, sodium carboxymethylcellulose or hydroxypropylmethylcellulose; lower alkanols, for example ethanol or isopropanol; lower
15 aralkanols; lower polyalkylene glycols or lower alkylene glycols, for example polyethylene glycol, polypropylene glycol, ethylene glycol, propylene glycol, 1,3-butylene glycol or glycerin; fatty acid esters such as isopropyl palmitate, isopropyl myristate or ethyl oleate; polyvinylpyrrolidone; agar; carrageenan; gum tragacanth or gum acacia, and petroleum jelly. Typically, the carrier or carriers will form from 10% to
20 99.9% by weight of the compositions.

Medicaments and pharmaceutical formulations of the present invention may be in a form suitable for administration by injection, in the form of a formulation suitable for oral ingestion (such as capsules, tablets, caplets, elixirs, for example), in the form of an ointment, cream or lotion suitable for topical administration, in a form suitable for
25 delivery as an eye drop, in an aerosol form suitable for administration by inhalation, such as by intranasal inhalation or oral inhalation, or in a form suitable for parenteral administration, that is, intradermal, subcutaneous, intramuscular or intravenous injection.

Solid forms of the medicaments and pharmaceutical formulations for oral administration may contain binders acceptable in human and veterinary pharmaceutical
30 practice, sweeteners, disintegrating agents, diluents, flavourings, coating agents, preservatives, lubricants and/or time delay agents. Suitable binders include gum acacia, gelatine, corn starch, gum tragacanth, sodium alginate, carboxymethylcellulose or polyethylene glycol. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose,
35 polyvinylpyrrolidone, guar gum, xanthan gum, bentonite, alginic acid or agar. Suitable

diluents include lactose, sorbitol, mannitol, dextrose, kaolin, cellulose, calcium carbonate, calcium silicate or dicalcium phosphate. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

Liquid forms of the medicaments and pharmaceutical formulations for oral administration may contain, in addition to the above agents, a liquid carrier. Suitable liquid carriers include water, oils such as olive oil, peanut oil, sesame oil, sunflower oil, safflower oil, arachis oil, coconut oil, liquid paraffin, ethylene glycol, propylene glycol, polyethylene glycol, ethanol, propanol, isopropanol, glycerol, fatty alcohols, triglycerides or mixtures thereof.

Suspensions for oral administration may further comprise dispersing agents and/or suspending agents. Suitable suspending agents include sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, poly-vinyl-pyrrolidone, sodium alginate or acetyl alcohol. Suitable dispersing agents include lecithin, polyoxyethylene esters of fatty acids such as stearic acid, polyoxyethylene sorbitol mono- or di-oleate, -stearate or -laurate, polyoxyethylene sorbitan mono- or di-oleate, -stearate or -laurate and the like.

For preparation of the medicaments and pharmaceutical formulations as injectable solutions or suspensions, non-toxic parenterally acceptable diluents or carriers may be used such as Ringer's solution, isotonic saline, phosphate buffered saline, ethanol and 1,2 propylene glycol.

Emulsions for oral administration may further comprise one or more emulsifying agents. Suitable emulsifying agents include dispersing agents as exemplified above or natural gums such as guar gum, gum acacia or gum tragacanth.

Topical formulations comprise an active ingredient(s) (e.g. i.e. antibodies and/or antigen-binding fragments thereof of the present invention) together with one or more acceptable carriers, and optionally any other therapeutic ingredients. Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin to the site of where treatment is required, such as liniments, lotions, creams, ointments or pastes, and drops suitable for administration to the eye, ear or nose.

When formulated as drops, the medicaments and pharmaceutical formulations may comprise sterile aqueous or oily solutions or suspensions. These may be prepared by dissolving the active ingredient in an aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and optionally including a surface active agent. The resulting solution may then be clarified by filtration, transferred to a suitable container and sterilised. For example, sterilisation may be achieved by filtration followed by transfer to a container by an aseptic technique. Examples of bactericidal and fungicidal agents suitable for inclusion in the drops are phenylmercuric nitrate or acetate (0.002%), benzalkonium chloride (0.01%) and chlorhexidine acetate (0.01%). Suitable solvents for the preparation of an oily solution include glycerol, diluted alcohol and propylene glycol.

When formulated as creams, ointments or pastes, the medicaments and pharmaceutical formulations may be semi-solid formulations of the active ingredient for external application. They may be made by mixing the active ingredient in finely-divided or powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid, with a greasy or non-greasy basis. The basis may comprise hydrocarbons such as hard, soft or liquid paraffin, glycerol, beeswax, a metallic soap; a mucilage; an oil of natural origin such as almond, corn, arachis, castor or olive oil; wool fat or its derivatives, or a fatty acid such as stearic or oleic acid together with an alcohol such as propylene glycol or macrogols.

The medicaments and pharmaceutical formulations may include any suitable surfactant such as an anionic, cationic or non-ionic surfactant such as sorbitan esters or polyoxyethylene derivatives thereof. Suspending agents such as natural gums, cellulose derivatives or inorganic materials such as siliceous silicas, and other ingredients such as lanolin, may also be included.

Methods for treating prostate, bladder, and/or pancreatic cancer

According to the present invention, antibodies and antibody-binding fragments thereof, which may be naked or conjugated to at least one cytotoxic agent, are suitable for the treatment of prostate, bladder, and/or pancreatic cancer. The antibodies and/or fragments thereof may be administered as components of pharmaceutical formulations or medicaments as are described herein.

The identification of subjects with or at risk of developing prostate, bladder, and/or pancreatic cancer is well known to those of ordinary skill in the art. For example, in the case of prostate cancer suitable methodologies include, but are not limited to, digital rectal exam, PSA-based assays, and prostate biopsy.

One of the distinguishing factors between benign prostate tumors (non cancerous) and malignant prostate tumors (cancerous) is the ability of the cancerous form to metastasise. Metastasis is the capacity for cancerous cells to spread (metastasise) to other parts of the body. Prostate cancer in patients is further categorised into stages according to the TNM Classification of Malignant Tumours (TNM), which categorises cancer based on the size and extent of the primary tumor (T), the spread of the cancer to nearby lymph nodes (N), and the presence of secondary tumor formed by the metastasis (M) of the primary tumor to other parts of the body (American Cancer Society). **Table 2** shows example definitions for each cancer stage.

Table 2. Definitions for cancer stages of the TNM system, adapted from the American Cancer Society.

Stage	Definition
Stage 0	Carcinoma in situ
Stage I, Stage II, and Stage III	Higher numbers indicate more extensive disease: Larger tumor size and/or spread of the cancer beyond the organ in which it first developed to nearby lymph nodes and/or tissues or organs adjacent to the location of the primary tumor
Stage IV	The cancer has spread to distant tissues or organs

The methods may be used for treating prostate, bladder, and/or pancreatic cancer in a subject. Non-limiting examples of prostate cancers that may be treated with the present invention include prostatic intraepithelial neoplasia, adenocarcinoma, leiomyosarcoma, and rhabdomyosarcoma. Non-limiting examples of bladder and/or pancreatic cancer that may be treated with the present invention include T categories for bladder cancer, N categories, and M categories, as defined in the American Joint Committee on Cancer (AJCC) 'TNM system', muscle invasive bladder cancer, and non-muscle invasive bladder cancer.

It will be understood that "treating" within the context of the methods described herein refers to the alleviation, in whole or in part, of symptoms associated with prostate, bladder, and/or pancreatic cancer, or slowing, inhibiting or halting of further progression or worsening of those symptoms, or the prevention of prostate, bladder, and/or pancreatic cancer. Accordingly, treating prostate, bladder, and/or pancreatic cancer may include, for example, inhibiting or preventing the metastasis of the cancer, a reduction in the speed and/or number of the metastasis, a reduction in tumour volume of the metastasised

prostate, bladder, and/or pancreatic cancer, a complete or partial remission of the metastasised prostate, bladder, and/or pancreatic cancer, or other therapeutic benefit(s).

The subject may be any animal that can benefit from the administration of the antibodies and/or antibody-binding fragments thereof. In some embodiments, the subject
5 is a mammal, for example, a human, a dog, a cat, a horse, a cow, a pig, a primate, or a rodent (e.g. a rat or mouse).

The methods may involve the administration of a “therapeutically effective amount” of antibodies and antibody-binding fragments thereof according to the invention. A “therapeutically effective amount” will be understood to refer to an amount of the
10 antibodies and/or fragments that alleviates, in whole or in part, symptoms associated with prostate, bladder, and/or pancreatic cancer, or slows, inhibits or halts further progression or worsening of those symptoms, or prevents or provides prophylaxis for prostate, bladder, and/or pancreatic cancer in a subject at risk for developing prostate cancer.

The therapeutically effective amount may vary depending upon the route of
15 administration and dosage form. Effective amounts of conjugated antibodies and/or fragments thereof according to the present invention typically fall in the range of about 0.001 up to 100 mg/kg/day, for example in the range of about 0.05 up to 10 mg/kg/day. Typically, the antibodies and/or fragments are selected to provide a formulation that exhibits a high therapeutic index. The therapeutic index is the dose ratio between toxic
20 and therapeutic effects which can be expressed as the ratio between LD₅₀ and ED₅₀. The LD₅₀ is the dose lethal to 50% of the population and the ED₅₀ is the dose therapeutically effective in 50% of the population. The LD₅₀ and ED₅₀ are determined by standard pharmaceutical procedures in animal cell cultures or experimental animals.

Generally, an effective dosage of conjugated antibodies and/or fragments thereof
25 according to the present invention is expected to be in the range of about 0.0001mg to about 1000mg of active component(s) (i.e. of conjugated antibodies and/or fragments thereof according to the present invention) per kg body weight per 24 hours; typically, about 0.001mg to about 750mg per kg body weight per 24 hours; about 0.01mg to about 500mg per kg body weight per 24 hours; about 0.1mg to about 500mg per kg body weight
30 per 24 hours; about 0.1mg to about 250mg per kg body weight per 24 hours; about 1.0mg to about 250mg per kg body weight per 24 hours. More typically, an effective dose range is expected to be in the range about 1.0mg to about 200mg per kg body weight per 24 hours; about 1.0mg to about 100mg per kg body weight per 24 hours; about 1.0mg to about 50mg per kg body weight per 24 hours; about 1.0mg to about 25mg per kg body
35 weight per 24 hours; about 5.0mg to about 50mg per kg body weight per 24 hours; about

5.0mg to about 20mg per kg body weight per 24 hours; about 5.0mg to about 15mg per kg body weight per 24 hours.

Alternatively, an effective dosage may be up to about 500mg/m² of active component(s) (i.e. of conjugated antibodies and/or fragments thereof according to the present invention). Generally, an effective dosage is expected to be in the range of about 25 to about 500mg/m², preferably about 25 to about 350mg/m², more preferably about 25 to about 300mg/m², still more preferably about 25 to about 250mg/m², even more preferably about 50 to about 250mg/m², and still even more preferably about 75 to about 150mg/m².

Typically, in therapeutic applications, the treatment would be for the duration of prostate, bladder, and/or pancreatic cancer disease in the subject. Further, it will be apparent to one of ordinary skill in the art that the optimal quantity and spacing of individual dosages will be determined by the nature and extent of the prostate, bladder, and/or pancreatic cancer being treated, the form, route and site of administration, and the nature of the particular individual being treated. Also, such optimum conditions can be determined by conventional techniques.

In many instances, it will be desirable to have several or multiple administrations of conjugated antibodies and/or fragments thereof according to the present invention. For example, a given dosage may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times. The administrations may be from about one to about twelve week intervals, and in certain embodiments from about one to about four week intervals. Periodic re-administration may be desirable in the case or risk of recurrent occurrence of prostate, bladder, and/or pancreatic cancer in a given subject.

It will also be apparent to one of ordinary skill in the art that the optimal course of treatment can be ascertained using conventional course of treatment determination tests.

The conjugated antibodies and/or fragments thereof according to the present invention can be formulated for various routes of administration, for example, by oral, parenteral (e.g. intradermal, intravenous, intraspinal, intraperitoneal, subcutaneous or intramuscular), nasal, rectal, topical, or by way of an implanted reservoir. Systemic or parenteral administration includes, but is not limited to, intraperitoneal, intramuscular, subcutaneous, and intravenous injections. In one embodiment, they are administered by a mucosal route. Non-limiting examples of acceptable routes of mucosal administration including intranasal, ocular, buccal, genital tract, rectal, intratracheal, skin, and the gastrointestinal tract.

In some embodiments, the treatment methods described herein antibodies and/or fragments thereof may be administered in combination with other agents or therapies. The antibodies and/or fragments thereof may be components of pharmaceutical formulations or medicaments as are described herein. Non-limiting examples of the additional agents or therapies include hormonal agents, LHRH agonists and antagonists, CYP17 inhibitors, taxane therapy, radioisotope treatment, immunomodulatory therapy. Examples include docetaxel, mitoxantrone, abiraterone acetate, enzalutamide, ketoconazole, corticosteroids, Sipuleucel-T, cabazitaxel, ²²³radium, gemcitabine and Bacille Calmette Guérin (BCG) immunotherapy. Additional radiation therapies also used include external beam radiation and brachytherapy. The antibodies and/or fragments thereof may be administered to the subject simultaneously with the additional agents or therapies. Additionally or alternatively, the antibodies and/or fragments may be administered to the subject before or after the additional agents or therapies are administered.

Examples

The present invention will now be described with reference to specific examples, which should not be construed as in any way limiting.

Example 1: analysis of antibodies from MIL-38 hybridoma populations

1.1 Materials and Methods

- MIL-38 antibody preparation

Preparations MIL-38 antibody hybridoma were obtained from the following sources:

(i) In house cell stocks of the BLCA-38 hybridoma were used to generate MIL-38 antibody preparations designated 33A.

(ii) An early passage (<6) frozen down of cells from the original deposit of the BLCA-38 hybridoma (HB11785) was used to perform single cell cloning and provide a number of clones to characterize. The MIL-38 1F5 clone was selected and deposited at CellBank Australia under deposit number CBA20140026.

(iii) The hybridoma stock used as a basis to generate the preparations described in (i) and (ii) above was prepared as described in U.S. patent no. 5,622,836 to Walker et al., the entire contents of which are incorporated herein by cross-reference.

For purification of MIL-38 antibody, frozen cell stocks were quickly thawed followed by resuspension in RPMI 1640 medium and allowed to grow at 37°C with 5% CO₂ for 24 hours. Cells were expanded, split and scaled up in a sequential process. At each step,

cells were resuspended in fresh medium and incubated at 37°C with 5% CO₂. After scale up, cells were transferred to sterile serum free medium and grown till the start of death phase. The supernatant was harvested to collect the MIL-38 antibody and filter sterilised. Antibody supernatant was stored at -80°C until required. Antibody was purified using
5 Pierce protein G according to the manufacturer's recommendations.

- *Western blot and Sypro gel analysis*

Protein extraction: DU-145 (MIL-38 antigen positive) or C3 (MIL-38 antigen negative) cells were cultured according to standard tissue culture techniques. Cell
10 membrane proteins were enriched using the Merck Millipore ProteoExtract Native Membrane Protein Extraction Kit (MPEK) according to the manufacturer's instructions.

- *Transfer*

Gels were transferred onto a nitrocellulose membrane for 10 min at 2.5 A and 25V
15 maximum using the Transblot Turbo system (Biorad).

- *Western blot*

Briefly, after transfer membranes were blocked with 5% skim milk in PBS-Tween (0.1%) for 2h at room temperature. Primary antibodies (1µg/ml in 5% skim milk – PBS-Tween (0.1%)) were applied and incubated overnight at 4°C. After washing (3x 10 min
20 PBS-Tween (0.1%)) membranes were incubated with secondary antibody (1:2000 sheep-anti-mouse HRP-labelled in 5% skim milk – PBS-Tween (0.1%)). After washing (3x 10 min PBS-Tween (0.1%)) antigen was detected by using ECL detection kit (Biorad) and imaging with LAS4000 mini (GE Life Science).

25

- *Sypro gels*

Gels were fixed in fixing solution (10% Ethanol, 7% acetic acid) for 2h before being transferred into Sypro®Ruby Protein Stain and incubated overnight at room temperature in the dark. Before imaging gels were rinsed and washed with destaining
30 solution (10% Ethanol, 7% acetic acid) for a minimum of 2h. Imaging was performed with a Pharos X Scanner.

- *Immunofluorescence assay (IFA)*

IFA: Cells were grown on coverslips until 75% confluent and placed in 6 well
35 plates. The cells were washed with PBS followed by fixing with acetone. Cells were

washed again with PBS followed by incubation with TBS then blocked with PBS containing 5% skim milk. Cells were then incubated in the dark with MIL-38, chimeric MIL-38 or Cetuximab followed by incubation with a Goat anti-mouse or Goat anti-human antibody labelled with FITC or Alexa488. Both antibodies were prepared in PBS containing 1% skim milk followed. Washing with PBS was performed between the primary and secondary antibody incubations. After secondary incubation, cells were washed with PBS containing DAPI and visualised for green fluorescence (MIL-38 positive).

- *SDS-PAGE electrophoresis*

SDS-PAGE: Samples were mixed with non-reducing SDS-containing sample buffer and loaded onto a 4-15% precast polyacrylamide gel (Criterion TGX; Biorad). Gels were run for 10 min at 80V and additional 50 min at 200V in Tris-Glycine running buffer.

- *Sequencing (DNA)*

Previous results indicated that the BLCA-38 cells deposited at ATCC accession number HB11785 contained two populations of cells. Subcloning identified two types of clones: AM3/Alfio II type (did not bind GPC-1) or AM4/Alfio I (bound GPC-1 strongly). The two populations were sequenced together with a low passage stock obtained from ATCC (termed "1-0 or original").

For sequencing runs 224945 (1-O) and 449295-1 (Alfio I), total RNA was extracted from frozen hybridoma cells and cDNA was synthesized from the RNA. PCR was then performed to amplify the variable regions (heavy and light chains) and constant regions of the antibody, which were then cloned into a standard cloning vector separately and sequenced. Total RNA was isolated from the hybridoma cells following the technical manual of TRIzol® Plus RNA Purification System. The total RNA was analysed by agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA using isotype-specific anti-sense primers or universal primers following the technical manual of SuperScript™ III First-Strand Synthesis System. The antibody fragments of VH, VL, CH and CL were amplified according to the standard operating procedure of RACE of GenScript. Amplified antibody fragments were separately cloned into a standard cloning vector using standard molecular cloning procedures. Colony PCR screening was performed to identify clones with inserts of correct sizes. No less than five single colonies with inserts of correct sizes were sequenced for each antibody fragment. VH and VL plasmids encoded the full-length variable regions of the antibody and a part of CH1 and CL. CH plasmid encoded a part of CH1 and full-length CH2 and CH3. CL plasmid

encoded a part of CL. In order to get full-length constant regions or heavy/light chain, the part of constant regions encoded by VH and VL plasmids and the part of constant regions encoded by CH and CL plasmids were amplified by PCR separately, and then overlap extension PCR was employed to obtain full-length DNAs. Five single colonies with correct VH, VL, CH and CL insert sizes were sent for sequencing. The isolated total RNA of the sample was run alongside a DNA marker (Marker III - TIANGEN, Cat. No. : MD103) on a 1.5% agarose/GelRed™ gel. Four microliters of PCR products of each sample were run alongside the DNA marker (Marker III) on a 1.5% agarose/GelRed™ gel. The PCR products were purified and stored at -20 °C until further use.

The V_H, V_L, C_H and C_L genes of five different clones were nearly identical. The consensus sequence, listed below, was determined to be the sequence of the antibody produced by the monoclonal hybridoma population (AM-4).

MIL-38 Mouse IgG₁ Heavy Chain DNA Consensus Sequence (SEQ ID NO: 1)

[illegible]

Individual regions of mouse heavy chain encoded sequence are highlighted with alternating shaded/unshaded text. Positions: 1-57 = leader sequence; 58-147 = framework region (HFR1); 148-162 = complementarity determining region (HCDR1); 163-204 = HFR2; 205-255 = HCDR2; 256-351 = HFR3; 352-378 = HCDR3; 379-411 = HFR4; 412-1383 = constant regions (CH1-CH3); 703-741 = hinge region (underlined); 1384-1386 = stop codon.

MIL-38 Mouse Kappa Light Chain DNA Consensus Sequence (SEQ ID NO: 2)

10 AAGAGTGGCTTACTCAGGTCTGGCGTTGGTGGCTGGCTGGCTTACAGTCCAGATGGACATCCAGATG
 ACTCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAACTGTCACCATCACATGTCTCAAGTCTCA
 TCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCA
 TGGTATCAGCAGAAACAGGGAAAATCTCCTCAACTCCTGGTCTATCTCTCAAGTCTCAAGTCTCA
 GGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAAT
 AGCCTGCAGCCTGAAGATTTTGGGACTTATTACTGTCAAGTCTCTCAAGTCTCAAGTCTCAAGTCTCA
 GGCACCAAGCTGGAAATCAAAAGGCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCA
 15 TAACATCTGGAGTCTCAGTGGTGGTCTCTGAGCACTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCA
 AGATTCAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCA
 TCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCA
 ACTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCAAGTCTCA
 TAG

Individual regions of mouse light chain encoded sequence are highlighted with alternating shaded/unshaded text. Positions: 1-60 = leader sequence; 61-129 = framework region (LFR1); 130-162 = complementarity determining region (LCDR1); 163-207 = LFR2; 208-228 = LCDR2; 229-324 = LFR3; 325-351 = LCDR3; 352-381 = LFR4; 382-702 = constant regions (CK); 703-705 = stop codon.

25 The heavy and light chain AM-4 MIL-38 consensus DNA sequences above translate to the following heavy chain and light chain amino acid sequences:

AM-4 MIL-38 Mouse IgG1 Heavy Chain Amino Acid Consensus Sequence (SEQ ID NO: 3)

30 MAWVNTLLFLMAAQSICAQIQLVQSGPELKKPGETVKISCKASGYAFTDYSPKHWVKQAPGKGLRWMGWINT
 ETGEPTYTDDFKGRFAFSLETSASTAFLQINNLRNEDTATYFCARHYDYGGFYWGQGLTVTVSAAKTTPTS
 VYPLAPGSAAGTNSMVTLGCLVKGYFPEPVTVTVNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSSET
 VTCNVAFASSTKVDKKIVPRDCGCKPCICTVFEVSSVFIFFPKPKDVLTTITLTPKVTCTVVDISKDDFEVQ
 FSWFVDDVEVHTAQTQPREEQFNSTFRSVSELIHQDWLNGKEFKCRVNSAFAFPAPIEKTLISKTGGRPKAF

QVYTIPTPPKEQMAKD~~V~~SLTCMITDFFPEDITVEWQWNGQPAENYKNTQFIMETDGSYFVYSKLN~~V~~QKSNWE
 AGNTFTCSVL~~REGLNHHTEKSLSHSPGK~~*

Individual regions of mouse IgG1 heavy chain sequence are indicated in the amino acid sequence above. Positions 1-19 = leader sequence; 20-49 = framework region (HFR1);
 50-54 = complementarity determining region 1 (HCDR1); 55-68 = HFR2; 69-85 =
 HCDR2; 86-117 = HFR3; 118-126 = HCDR3; 127-137 = HFR4 (also called the joining region or J-region); 138-461 = IgG1 chain constant regions (CH1 – CH3) & stop codon (*). Hinge region – is underlined in the sequence above.

AM-4 consensus MIL-38 Light Chain Amino Acid Consensus Sequence (SEQ ID NO: 4)

MSVL~~TQVLA~~ILLL~~WLTGAF~~CDIQMTQSPASLSASVGETVTITCRASGNVHNYLAWYQOKQCKSPQLLVYTAK
 ELAEGVPSRFSGSGSGTQYSLKINSLOPEDFGTYYCQHFWSNPWTFGGGTKLEIKRADAAFTVSIFPPSSEQ
 LTSGGASVVCFLN~~INFYFKDINVKWKIDG~~SERQNGVLNSWTDQDSKDYMSSTLT~~LT~~KDEYERHNSYTCEA
 THKTSTSPIVKGFNRNEC*

Individual regions of light chain amino acid sequence are indicated as labelled: Positions 1-20 = Leader sequence; 21-43 = framework region (LFR1); 44-54 = complementarity determining region 1 (LCDR1); 55-69 = LFR2; 70-76 = LCDR2; 77-108 = LFR3; 109-117 = LCDR3; 118-127 = LFR4; 128-234 = kappa constant region (CK) & stop codon(*)

Example 2: preparation and testing of chimeric MIL-38 antibodies

2.1 Materials and Methods

- Preparation of chimeric antibodies

Two optimised cDNA sequences were developed for cloning purposes. These were based on the AM-4 Heavy chain and Light chain consensus sequences identified above in Example 1.

The first optimised cDNA sequence was used in the generation of a mouse-human chimeric heavy chain sequence:

CHO codon Optimized cDNA Sequence #1 – mouse-human chimeric heavy chain 1404 bp (SEQ ID NO: 5)

Individual regions of mouse-human chimeric heavy chain encoded sequence are highlighted with alternating shaded/unshaded text. Positions: 1-57 = leader sequence; 58-147 = framework region (FR1); 148-162 = complementarity determining region (CDR1); 163-204 = FR2; 205-255 = CDR2; 256-351 = FR3; 352-378 = CDR3; 379-411 = FR4; 412-1401 = human constant regions (CH1-CH3); 706-750 = hinge region (underlined); 1402-1405 = stop codon.

30

35

47

GGCGTGCCCAGCAGGTTCTCTGGCTCCGGCAGCGGCACACAGTATAGCCTGAAGATCAAC
TCTCTGCAGCCTGAGGATTTTGGCACCTACTATTGCAGCATTTGGTAAATCAAGATTTTGGCGGC
GGCACCAAGCTGGAGATCAAGGACAAATCCGGCTTCGGGATTCCTCCCAAGAGCA
5
GATGAGATATCTGAGATGGGATTCTGAAAGCTTAAGGAGATCAATATATATATAT
ATAGCTCTTCAGCACTGACCTCTCCAGGCGGACTACGAGAACTCAAGCTATAGCTTCAAGC
ATTGACAGTTTGTAGGCACTTCAAGCTTCTCAATGTCGAAATTTGA

Individual regions of mouse-human chimeric light chain encoded sequence are highlighted with alternating shaded/unshaded text. Positions: 1-60 = leader sequence; 61-129 = framework region (LFR1); 130-162 = complementarity determining region (LCDR1); 163-207 = LFR2; 208-228 = LCDR2; 229-324 = LFR3; 325-351 = LCDR3; 352-381 = LFR4; 382-702 = human constant region (CK); 703-705 = stop codon.

Chimeric MIL-38 Mouse Human C_H1-C_H3 chains were transiently expressed in suspension CHO-3E7 cells using serum free medium, followed by one-step purification.

CHO-3E7 cells were grown in serum free FreeStyle™ CHO Expression Medium (Life Technologies, Carlsbad, CA, USA). The cells were maintained in Erlenmeyer Flasks (Corning Inc., Acton, MA) at 37°C with 5% CO₂ on an orbital shaker (VWR Scientific, Chester, PA). On the day of transfection, DNA and PEI (Polysciences, Eppelheim, Germany) were mixed at an optimal ratio and then added into the flask with cells ready for transfection. The supernatant collected on day 6 was used for further purification.

Cell culture broth was centrifuged and followed by filtration. Filtered supernatant was loaded onto a 5 ml Protein A CIP column (GenScript, Cat.No.L00433) at 3.0 ml/min. After washing and elution with appropriate buffer, the fractions were collected and neutralized with 1M Tris-HCl, pH 9.0. The purified protein was analyzed by SDS-PAGE, Western blot by using standard protocols for molecular weight, yield and purity measurements.

- Chimeric MIL-38 antibody assays (Slide Immunofluorescence)

The MIL-38 chimeric antibody was used in immunofluorescence assays with DU-145 cells. The murine MIL-38 prep 33A was used as a positive control for GPC-1 antigen staining, while Cetuximab (a chimeric antibody targeting the EGFR) was used as a positive control for staining of human IgG constant regions. A slide with no primary antibody was used as a negative control. Staining was performed essentially as described

in Section 1.1 with the exceptions that secondary antibodies were labelled with Alexafluor 488 and that anti-human antibodies were used to stain the chimeric and cetuximab samples.

5 - *Chimeric MIL-38 western blots*

The reactivity of the chimeric MIL-38 and murine MIL-38 towards DU-145 and C3 MPEK extracts as well as to recombinant NS0-produced GPC-1 antigen was tested by Western blot. Western blots were probed either by murine MIL-38 or chimeric MIL-38. Chimeric MIL-38 was detected by goat anti-human secondary antibody followed by a
10 sheep-anti-goat HRP antibody. As a control, murine MIL-38 was detected by goat anti-mouse secondary antibody followed by a sheep-anti-goat HRP antibody. Equivalent reactivity was observed for chimeric MIL-38 and murine MIL-38 when detected under equivalent conditions.

15 **2.2 Results**

- *Expression of chimeric antibody sequences*

The recombinant plasmids encoding heavy chain and light chain of Chimeric MIL-38 Mouse Human CH1-CH3 Chain were transiently transfected into suspension CHO-3E7 cell cultures. The target protein was captured from the cell culture supernatant by
20 Protein A CIP 5 ml column and followed by buffer exchange. The purified protein was analyzed by SDS-PAGE and Western blot.

- *Chimeric antibody sequences*

Optimised cDNA sequence #1 (**SEQ ID NO: 5**) was used to generate a chimeric
25 MIL-38 antibody heavy chain with the following amino acid sequence:

Chimeric MIL-38 Mouse Human CH1-CH3 Chain Sequence Mouse VH-Human CH1-CH3 Chain (heavy chain) (SEQ ID NO: 7)

```

30 MANVNTLLFLMAAQSIQAQIQLVQSGPELKKPGETVKISCKASGYAFTDYSMDWVKQAPGKGLRWMGWINT
ETGEPTYTDDFKGRFAFSLETSASTAFLQINNLRNEDTATYFCARHYDYGGEFYWGQGTLLTVTVSSASTKGPS
VFPLAPESKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ
TYICNVNHPSENTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPPKPKDTLMISRTPEVTCWVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPTEKTIISKAK
GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTFPVLDSDGSEFFLYSKLTV
35 DKSRNQQGNVFSCVMHEALHNHYTQKSLSLSPGK*
```

Individual regions of mouse-human chimeric heavy chain sequence are indicated in the amino acid sequence above: Positions 1-19 = leader sequence; 20-49 = framework region (HFR1); 50-54 = complementarity determining region 1 (HCDR1); 55-68 = HFR2; 69-85 = HCDR2; 86-117 = HFR3; 118-126 = HCDR3; 127-137 = HFR4 (also
 5 called the joining region or J-region); 138-467 = IgG1 chain constant regions (CH1-CH3), & stop codon (*). Hinge sequence - human IgG1 heavy chain hinge sequence is underlined above.

Optimised cDNA sequence #2 (SEQ ID NO: 6) was used to generate a chimeric
 10 MIL-38 antibody light chain with the following amino acid sequence:

Chimeric MIL-38 Mouse-Human Kappa Light Chain Sequence: Mouse VK-Human CK sequence (SEQ ID NO: 8)

15

```
MSVLTQVLALLLLNLTCARCDIQMTQSPASLSASVGETVTITCRASGVNHYLLAWYQQKQKSPQLLVYTAK
TLADGVPSRFSGSGSGTQYSLKINSLOPEDFGTTYCOHFWSNPWTFFGGGKLEIKRTVAAPSVFIFPPSDEQ
LKSGTASVVCLLNNFYFPEAKVQWKNVDNALQSGNSQESVTEQDSKDSITYLSGTTLTLSKADYEKHKVYACEV
THQGLSPVTKSFNRGEC*
```

Individual regions of mouse-human chimeric light chain amino acid sequence are indicated as labelled: Positions 1-20 = Leader sequence; 21-43 = framework region (LFR1); 44-54 = complementarity determining region 1 (LCDR1); 55-69 = LFR2; 70-76 = LCDR2; 77-108 = LFR3; 109-117 = LCDR3; 118-127 = LFR4; 128-234 = kappa constant region (CK) & stop codon(*)

25

- Chimeric MIL-38 antibody assays (Slide Immunofluorescence)

Figures 1A-D show bright field images of cells. Figure 1E shows staining of the 33A positive control. Figure 1F shows staining of the chimeric MIL-38 antibody. Figure 1G shows staining of using a commercial chimeric (mouse/human) monoclonal antibody (Cetuximab) positive control, and Figure 1H shows the no primary antibody negative control staining. Strong staining was observed in Figures 1E, F and G and no staining was observed in Figure 1H. These results demonstrate that the chimeric MIL-38 antibody successfully binds DU-145 cells in IFA, indicating that the binding specificity of the parental murine MIL-38 antibody has been maintained.

35

- *Chimeric MIL-38 antibody assays (Western blots)*

Figure 2A shows a western blot probed with murine MIL-38, followed by anti-mouse HRP secondary antibody. Exposure time for the Western blot shown in **Figure 2A** was 30 seconds. **Figure 2B** shows a western blot probed with chimeric MIL-38, followed by goat anti-human secondary antibody. The complex was detected using a sheep-anti-goat HRP antibody. Exposure time for the Western blot shown in **Figure 2B** was 30 minutes. **Figure 2C** shows a western blot probed with murine MIL-38, followed by goat anti-mouse antibody. The complex was detected using a sheep-anti-goat HRP antibody. Exposure time for the Western blot shown in **Figure 2C** was 30 minutes.

The murine MIL-38 anti-mouse recognises the antigen in DU-145 lysates and recombinant GPC-1 NS0. Reactivity was not observed in C3 lysates as expected (**Figure 2A**).

A three-antibody detection method was required to test reactivity of the chimeric MIL-38 with DU-145 and C3 extracts as well as recombinant NS0 GPC-1 (**Figure 2B**). A control western blot using a three antibody detection method was also performed with murine MIL-38 (**Figure 2C**). When a three antibody detection method was used, detection was far less sensitive than using the standard two antibody method (for the Western blots shown in **Figures 2A** and **C**, exposure time used for **Figure 2A** was 30 seconds, whereas that used for **Figure 2C** was 30 minutes).

As shown in **Figure 2B**, the Chimeric MIL-38 recognises the recombinant GPC-1 NS0 antigen and shows comparable reactivity to murine MIL-38 when detected using this method (compare **Figures 2B** and **C**).

2.3 Discussion

The Chimeric MIL-38 antibody was successfully expressed and purified in suspension CHO-3E7 cells. The H and L chains of target antibody were detected with estimated molecular weights of ~55 kDa (Cal.M.W. ~52 kDa) and 28 kDa (Cal.M.W. ~26 kDa) based on SDS-PAGE and Western blot analysis.

Equivalent reactivity between the chimeric MIL-38 and the murine parent was observed in IFA and western blotting, indicating that binding specificity has been maintained in the construction of the chimeric antibody.

Example 3: preparation and single chain variable fragment (scFv) MIL-38 antibody**3.1 Materials and Methods***- Preparation of scFv antibody*

An optimised cDNA sequence was developed for cloning purposes. This was based on the AM-4 Heavy chain and Light chain consensus sequences identified above in **Example 1**. The heavy chain variable region was positioned at the N-terminus and linked to the light chain variable region using a flexible to serine/glycine linker. The scFv protein incorporated a C-terminal tag consisting of six histidines (hexa his) to facilitate purification of the recombinant protein and a C-terminus cysteine residue to facilitate Cys-based labeling.

An optimised cDNA sequence was used to generate a mouse-human chimeric heavy chain amino acid sequence:

scFv MIL-38 amino acid sequence (SEQ ID NO: 9)

MCWSCIILFLVATATEVHSQIQLVQSGPELKKPGETVKISCKASGYAFTDYSMTWVKQAPGKGLRWMGWINT
ETGEPTTYTDDFKGRFAFSLETSASTAFLQINNLRNEDTATYFCARHYDYGGEPTWGGTLVTVSSGGGSGG
GGGGGGSDIQMTQSPASLSASVGETVTITCRASGNVHNYLAWYQOKQKSPQLLVYTAKTLAGVPSRFSG
SGSGTQYSLKINSLOPEDFGTYCQHFWSENPWFEGGGTKLEIKHHHHHC*

Individual regions of mouse-human chimeric heavy chain sequence are indicated in the amino acid sequence above: Positions 1-19 = leader sequence; 20-49 = framework region (HFR1); 50-54 = complementarity determining region 1 (HCDR1); 55-68 = HFR2; 69-85 = HCDR2; 86-117 = HFR3; 118-126 = HCDR3; 127-137 = HFR4 (also called the joining region or J-region); 138-152 = gly/ser flexible linker sequence, 153-175 = framework region (LFR1); 176-186 = complementarity determining region 1 (LCDR1); 187-201 = LFR2; 202-208 = LCDR2; 209-240 = LFR3; 241-249 = LCDR3; 250-259 = LFR4; 260-265 = Hexa His tag & stop codon (*). Linker sequence – Gly/ser linker sequence is underlined above.

ScFv MIL-38 was transiently expressed in CHO cells followed by one-step purification utilizing the hexa-histidine tag and a nickel affinity column.

- *FLOW cytometry*

Murine 1F5 MIL-38 antibody or the scFv MIL-38 construct were labelled with CY5 fluorophor using standard methods. These antibodies were then used to stain DU-145 prostate cancer cells.

5 Briefly, DU-145 cells were detached using PBS/2mM EDTA. Following centrifugation, cells were incubated with antibodies or control solutions in FACS wash solution (PBS/5% fetal calf serum) on ice for 45 min. Following washing with FACS wash solution, cells were resuspended in PBS then assessed for cell binding using FLOW cytometry.

10 To examine the specificity of binding, DU-145 cells were pre-incubated with unlabelled murine MIL-38 antibody prior to incubation with CY5-labelled MIL-38.

Figure 3A shows FLOW histograms of DU-145 cells alone or with a CY5-labelled isotype control. **Figure 3B** show FLOW histograms of DU-145 cells that were either untreated or labelled with either CY5-labelled MIL-38, or cells that had been pre-
15 incubated with unlabelled MIL-38 prior to incubation with CY5-labelled MIL-38. Specificity of binding is indicated by the lower fluorescence intensity seen for the cells pre-incubated with CY5-labelled MIL-38. **Figure 3C** show FLOW histograms of DU-145 cells that were either untreated or labelled with either CY5-labelled MIL-38, or CY5-labelled scFv MIL-38.

20

3.3 Discussion

The MIL-38 antibody was successfully converted to an scFv format. Binding to DU-145 cells was lower than the parent MIL-38 murine antibody.

25 **Example 4: Internalisation of murine 1F5 MIL-38 and chimeric MIL-38 antibody in DU-145 prostate cancer cells**

4.1 Materials and Methods

- *FLOW cytometry of MDA-MB-231, T-24, PANC-1 or DU-145 cells*

30 Cells were detached with PBS-EDTA then incubated with MIL-38 primary antibody on ice for 45 min in FACS wash buffer (PBS +5% FCS). Cells were washed three times in FACS wash buffer then incubated with AlexaFluor 488 secondary antibody for 30 min on ice in the dark. Following washing 3 times with FACS wash buffer cells were transferred to FACS tubes and assayed using FLOW cytometry.

35

- *Antibody internalisation assay*

MDA-MB-231 or DU-145 cells were seeded in 8-well chamber slides (5) and grown for 24 hours in media containing 10% FCS. Cells were changed to serum free medium for 36 hours. Slides were put on ice, the medium was removed and 200µL of 10ug/mL of cold antibody solution in serum free medium were added per well. Cells were incubated for 30 minutes on ice, then washed once with cold serum free medium, 200µL of serum free medium were added per well, and cells were incubated at 37°C, 5% CO₂ for 0, 5, 15 and 30 min. The medium was then removed and cells were fixed with 3% PFA in PBS for 15 min at room temperature. After fixation, cells were washed once with PBS and permeabilised with cold Triton X-100 0.1% in PBS for 5min. Cells were then washed with PBS to remove excess of detergent, and then blocked with 5% skim milk in Tris Buffered Saline (TBS, pH 7.5) for 30 min. The corresponding secondary antibody (goat anti-mouse-Alexa488 and goat anti-human-Alexa488) was diluted (4µg/mL and 2 µg/mL respectively) in PBS. 200uL/well to the secondary antibody solution was added per well, and incubated for 45min at room temperature in the dark. Cells were washed once with PBS, then incubated with 200µL per well of 1µg/mL Hoechst (nuclear counterstaining) for 5 minutes, then washed twice with PBS. Chambers were removed, and slides were mounted using 70% glycerol in water as mounting media.

For T-24 cells, cells were seeded in 8-well chamber slides and grown for 3 days, until they reached about 50% confluency. Cells were incubated for 72 hours in serum free medium. Slides were then processed as described above for MDA-MB-231 and DU-145 cells.

For the PANC-1 timecourse, cells were treated as described above for DU-145 cells. Duplicate slides were made, and the cells fixed following 15 min or 60 min at 37°C.

4.2 Results

Figure 4A shows FLOW cytometry histograms for MDA-MB-231 and controls (no primary antibody or isotype only), while **Figure 4B** shows relative reactivity of the 1F5 MIL-38 murine antibody to MDA-MB-231, T-24 or DU-145 cells. **Figure 4C** shows reactivity of the 1F5 MIL-38 murine antibody to PANC-1 cells.

Figure 5A shows localization of the murine MIL-38 antibody (green) following 30 min of incubation with DU-145 cells. Cell nuclei are shown in blue. **Figure 5B** shows localization of the chimeric MIL-38 antibody (green) following 30 min of incubation with DU-145 cells. Cell nuclei are shown in blue. **Figure 5C** shows lack of binding or internalisation of chimeric MIL-38 following 30 min of incubation with MDA-MB-231

cells. **Figure 5D** shows localization of the chimeric MIL-38 antibody (green) immediately following incubation with T-24 cells. Cell nuclei are shown in blue. **Figure 5E** shows localization of chimeric MIL-38 antibody (green) in PANC-1 cells after 15 minutes during a 60 minute timecourse. **Figure 5F** shows internalization of anti-human antibody labelled with Alexa-Fluor 488 in PANC-1 cells after completion of the 60 minute timecourse.

4.3 Discussion

MIL-38 antibodies bind to the GPC-1 heparan sulfate proteoglycan antigen (**Figures 1 and 2**). GPC-1 is expressed on the surface of prostate cancer cells (**Figure 1**) and has also been reported to be expressed on a variety of other cancer cell lines including ovary and bladder (Walker et al 1989, Russell et al 2004) and breast (Matsuda et al 2001). GPC-1 has also been reported to internalise and recycle to the cell surface via caveolae and endosomal trafficking in T-24 bladder cancer cells (Mani et al 2006).

GPC-1 may therefore be able to transport bound MIL-38 antibodies into the cell via these trafficking mechanisms to enable delivery of toxic moieties such as drugs or radionuclides. The target of the BLCA-38 antibody has recently been identified as GPC-1 (PCT application no: PCT/AU2015/000018). However, the BLCA-38 antibody (a parent cell line to the 1F5 murine MIL-38 antibody) has been reported as not undergoing internalisation (Khatri 2010). BLCA-38 cells were recently shown to be bi-clonal with only one cell population recognising GPC-1 (PCT/AU2014/000999). The 1F5 murine MIL-38 subclone and the chimeric MIL-38 antibody both recognise GPC-1, however their internalization has not to date been investigated in a range of cell types.

DU-145 prostate cancer cells show higher antibody binding than MDA-MBA-231 breast cancer cells or T-24 bladder cancer cells and chMIL-38 is also able to bind PANC-1 cells (**Figure 4**). Both murine MIL-38 monoclonal antibody and the chimeric MIL-38 antibody were able to internalise into DU-145 cells with strongest internalization seen at 30 min (**Figure 5A and B**). T-24 cells also demonstrate internalization of the chimeric MIL-38 (**Figure 5D**), however MDA-MB-231 cells do not show detectable binding or internalization of MIL-38 under these conditions (**Figure 5C**) despite expressing the MIL-38 antigen (**Figure 4**). PANC-1 cells show binding of MIL-38 to the cell membrane at 15 min post incubation, then almost complete internalisation of the antibody 60 min after incubation (**Figure 5E and F**).

GPC-1-mediated internalisation of MIL-38 antibodies therefore depends on more than expression of the MIL-38 antigen and may depend on the level of expression or the

cell type. The role of cell type is supported by a recent report describing alterations of endosomal function in prostate cancer compared to normal prostate (Johnson et al 2014 Mol Cancer Res. 2014 Dec;12(12):1851-62).

The chimeric MIL-38 antibody was internalised after binding GPC-1 on the cell
5 surface of either prostate, bladder or pancreatic cancer cell lines. The strong internalisation of chMIL-38 indicates its suitability as an antibody drug conjugate (ADC).

Example 5: Targetting of MIL-38 antibodies to DU-145 xenografts

10 5.1 Materials and Methods

- Xenograft model

Nude mice were injected subcutaneously in the left flank with 1×10^6 DU-145 cells. The cells were allowed to grow for approximately four weeks, then 100µl of 5µM Cy-5-
15 labelled antibody in PBS was injected i.v. into the tail vein. Optical images were taken 24hrs post injection using a Bruker MS-FX Pro scanner. Three mice were injected with Cy-5 labelled 1F5 murine MIL-38 and three mice were injected with Cy-5 labelled chimeric MIL-38.

20 5.2 Results

Both 1F5 MIL-38 (**Figure 6A**) and chimeric MIL-38 (**Figure 6B**) demonstrated strong targeting to the DU-145 xenografts in each of the three mice tested per group, with minimal off target binding. Some minor accumulation was also noted at the ear puncture marks, possibly due to inflammation caused by the puncture wound (note no localisation
25 to the unpunctured ears).

5.3 Discussion

Previous data using radio-conjugated BLCA-38 indicated targeting to prostate cancer xenograft (Carter et al 1994), however due to the biclonal nature of the BLCA-38
30 antibody it is unclear if the 1F5 clone would show similar targeting or whether the AM3-like clone is responsible for the tumour localization seen.

The results shown in **Figures 6A** and **6B** indicate that both the 1F5 and chimeric forms of MIL-38 localise to DU-145 tumour xenografts, implying that the 1F5 antibody clone isolated from BLCA-38 mixed population is responsible for tumour targeting and

that the second form of antibody present in the BLCA-38 stocks is not required for tumour localization.

The tumour-specific localization of the 1F5 and chimeric MIL-38 antibodies suggests that they would be good agents to deliver anti-prostate cancer therapies such as antibody drug conjugates or radio-immunotherapies.

Example 6: Cell growth inhibition assays

6.1 Materials and Methods

10 - Cell growth inhibition assay

This assay is designed to screen antibodies that may internalise and deliver toxins to cells resulting in cell growth inhibition and/or cell death. Antibodies that induce cell growth inhibition are candidates to convert to an antibody-drug conjugate (ADC) format with the toxin payloads identified.

15 The assay involves first the incubating the test antibody and target cells in the presence of Protein G that has been linked to different toxins suitable for use in ADC technology. An antibody dose response curve is established with fixed final concentration of protein G-toxin.

The following protein G combinations were chosen:

20 **Table 3: protein G combinations**

Preloaded Protein G	Linker	Working Concentration
PSGD (preloaded with DM1)	Non-cleavable	10 nM
PSGE (preloaded with MMAE)	Cleavable	50 nM
PSGF (preloaded with MMAF)	Non-cleavable	100 nM
PSGDuc (preloaded with Duocarmycin)	Cleavable	50 nM

The assay format was performed as follows:

25 Cells were plated in 96 well at 20-30 % confluency in 100µl culture medium then incubated overnight. Antibodies were serially diluted, starting from the concentrations indicated in the graph at 19:60 ratio, then added to the 96 well plate containing cells.

Preloaded protein G-drug conjugate was added to treated wells at concentration indicated above. Cells were then incubated for 3 days, then measurement of cell viability was performed using CelltitreGlo.

5 The following antibodies were tested:

a. Chimeric MIL-38 (human IgG1)

b. Murine 1F5 MIL-38 (murine IgG1, high GPC-1 binding subclone of BLCA-38)

10 c. Murine BLCA-38 (murine IgG1, mixed population consisting of 1F5 – type and AM3 type antibodies)

d. Murine AM3 (murine IgG, low GPC-1 binding subclone of BLCA-38)

e. Erbitux (control human IgG1 chimeric anti-EGFR antibody)

15 6.2 Results

Two cell lines were chosen for inclusion in the assay DU-145 and MDA-MB-231. Both are GPC-1 positive, but exhibit different levels of MIL-38 reactivity and internalization. The antibodies chosen represent a range of different characteristics:

20 Table 4: antibodies used

Antibody	DU-145		MDA-MB-231		Isotype
	Binding	Internalisation	Binding	Internalisation	
Chimeric	++++	++++	Not tested	-	Human IgG1
BLCA-38	++++	Negative*	++	Not tested	Murine IgG1
AM3	-/+	Not tested	Not tested	Not tested	Murine IgG1
1F5	++++	++++	++	Not tested	Murine IgG1
Erbitux	Positive control	Assumed positive	Positive control	Assumed positive	Human IgG1

*BLCA-38 is reported not to internalise (Khatri et al 2010).

MDA-MB-231 cells were insensitive to all anti-GPC-1 antibody/toxin combinations (**Figures 7A to 7D**), whereas anti-EGFR/toxin complexes were able to inhibit growth of MDA-MB-231 cells (**Figure 7E**). The MDA-MB-231 cells required internalisation of toxins for growth inhibition as evidenced by lack of inhibition with Erbitux alone (**Figure 7E**). It is unclear if the insensitivity of MDA-MB-231 cells to anti-GPC-1 antibodies was due to the expression level of GPC-1, the ability of the GPC-1/anti-GPC-1/Protein G toxin complex to internalise or differential processing for anti-GPC-1 vs anti-EGFR complexes.

DU-145 cells were sensitive to all the toxins used in this assay when targeted by Erbitux, but showed no inhibition with Erbitux alone (**Figure 7E**). Chimeric MIL-38 was able to induce growth inhibition in DU-145 cells with PSG preloaded with MMAE and MMAF toxins but not Duocarmycin or DM1, demonstrating that the particular toxin involved also had an effect (**Figure 7A**). In contrast, 1F5 MIL-38, BLCA-38 and AM3 were unable to induce cell growth inhibition in these assays.

The concentrations of protein G-duocarmycin used in experiments depicted in **Figures 7A-7E** proved to be toxic to the DU-145 cells even in the absence of antibody, therefore a titration was performed to determine an appropriate lower concentration for DU-145 cells (**Figure 7F** panel 1). Erbitux was able to induce cell growth inhibition in a dose-dependent manner under these conditions, but none of the MIL-38 antibodies were able to cause growth inhibition under these conditions.

Internalisation of the antibody alone does not predict growth inhibition: the chimeric MIL-38 and the 1F5 murine MIL-38 internalise to equivalent levels (**Figures 5A and B**). Growth inhibition is observed for chimeric MIL-38 (**Figure 7A**) yet no growth inhibition is observed with 1F5 murine antibody (**Figure 7D**).

6.3 Discussion

A number of key questions were assessed in these assays:

1. If a cell line expresses the GPC-1 antigen (regardless of expression level) it will be sensitive to anti-GPC-1 antibodies?
2. If an antibody internalises it will be able to cause growth inhibition via internalization of a GPC-1/anti-GPC-1 antibody/protein G toxin complex?

3. Equivalent levels of antibody internalization should lead to equivalent growth inhibition.

4. Relative sensitivity of a cell line/antibody/toxin combination.

5

MIL-38-derived antibodies display a distinct activity profile compared to the positive control in these assays (Erbix). Firstly chimeric MIL-38 in combination with the toxins tested shows no activity towards the breast cancer cell line MDA-MB-231 despite expression of GPC-1 on this cell line. In contrast Erbix is active against MDA-MB-231 cells with all toxins tested.

Secondly, chimeric MIL-38 shows growth inhibition with MMAE and MMAF toxins for DU-145 cells, whereas murine 1F5, AM3 or BLCA-38 shows no activity. This is particularly unexpected in the case of 1F5 where equivalent internalization has been demonstrated (**Figures 5A and 5B**), suggesting that the differential activity observed is due to the chimeric framework.

It is clear from these results that internalization of either the antigen alone or the antibody do not predict the potential activity of given antibody/antigen complex and that empirical data must be obtained to determine the potential utility of a given molecule.

The observation that MDA-MB-231 cells that express the lowest levels of GPC-1 antigen do not show cell growth inhibition with chimeric MIL-38 despite expressing the GPC-1 antigen suggests that an ADC version of the chimeric MIL-38 may be able to target prostate cancer cells specifically and not affect other tissues with low GPC-1 antigen expression. The identification of growth inhibition with chimeric MIL-38 but not 1F5 suggests that the chimeric format provides unexpected advantages for the deployment of ADC-based strategies.

Example 7: Development of a scalable chimeric expression system

7.1 Materials and Methods

- Cell line development

Production of sufficient chimeric antibody for clinical trials and ultimately commercial supply requires a production system capable of high antibody expression and good recoveries following purification. Production of the chimeric antibody described in Example 1 was performed using transient expression, a technique unsuitable to large scale

GMP production. Therefore a suitable expression/purification system was developed with the following characteristics:

1. Use of an established gene transduction technology.
2. Use of a cell line of sufficient quality and lineage suitable for clinical grade
5 production
3. Use of cell growth medium, bioreactors and purification techniques suitable for scalable GMP production of final product.

Catalent was selected as the manufacturer due to their extensive experience with
10 GMP production of antibodies and their proprietary GPEX retrovector expression technology.

A stable cell pool was developed using the chimeric MIL-38 heavy and light chains coding sequences incorporated into Catalent's GPEX expression retrovectors.

Following selection, a stable pool was established and used for 25L scale up
15 production of chimeric MIL-38. The cell supernatant was purified using processes suitable for scalable production.

7.2 Results

A stable pool was established and used for an early stage production run. A 25L
20 production run was performed as an 18-day fed-batch using the stable cell pool. Viable cell density (**Figure 8A**), cell viability (**Figure 8B**) and antibody production (**Figure 8C**) showed good performance with expression levels of 0.8 g/L obtained at this early stage of development.

The supernatant harvest from the fed batch was clarified, then purified using
25 scalable techniques used for antibody purification, host cell protein and virus reduction (**Table 5**). Yield of approximately 50% were obtained using non-optimised purification, indicating that a scalable process had been developed.

Table 5: yields and recovery during purification of chimeric MIL-38.

Step	Volume (L)	Conc. (mg/mL)	Total (g)	Step Yield (%)	Overall Yield (%)
Harvest	25.0	0.81	20.3	100	100
Clarification	24.7	0.77	18.9	93	93
Protein A / Low pH Hold	2.4*	6.6	15.9	84	78
Q Flow through	2.22	5.2	11.5	94	73
CEX Bind and Elute	0.50	16.1	8.0	75**	55
Viral Filtration	0.77	9.2	7.1	89	49
Formulation	0.78	9.3	7.3	103	50

7.3 Results

A stable CHO cell pool expressing chimeric MIL-38 was established using the
 5 GPEX technology and used for an early stage production run. Good expression was
 observed during the initial fed batch production run and good recoveries were achieved
 using standard purification processes known to be suitable for GMP production.

The stable pool will form the basis for future cell line development, incorporating
 clone isolation and master and working cell bank construction. The purification process
 10 used can be further optimized to increase yields.

Use of well established cell lines, expression vectors, cell growth systems and
 purification techniques means that the final antibody product obtained from the 25L pilot
 batch should possess very similar characteristics to that produced from the final
 production process. The yields obtained to date indicate that the product can be produced
 15 at costs consistent with other marketed antibody products.

Example 8: Development of a chimeric MIL-38 DOTA conjugate for imaging and radiotherapy

5

8.1 Materials and Methods

- *Conjugation of MIL-38 with DOTA for ^{177}Lu labeling*

DOTA was conjugated to chimeric MIL-38 at a 10:1 ratio as follows. p-SCN-Bn-DOTA was prepared in 0.1M PBS (pH 8.5) buffer, then chimeric MIL-38 (cMIL-38) was added to the mixture. The mixture was allowed to react for 20 hours at room temperature in the dark.

The conjugated mAb mixture was purified using a pre-conditioned mini PD-10 gel column. Fractions were screen fractions for activity by HPLC.

15 - *Labeling of MIL-38 with ^{177}Lu*

0.472.1 mCi of ^{177}Lu in 10 μL of 0.05N HCl was added to 0.2 mg of DOTA-cMIL-38, then the pH adjusted to pH 7.5. The mixture was incubated at 50°C for 60 min in the dark, then cooled to room temperature and let sit for 20 min.

The radiolabelled MIL-38 DOTA was purified using a pre-conditioned mini PD-10 gel column. The column was preconditioned with 2mL of 1x PBS and the effluent discarded. This was repeated four times in total, then the radioactive solution was added to the centre of the column. 0.2 ml of PBS was added and the fraction collected in a low binding Eppendorf tube. This was repeated another 19 times, then the activity in each fraction was determined using a dose calibrator. A sample of the radioactive mAb was analysed using radio-HPLC for confirmation analysis.

25

- *Mock labeling of MIL-38 DOTA and FLOW cytometry*

To confirm that binding of chimeric MIL-38 to DU-145 cells was retained following conjugation with DOTA and after the radio-labeling process, MIL-38 DOTA was put through a “mock” radiolabeling process in which it was incubated with the same buffers and exposed to the same temperatures that are used during the radio-labeling process described above. The PD-10 column purification steps were omitted.

30

The chimeric MIL-38, MIL-38 DOTA and mock-labelled MIL-38 DOTA were then each used as the primary antibody for FLOW cytometry analysis.

35

- *Conjugation of chMIL-38 with DOTA for scale up manufacture and ^{67}Ga labelling*

Conjugation of chMIL-38 to p-SCN-Bn-DOTA (DOTA) was undertaken following the method outlined by Forrer et al. titled “in vitro characterization of ^{177}Lu -radiolabelled chimeric anti-CD20 monoclonal antibody and a preliminary dosimetry study”. Three conditions were used: 5, 10 and 20 fold excess of DOTA. Briefly, three vials of chMIL-38 (9.3mg/ml) were combined and buffer exchanged from 0.01M PBS to 0.2M Na_2CO_3 , pH9.5. The chMIL-38 solution was then concentrated to a final volume of 433 μl , giving an effective concentration of 64.4 mg/ml. The material was split into three 144 μl aliquots then reacted with DOTA (5, 10 or 20 equivalents). Each reaction mixture was maintained at 37°C for 1 hour. Samples were then washed with 5 ml of Na_2CO_3 buffer pH 9.5 then buffer exchanged with 0.25M ammonium acetate, pH7.

Mass spectral analysis indicated that the number of DOTA attached was 4.3 DOTA: MAb (5 fold reaction excess), 4.6 DOTA: MAb (10 fold reaction excess) and 7.5 DOTA: MAb (20 fold reaction excess).

- *^{67}Ga labeling of chMIL-38 DOTA*

^{67}Ga -labeling of chMIL-38 DOTA was performed using the product generated from the 20 fold reaction excess containing 7.5 DOTA:MAb ratio. Reaction conditions: 50 μl $^{67}\text{GaCl}_3$ in 0.02M HCl, 100 μl 0.1M NaOAc buffer at pH 5.0, chMIL-38 DOTA: 5 to 25 μl of antibody (46-230 μg) was used. The mixture was incubated at 40°C for 1 or 16 hours. The radiochemical yield and derived specific activity were calculated.

- *Assessment of DOTA conjugation and labelling conditions on MIL-antigen binding by FLOW cytometry and direct binding ELISA*

To confirm that the reaction conditions used to conjugate DOTA to chMIL-38 using 5, 10 or 20 fold excess of DOTA did not significantly alter binding of chMIL-38 DOTA to GPC-1 two assays were used: FLOW cytometry and a direct binding ELISA. FLOW cytometry was performed as follows. DU-145 or T-24 cells were detached using 2mM EDTA (10 min at 37°C). Cells were pelleted, and then 2×10^5 cells per tube were prepared. Cells were incubated with primary antibody (1 $\mu\text{g}/\text{ml}$, 50 μl) on ice for 45 min, then washed three times in FACS wash buffer (1 x PBS solution). Cells were then incubated with 50 μl of secondary antibody (1 $\mu\text{g}/\text{ml}$ AlexaFluor 488 goat anti- human (H+L)) for 45 min. Cells were washed three times in FACS wash buffer then run through BD LSRFortessaTM – Cell analyser using software BD FACSDIVATM.

The GPC-1 direct binding ELISA was performed as follows. 96 well plates were coated with 10mM Carbonate buffer, pH 9 for 15 mins. The carbonate buffer was removed and wells were coated overnight at room temperature with 1.5 µg/ml recombinant human GPC-1 (R&D systems) in 10mM Carbonate buffer, pH 9. The following day wells were washed with 5mM carbonate buffer, pH 9 then blocked with 10% casein in PBS for 1 hours. The primary antibody was serially diluted starting at 25 ng/mL to 0.39 ng/mL then incubated 1 hours at room temperature. Plates were then washed four times with PBS-Tween 20 (0.1%). Secondary antibody Thermofisher rabbit anti-human was diluted 1:8000 in PBS-Tween, 10% casein then incubated for 1 hours at room temperature. Plates were washed four times with PBS-Tween and 100 µl TMB added. Plates were developed for 2 min and read at λ_{450} nm.

8.2 Results

Figure 9A shows an A280 absorbance trace of gel filtration standards used to calibrate the HPLC. **Figure 9B** shows an A280 absorbance trace of un-conjugated chimeric MIL-38. **Figure 9C** shows an A280 absorbance trace of chimeric MIL-38 following DOTA conjugation and PD-10 column clean up. **Figure 9D** shows a radio-HPLC trace of ^{177}Lu activity following labeling of MIL-38 DOTA with ^{177}Lu and PD-10 column clean up. **Figure 9E** shows the A280 absorbance trace corresponding to **Figure 9D**.

Using the conjugation, labeling and purification procedures described above, a single peak of ^{177}Lu radiolabelled chimeric MIL-38 DOTA is obtained.

Figure 10 shows the FLOW histograms for chimeric MIL-38, chimeric MIL-38 DOTA and mock-labelled chimeric MIL-38 DOTA. DOTA conjugation slightly reduces the Fluorescence signal observed compared to untreated chimeric MIL-38. Mock labelling does not affect the fluorescence detected when compared to MIL-38 DOTA.

Alternate conjugation conditions were used for scale up production of chMIL-38 DOTA and for labelling with ^{67}Ga . ^{67}Ga has a half-life of ~3.3 days and is therefore a better isotope to predict the *in-vivo* biodistribution of chMIL-38 DOTA-labelled chMIL-38 than isotopes such as ^{68}Ga (half life ~68 min).

Figure 11 shows labeling of DOTA-conjugated MIL-38 with ^{67}Ga . **Figure 11A** shows a size exclusion chromatography HPLC chromatogram of the radioactivity of MIL-38 labelled with ^{67}Ga . The star indicates free ^{67}Ga remaining in the reaction mixture. **Figure 11B** shows a stability study of ^{67}Ga -labelled MIL-38 DOTA. The size exclusion chromatography HPLC chromatogram of the radioactivity of MIL-38 labelled with ^{67}Ga

is shown together with the corresponding UV trace. The ^{67}Ga -MIL-38 preparation had free ^{67}Ga removed, then was allowed to remain at room temperature for two weeks prior to undergoing SEC HPLC chromatograph. While there is some detachment of the ^{67}Ga from the chMIL-38 DOTA (indicated by the second peak at ~10 min elution time), the majority of the antibody and radioactivity remained intact under these conditions indicating good stability.

To maximise specific activity of the ^{67}Ga -labelled chMIL-38 DOTA, the effects of the ratio of chMIL-38 DOTA to $^{67}\text{GaCl}_3$ and incubation time (1 hours vs 16 hours) were examined. As shown in Table 6 the amount of antibody in the reaction mix was the most important variable in determining radiochemical yield (RC) and the derived specific activity (SA).

Table 6. Effects of antibody mass and incubation times on radiochemical yield and derived specific activity for chMIL-38 labelling with ^{67}Ga

Reaction ID	Reaction Time (h)	^{67}Ga Activity (kBq)	Mass of Antibody (ng)	RC Yield (%)	Derived SA (MBq/mg)
NG107104-1	1	40.4	230	98	170
NG107104-2	1	39.5	92	79	340
NG107104-3	1	37.4	46	72	585
NG107104-4	16	37.8	230	97	136*
NG107104-5	16	38.1	92	83	293*
NG107104-6	16	38.1	46	84	594*

* Decay corrected value (16h decay, x0.853)

As the conditions used for conjugation of DOTA to chMIL-38 and for labelling with ^{67}Ga labelling may affect the binding activity a series of experiments was designed to examine the effects of these procedures on chMIL-38 binding to the GPC-1 antigen in two distinct assays: FLOW cytometry (targeting the cell-bound antigen) and a direct binding ELISA (targeting a surface bound recombinant form of GPC-1).

The effects of the labelling conditions were determined by "mock-labeling" as described previously. As well, the intra- and inter-assay reproducibility of these assays was assessed.

Figure 12 shows binding of chimeric MIL-38, MIL-38 DOTA prepared using three different DOTA conjugate ratios and mock radio-labelled MIL-38 DOTA to DU-145 or T-24 cells using FLOW cytometry, as well as binding in a direct antigen binding ELISA. **Figure 12A** shows binding of un-labelled chimeric MIL-38 or chimeric MIL-38 that had

been conjugated to DOTA using either a 5, 10 or 20 fold molar excess of DOTA. Essentially equivalent binding curves were obtained indicating that the DOTA conjugation did not cause a reduction in MIL-38 binding to DU-145 cells. **Figure 12B** shows FLOW cytometry binding to T-24 cells of three separate batches of chMIL-38 DOTA (20 fold excess) that had each undergone a mock-labelling reaction. Essentially identical binding curves were observed indicating good inter-batch reproducibility. **Figure 12C** shows FLOW cytometry binding to T-24 cells of three separate FLOW reactions of chMIL-38 DOTA (20 fold excess) all prepared from one batch that had undergone a mock-labelling reaction. Essentially identical binding curves were observed indicating good intra-batch reproducibility. **Figure 12D** shows overlaid FLOW cytometry profiles of binding to T-24 cells for secondary antibody alone (red trace), chMIL-38 DOTA (green trace), one sample from the inter-batch reproducibility (Batch 1, blue trace) and one sample from the intra-batch reproducibility (Intra 1, orange trace). Essentially identical binding curves were observed indicating that the mock-labelling process did not affect binding to cells compared to the chMIL-38 DOTA control. **Figure 12E** shows binding of chMIL-38 and chMIL-38 that had been conjugated to DOTA using either a 5, 10 or 20 fold molar excess of DOTA to recombinant GPC-1 in a direct binding ELISA. **Figure 12F** shows direct binding to recombinant GPC-1 of chMIL-38 DOTA, the 3 different preparations of mock-labelled chMIL-38 DOTA prepared for the inter-batch comparison and 3 different reactions from the intra-batch preparation.

The conjugation of DOTA to chMIL-38 reduced its binding to GPC-1 in the direct assay compared to the un-conjugated chMIL-38, although this effect was only observed at lower antibody concentrations and was not dependent on the DOTA ratio used in the experiment or the number of DOTA molecules attached (**Figure 12E**). The mock-radiolabelling conditions tested did not affect the binding of chMIL-38 DOTA compared to chMIL-38 DOTA that was not subjected to mock labelling and the assay showed good inter and intra-batch reproducibility (**Figure 12F**).

8.3 Discussion

A method has been successfully developed to conjugate chimeric MIL-38 with the DOTA chelation molecule. A chimeric MIL-38 DOTA antibody can be labelled with agents such as ^{67}Ga for imaging, or ^{177}Lu for imaging and radio immunotherapy treatment. Other agents such as Gadolinium or ^{90}Y trium can also be chelated with the DOTA chelator.

FLOW cytometry and direct binding data indicates that the conjugation and labeling processes have minimal effect on the ability of chimeric MIL-38 to bind DU-145 cells.

Example 9: Chimeric MIL-38 localization in a mouse xenograft model

5

9.1 Materials and Methods

- *Antibody labelling and internalisation*

Chimeric MIL-38 was labelled with the Cy5 fluorophor for visualisation. Retention of binding activity post labelling was confirmed by FLOW cytometry using DU-145 cells.

10 ChMIL-38 Cy5 demonstrated clear internalisation into DU-145 cells.

- *Xenografts and antibody infusions*

Three 8-week-old male Balb/c nude mice were injected subcutaneously with 5×10^6 DU-145 cells in 100 μ L serum free media (RPMI) into the right flank of each mouse. At 15 26 days all mice had palpable tumours (approximately 4 mm across). Antibody solution (approx. 150 μ g/injection) was injected via the tail vein and then imaged using the Bruker In-Vivo Xtreme Optical Scanner at 0, 4 hours, 24 hours, 48 hours, 72 hours and 144 hours.

20

9.2 Results

Tumour localisation was seen at 4 hours in two of three mice and was clearly evident at 24 hours post infusion in all three mice tested (**Figure 13A**). Tumour localisation was maintained at 48 and 72 hours while non-tumour signal decreased. Optimal tumour to background signal was observed at 144 hours at which time 25 background signal had cleared. The chMIL-38 Cy5 infusions were well tolerated. **Figure 13B** shows a Multi-modal Animal Rotation System (MARS) image of a representative mouse from **Figure 13A** after 144 h.

Example 10: Biodistribution study of ^{177}Lu DOTA MIL-38

30

10.1 Materials and Methods

- *Biodistribution study*

15 Male Wistar rats were injected at T_0 with 5 planned exsanguination time points for dissection and measurement of key organ radioactivity accumulation. Planned time 35 points were 6 hours, 24 hours, 48 hours, 7 days and 14 days.

DOTA conjugated MIL-38 antibody, supplied by Singapore Radiopharmaceuticals was radiolabelled with ^{177}Lu and passed bench top quality assurance testing.

10.2 Results

A standardised plot of % of injected activity per gram of organ at the different timepoints (**Figure 14**) indicates a safe radioimmunotherapy profile without evidence of toxic accumulation in analysed tissues and organs. Of note, key excretory organs (liver, kidneys) do not display evidence of toxic radioactive accumulation (**Figure 15**).

No adverse events were noted with the test animals.

10.3 Discussion

^{177}Lu -labelled chimeric MIL-38 DOTA showed good tolerability with no evidence of toxic accumulation in tissues or organs, in particular excretory organs. The agent was well tolerated with no adverse events noted.

These results in normal rats combined with the tumour targeting seen with fluorescently labelled chimeric MIL-38 (**Figures 6 and 13**) suggest that the MIL-38 DOTA conjugate is likely to have applicability for both imaging and radioimmunotherapy of prostate cancer.

Example 11: PET imaging study of ^{64}Cu chMIL-38 DOTA and NOTA in xenografts

11.1 Materials and Methods

The aim of this study was to examine *in vivo* targeting of radio-labelled chMIL-38 to DU-145 xenografts using PET-CT and ex vivo organ analysis. As neither ^{67}Ga nor ^{177}Lu can be used for PET-CT imaging ^{64}Cu was used as the radiolabel. NOTA is the preferred chelator for ^{64}Cu , hence chimeric MIL-38 was also conjugated to the NOTA chelator and ^{64}Cu was used as the imaging isotope. Imaging was performed using a Siemens Inveon PET-CT scanner. Both ^{64}Cu chMIL-38 NOTA and ^{64}Cu chMIL-38 DOTA were used for imaging and biodistribution purposes.

8-week-old male Balb/c nude mice were injected subcutaneously with 5×10^6 DU-145 cells in 100 μL serum free media (RPMI) into the right flank of each mouse. There was no evidence of ulceration at the time of dosing, the animals were closely monitored and remained in good condition apart from the tumours. At 38 days all mice had palpable tumours (approximately 4 mm across). Antibody solutions were injected via the tail vein

at 54 days post inoculation and then imaged using the Siemens Inveon PET-CT instrument. Two mice were infused with ^{64}Cu chMIL-38-DOTA and three mice with ^{64}Cu chMIL-38-NOTA. Each mouse received approximately 100 μg of chMIL-38 with activities of 3.1-3.4MBq (chMIL-38 DOTA) or 4-4.8MBq (chMIL-38 NOTA).

5 The PET Images were reconstructed using an ordered-subset expectation maximization (OSEM2D) algorithm and analysed using the Inveon Research Workplace software (IRW 4.1) (Siemens) that allows fusion of CT and PET images and definition of regions of interest (ROIs). CT and PET datasets of each individual animal were aligned using IRW software (Siemens) to ensure good overlap of the organs of interest.

10 Mice were imaged at 24 hours and 48 hours, then the major organs were removed from the mice and the amount of antibody present was measured by γ -analysis.

11.2 Results

In vivo imaging showed good tumour accumulation and long term localisation (over 2 days) for the chMIL38 NOTA antibody as was observed for the Cy5-labelled chMIL-38 (Figure 16). There was far higher binding and accumulation in the tumour for the NOTA-labelled antibody compared to the DOTA-labelled antibody (Figure 17). This may be due to either instability of the chelator *in vivo* (for the case of the DOTA), chelator-induced non-specific uptake in other organs, or loss of ^{64}Cu from the DOTA due to the lower binding efficiency. The NOTA-labelled antibody showed high accumulation in the tumour, approximately equal to that observed for other antibody systems.

20 *Ex vivo* analyses showed biodistribution at 48 hours for the chMIL38 antibody labelled using the two different approaches (Figure 17). For chMIL-38 NOTA there was almost equal signal in the liver as the tumour indicating effective targeting. Imaging to longer time points would be expected to show almost exclusive tumour delineation.

Example 12: Targeting and tolerability study of goat anti-GPC-1 in xenografts

12.1 Materials and Methods

MIL-38 does not recognise mouse GPC-1, therefore there is a possibility that the tumour-specific localisation of chMIL-38 and the good tolerability seen in other studies may be due to the chMIL-38 only targeting human GPC-1 on the xenograft tumours and not targeting endogenous murine or rat GPC-1.

A goat anti-GPC-1 antibody was identified that recognises mouse recombinant GPC-1 on western blots and can immunoprecipitate mouse recombinant GPC-1 suggesting that it recognises the native (cell surface) form of mouse GPC-1. The Goat

anti-GPC-1 antibody was therefore used to confirm that the safety and tolerability results seen for chMIL-38 were also seen with an antibody that recognised mouse GPC-1.

The FDA guidance on exploratory Investigational New Drugs suggests a maximum microdose for proteins of <30nmols. For a full-length monoclonal antibody this is approximately 4.5 mg protein. ~0.3mg of antibody in a mouse is equivalent to a Human Equivalent Dose (HED) of 4.5 mg antibody with a safety factor of 22 or 1 mg antibody with a safety factor of 100 (see **Table 7**). 300µg of Goat anti-GPC-1 was used as the highest dose per infusion.

6 BalbC mice with DU-145 xenografts were dosed with goat anti-GPC-1 labelled with Cy5 via the tail vein. Three were dosed with 150µg of antibody and three were dosed with 300µg of antibody.

Table 7. Calculation of goat anti-GPC-1 dose for tolerability study.

Human Equivalent Dose (HED) Calculations		
Proposed Human Total Dose (mg)	1	4.5
Safety Factor	100	22.2
Equivalent Total Dose (mg)	100	100
Avg. Weight (kg)	75	75
Dose (mg/kg)	1.33	1.33
Conversion Factor**	12.3	12.3
Animal Dose (mg/kg)	16.4	16.4
Weight of mouse (g)	20	20
Dose per mouse (µg)	328	328

*recommended margin for safety factor is 100 See

Guidance for Industry on Estimating the Maximum

Safe Starting Dose in Initial Clinical Trials for

Therapeutics in Adult Healthy Volunteers

**for human: mouse refer pg 77 Guidance for Industry

Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for

Therapeutics in Adult Healthy Volunteers

Chimeric MIL-38 does not bind recombinant mouse GPC-1

Goat Anti-GPC-1 binds both recombinant human and mouse GPC-1

Goat Anti-GPC-1 can act as a surrogate in a mouse tolerability study

15

12.2 Results

- Targeting

In vivo imaging showed tumour accumulation and long-term localisation (over 5 days) for the goat anti-GPC antibody. This was particularly apparent in mouse 2.2 and 2.3 images (**Figures 18A and 18B**) and the MARS image (**Figure 18C**).

20

Ex vivo analyses (**Figure 18B**) showed that the goat anti-GPC antibody exhibited strong signals in the tumours. Some residual signal in the gut of all animals suggested that the antibody was being cleared most likely through the biliary system.

5 - Safety and tolerability

Mice were assessed during the study according to appearance, clinical signs, unprovoked behavior and response to external stimuli (**Table 8**). Weight was also monitored.

None of the mice showed any significant health effects due to the antibodies used based on the score sheet used in this study. A single mouse (mouse 2.3) showed minor sluggishness following injection, but this was attributed to a minor burn on the tail incurred during injection. This mouse subsequently recovered during the course of the study.

Minimal variation in weight for each mouse was observed over the course of the study.

Table 8. Assessment of safety and tolerability.

Indicators	Score of each animal in group					Scoring of independent variables:
Date						
Appearance:						0. Normal, coat is smooth, fur flat and often has a sheen, eyes are clear and bright. 1. Slightly ruffled coat but no other noted changes 2. Moderate ruffled coat, eyes and nose may have discharges 3. Very ruffled coat, external orifices uncleaned, abnormal posture, may look hunched up, eyes look pale and pupils enlarged
Clinical Signs						0. Respiration appears normal, body temperature feels normal on handling, no twitching behaviour, normal bowel movements 1. Small changes in above parameters 2. Body temperature appears above normal, respiration rapid and shallow breathing, twitching behaviour, altered bowel movement (change in consistency or amount) 3. Marked increasing in body temperature, respiration noisy, convulse.
Unprovoked behaviour						This behaviour is best observed from a distance and before any handling is attempted. 0. Normal behaviour pattern 1. Minor changes 2. Abnormal behaviour, less mobile and less alert than normal, inactive when hyperactivity would be expected. 3. Unrelated vocalisation, self mutilation, respiratory grunts, very restless or does not move at all.
Behavioral responses to external stimuli						Often mice will show inequities with whisker twitching and sniffing, or attempts to escape if frightened. Mice can have good body tone and paw grip on handling. If the abdominal area of the body is painful then gently pressure and observation is a useful measure to pain. 0. Behavioural responses normal for the expected conditions 1. Shows some minor depression or minor exaggeration of responses. 2. Shows moderate signs of abnormal responses, there may be a change of behaviour 3. Reacts violently to stimuli or nonocular responses may be very weak as in a pre-coma state

Total scores of:

0-4 – Normal, no action required

5-10 – Monitor carefully, evidence of suffering

10 and above – ample evidence of severe pain, euthanase the animal

One score of 3 in any of the criteria – euthanase immediately.

5 - *Discussion*

The Goat anti-GPC-1 showed almost identical targeting to the chMIL-38 results seen in **Figure 13B**, particularly at the 120 hours time point. This suggests that there is limited binding to other organs, implying that GPC-1 is either not cell-surface expressed or expressed at low levels on the cell surface without high expression in any particular organ.

The goat anti-GPC-1 antibody was well tolerated at both doses.

Example 13: Detection of glypican-1 antigen using AM4 MIL-38 antibodies

Experiments performed by the present inventors determined that an original deposit of the hybridoma for MIL-38 antibody (ATCC accession no. HB11785: murine hybridoma BLCA-38), then referred to as the “BLCA-38 hybridoma” is a mixed population of hybridoma cells that produces two distinct antibody populations, referred to here as “AM3” and “AM4”. Hybridoma cells responsible for producing each different antibody population were separated, and the “AM4” hybridoma cells were deposited on 22 August 2014 at CellBank Australia (CBA) under accession number CBA20140026.

Ninety-six well plates were coated with MIL-38 preps AM3 or AM4 (1 µg/well) in carbonate buffer pH 9.5 overnight. Plates were blocked with PBS-Tween (0.1%) containing 5% skim milk at 37°C and washed. Antigen (GPC-1 NS0) was diluted in Buffer II (20mM HEPES pH 7.5, 0.5mM EDTA, 0.5% Triton X-100) with the addition of 150mM NaCl and incubated overnight at 37°C. Detection was performed with biotinylated AM4 antibody followed by detection with avidin HRP (1µg/mL). TMB (Sigma cat no T0440) was added and stopped with TMB stop solution (Sigma S5814). Absorbance was read at 450nm. Results are shown in **Figure 19A**.

In a second experiment, ninety-six well plates were coated with MIL-38 preps 34A (a mixture of AM3 and AM4 antibodies) or AM4 (2.5 µg/well) in PBS pH 7.2 for 1h at room temperature. Plates were blocked with Blocker Casein (Thermo) in PBS-Tween (0.05%) for 1h at 37°C. Following washing, antigen (GPC-1 NS0) was diluted in TBS pH 7.2 containing 50mM Tricine and 150 mM NaCl and incubated at 37°C for 1h. Detection was performed with biotinylated AM4 clone 1F5 followed by detection with avidin HRP

(1µg/mL). TMB (Sigma cat no T0440) was added and stopped with TMB stop solution (Sigma S5814). Absorbance was read 450nm. Results are shown in **Figure 19B**.

The first ELISA described above was developed using MIL-38 to capture NS0 produced GPC-1 (i.e. MIL-38 antigen). This experiment compared monoclonal AM3
5 MIL-38 and monoclonal AM4 MIL-38 for capture. AM3 did not function as a capture agent in a sandwich ELISA assay, whereas AM4 was shown to do so (**Figure 19A**).

The second ELISA described above compared the ELISA signal obtained when a mixed population of MIL-38 (AM3 and AM4) was compared to that obtained from a monoclonal AM4 1F5 clone. Using AM4 1F5 as a capture agent provided a higher ELISA
10 signal than using the mixed 34A antibody population (**Figure 19B**).

The sandwich ELISA results demonstrate that only the AM4-like forms of the monoclonal MIL-38 antibody have utility in detecting glypican-1 antigen as a capture reagent and that a capture agent containing a monoclonal population provides a superior ELISA signal to that consisting of a mixed population.

15

Example 14: Sequence analysis of AM4 and AM3 MIL-38 antibody populations

Materials and Methods

- Heavy and light chain sequencing (DNA)

20 Three separate sequencing runs were performed. The first run (coded 224945) utilised bi-clonal hybridoma cells from a mixed (AM4 and AM3) preparation named 1-O. The second run (coded 449295-1) utilised cells from Alfio I a hybridoma stock that was used to generate AM4. The third run (coded 449295-5) utilised cells from Alfio II, a hybridoma stock that was used to generate AM3.

25 For sequencing runs 224945 (1-O) and 449295-1 (Alfio I), total RNA was extracted from frozen hybridoma cells and cDNA was synthesized from the RNA. RT-PCR was then performed to amplify the variable regions (heavy and light chains) and constant regions of the antibody, which were then cloned into a standard cloning vector separately and sequenced.

30 Total RNA was isolated from the hybridoma cells following the technical manual of TRIzol® Plus RNA Purification System. The total RNA was analysed by agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA using isotype-specific anti-sense primers or universal primers following the technical manual of SuperScript™ III First-Strand Synthesis System. The antibody fragments of VH, VL, CH and CL were
35 amplified according to the standard operating procedure of RACE of GenScript.

Amplified antibody fragments were separately cloned into a standard cloning vector using standard molecular cloning procedures.

Colony PCR screening was performed to identify clones with inserts of correct sizes. No less than five single colonies with inserts of correct sizes were sequenced for each antibody fragment.

V_H and V_L plasmids encoded the full-length variable regions of the antibody and a part of C_{H1} and C_L . C_H plasmid encoded a part of C_{H1} and full-length C_{H2} and C_{H3} . C_L plasmid encoded a part of C_L . In order to get full-length constant regions or heavy/light chain, the part of constant regions encoded by V_H and V_L plasmids and the part of constant regions encoded by C_H and C_L plasmids were amplified by PCR separately, and then overlap extension PCR was employed to obtain full-length DNAs. Five single colonies with correct V_H , V_L , C_H and C_L insert sizes were sent for sequencing.

Sequencing run 449295-5 (Alfio II) encountered difficulty obtaining sequence corresponding to the expected IgG1 heavy chain sequence. Two RNA preparations were performed. For the 1st batch of cells, oligo-dT primer and CDS III primers were used for reverse transcription (RT). VH/CH and VK/CK were amplified by PCR using IgG1 and IgK specific primers, partial mouse β -actin gene was amplified as positive control. Normal light chain bands were obtained easily while only weak VH could be observed on the gel. Five individual colonies with correct VK and CK insert sizes were sent for sequencing. The VK and CK genes of five different clones were found to be nearly identical. The consensus light chain sequences from the Alfio II hybridoma is listed below. One unproductive heavy chain sequence was obtained from eight randomly sequenced VH positive clones, shown as below. Three kinds of heavy chain constant region sequences were obtained from ten randomly sequenced CH positive clones (one IgG₁ C_H , one IgG_{2a} C_H and eight IgG_{2b} C_H). In order to avoid the influence of potential class switching, amplification of the C_H using IgM specific primer was performed, but no target PCR product was obtained. There was also no target PCR product when full-length heavy chain (V_H - C_H) was amplified using heavy chain FR1 degenerate primers.

As no productive heavy chain could be obtained after several attempts, isolation of heavy chain sequence from the 2nd vial of Alfio II cells was attempted. For the 2nd vial of cells, oligo-dT primer was used for reverse transcription initially. V_H was amplified using IgG1, IgG2b, IgM, IgA specific primers and IgG degenerate primer, respectively, and VK was amplified using IgK specific primers. Productive light chain and unproductive heavy chain, which were identical with previous results, were obtained. Reverse transcription using Random 6 mers primer was also attempted without success.

In summary, multiple attempts to isolate light chain and heavy chain sequence were made. One rearranged light chain sequence was consistently obtained after different attempts on two batches of cells. However, only weak VH target PCR products were observed and sequencing did not result in any consistent heavy chain sequence.

Results

- Sequencing (DNA)

The isolated total RNA of the sample was run alongside a DNA marker (Marker III - TIANGEN, Cat. No. : MD103) on a 1.5% agarose/GelRedTM gel.

Four microliters of PCR products of each sample were run alongside the DNA marker (Marker III) on a 1.5% agarose/GelRed™ gel. The PCR products were purified and stored at -20 °C until further use.

The V_H, V_L, C_H and C_L genes of five different clones were nearly identical. The consensus sequence (**SEQ ID NO: 1** – AM4 MIL-38 Mouse IgG₁ Heavy Chain DNA Consensus Sequence; **SEQ ID NO: 2** – AM4 MIL-38 Mouse Kappa light Chain DNA Consensus Sequence) was determined to be the sequence of the antibody produced by the monoclonal hybridoma population (AM-4).

The heavy and light chain AM4 MIL-38 consensus DNA sequences above translate to the AM4 heavy chain amino acid sequence (**SEQ ID NO: 3** - AM4 MIL-38 Mouse IgG1 Heavy Chain Amino Acid Consensus Sequence) and the AM4 light chain amino acid sequence (**SEQ ID NO: 4** - AM4 consensus MIL-38 Light Chain Amino Acid Consensus Sequence).

AM3 consensus sequences

25 No consistent heavy chain sequence could be obtained from the AM3-like Alfio II cells. The light chain sequence obtained from sequencing run 449295-5 (Alfio II) was consistently obtained and showed clear differences in both the framework regions and the complementarity-determining regions compared to the sequence for the other two sequencing runs.

AM3 MIL-38 Kappa Light Chain DNA Consensus Sequence (SEQ ID NO: 10)

GACATTGTGATGACCCAGTCTCAAAAGTTCATGTCCACATCAATAGGAGACAGGGTCAGCGTCACCTGC
 CCAGCTGCAATCTGTTCTCCATCTAGCTGGTTTCAGCAGAAACCAGGGCAATCTCCTAAAGCACTGATT
 35 TACGGAGTCACTGATCGCTTCACAGGSCAGTGGATCTGGGACAGATTTCACT

5

10

AM3 MIL-38 Light Chain Amino Acid Consensus Sequence (SEQ ID NO: 11)

15

20

30

35

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from
5 the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

CLAIMS

1. Use of an anti-glypican-1 (anti-GPC1) antibody and/or an antigen-binding fragment thereof in the preparation of a medicament for treating prostate, bladder, and/or pancreatic cancer in a subject, wherein:

the antibody and/or fragment is conjugated to at least one cytotoxic agent that is toxic to a prostate, bladder, and/or pancreatic cancer cell in the subject, and

the antibody and/or fragment comprises:

(a) a heavy chain variable region comprising:

a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 3**;

a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 3**;

a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 3**; and

(b) a light chain variable region comprising:

a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 4**;

a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 4**;

a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 109-117 of **SEQ ID NO: 4**; and

the medicament does not comprise an antibody with a light chain variable region comprising:

a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 48-58 of **SEQ ID NO: 11**;

a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 74-80 of **SEQ ID NO: 11**;

a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 11**.

2. The use according to claim 1, wherein the anti-glypican-1 (anti-GPC1) antibody is produced by a hybridoma cell as deposited at Cellbank Australia under accession number CBA20140026, or progeny thereof.

3. The use according to claim 1 or claim 2, wherein the anti-glypican-1 (anti-GPC1) antibody is:

- (i) a humanised anti-GPC1 antibody,
- (ii) a chimeric anti-GPC1 antibody,
- (i) a human anti-GPC1 antibody,
- (ii) a monoclonal anti-GPC1 antibody,
- (iii) a multimeric anti-GPC1 antibody,
- (iv) a synthetic anti-GPC1 antibody, or
- (v) a chimeric antibody comprising a heavy chain constant region comprising or consisting of an amino acid sequence as defined in residues 138-467 of **SEQ ID NO: 7**; and a light chain constant region comprising or consisting of an amino acid sequence as defined in residues of 128-234 **SEQ ID NO: 8**.

4. The use according to any one of claims 1 to 3, wherein the antigen-binding fragment thereof is: a single chain variable fragment (scFv), a single chain variable fragment (scFv) comprising a sequence as defined in **SEQ ID NO: 9**, a variable domain (Fv) fragment, a fragment antigen binding (Fab) fragment, a F(ab)₂ fragment, a peptide, or a proteolytic fragment containing an epitope binding region.

5. The use according to any one of claims 1 to 4, wherein the cytotoxic agent is selected from the group consisting of:

- (a) an adrenocortical suppressant, an alkylating agent, an alkyl sulfonate, an anthracycline, an anti-angiogenic agent, an antibiotic, an antimetabolite, an antimitotic, an auristatin, a calicheamycin, a camptothecin, a COX-2 inhibitor, an enzyme inhibitor, an epipodophyllotoxin, an ethylenimine derivative, a folic acid analog, an HDAC inhibitor, a heat shock protein (HSP90) inhibitor, a hormone antagonist, a maytansinoid, a methyl hydrazine derivative, an mTOR inhibitor, a nitrogen mustard, a nitrosourea, a platinum coordination complex, a pro-apoptotic agent, a proteasome inhibitor, a purine analog, a pyrimidine analog, a radioisotope, a substituted urea, a taxane, triazene, a tubulin inhibitor, a tyrosine kinase inhibitor, and a vinca alkaloid;

(b) afatinib, aplidin, anastrozole, anthracyclines, AVL-101, AVL-291, axitinib, azaribine, bendamustine, bleomycin, bortezomib, bosutinib, bryostatin-1, busulfan, camptothecans, carboplatin, calicheamycin, camptothecin, carboplatin, carmustine, celecoxib, chlorambucil, cisplatin, cladribine, COX-2 inhibitors, crizotinib, cyano-morpholino doxorubicin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, dasatinib, daunorubicin, dinaciclib, 3',5'-O-dioleoyl-FudR (FUDR-d0), DM1, DM3, DM4, docetaxel, doxorubicin, doxorubicin glucuronide, duocarmycin, endostatin, entinostat, epidophyllotoxin, epirubicin glucuronide, erlotinib, estramustine, estrogen receptor binding agents, etoposide glucuronide, etoposide phosphate, etoposide (VP16), exemestane, farnesyl-protein transferase inhibitors, fingolimod, flavopiridol, floxuridine (FUDR), fludarabine, 5-fluorouracil, flutamide, fostamatinib, ganetespib, GDC-0834, gefitinib, gemcitabine, GS-1101, 10-hydroxycamptothecin, hydroxyurea, ibrutinib, idarubicin, idelalisib, ifosfamide, imatinib, irinotecan (CPT-11), lapatinib, lenolidamide, leucovorin, LFM-A13, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mithramycin, mitomycin, mitotane, mitoxantrone, monomethylauristatin D (MMAD), monomethylauristatin E (MMAE), monomethylauristatin F (MMAF), navelbine, neratinib, nilotinib, nitrosurea, olaparib, paclitaxel, PCI-32765, pentostatin, plicomycin, a 2-PDox pro-drug (pro-2-PDox), procarbazine, PSI-341, 2-pyrrolinodoxorubicine (2-PDox), raloxifene, semustine, SN-38, sorafenib, streptozocin, SU1 1248, sunitinib, tamoxifen, temazolomide, teniposide, thalidomide, thioguanine, thiotepa, topotecan, transplatinum, uracil mustard, vatalanib, vinblastine, vinca alkaloids, vincristine, vinorelbine, and ZD 1839.

6. The use according to any one of claims 1 to 5, wherein the cytotoxic agent is:

- (a) a radioisotope;
- (b) a radioisotope selected from the group consisting of: ^{90}Y , ^{188}Re , ^{166}Ho , ^{165}Dy , ^{109}Pd , ^{111}Ag , ^{186}Re , ^{198}Au , ^{153}Sm , ^{64}Cu , ^{177}Lu , ^{131}I , ^{125}I , ^{67}Cu , ^{175}Yb , ^{166}Dy , ^{169}Er , ^{212}Bi , ^{213}Bi , ^{225}Ac , ^{212}Pb , ^{66}Ga , ^{67}Ga , ^{68}Ga , ^{64}Cu , ^{86}Y , $^{94\text{m}}\text{Tc}$, and ^{89}Zr .

7. The use according to any one of claims 1 to 6, wherein the subject is a mammalian subject or a human subject.

8. An isolated antibody population comprising:

first anti-glypican-1 (anti-GPC1) antibodies and/or antigen binding fragments thereof, that are conjugated to at least one cytotoxic agent which is toxic to a prostate, bladder, and/or pancreatic cancer cell, wherein the first anti-glypican-1 (anti-GPC1) antibodies comprise:

- (a) a heavy chain variable region comprising:
 - a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 50-54 of **SEQ ID NO: 3**;
 - a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 69-85 of **SEQ ID NO: 3**;
 - a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 118-126 of **SEQ ID NO: 3**; and
 - (b) a light chain variable region comprising:
 - a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 44-54 of **SEQ ID NO: 4**;
 - a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 70-76 of **SEQ ID NO: 4**;
 - a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 109-117 of **SEQ ID NO: 4**;
- and wherein the antibody population does not contain second antibodies comprising a light chain variable region comprising:
- a complementarity determining region 1 (CDR1) comprising or consisting of an amino acid sequence defined by positions 48-58 of **SEQ ID NO: 11**;
 - a complementarity determining region 2 (CDR2) comprising or consisting of an amino acid sequence defined by positions 74-80 of **SEQ ID NO: 11**;
 - a complementarity determining region 3 (CDR3) comprising or consisting of an amino acid sequence defined by positions 113-121 of **SEQ ID NO: 11**.

9. The antibody population according to claim 8, wherein the first anti-glypican-1 (anti-GPC1) antibodies and/or antigen binding fragments thereof are any one or more of monoclonal antibodies, humanised antibodies, chimeric antibodies, multimeric antibodies, synthetic antibodies, and/or chimeric antibodies comprising:

- (a) a heavy chain constant region comprising or consisting of an amino acid sequence as defined in residues 138-467 of **SEQ ID NO: 7**; and
- (b) a light chain constant region comprising or consisting of an amino acid sequence as defined in residues of 128-234 **SEQ ID NO: 8**.

10. The antibody population according to claim 8 or claim 9, wherein the antigen binding fragments are any one or more of single chain variable fragments (scFv), single chain variable fragments (scFv) comprising a sequence as defined in **SEQ ID NO: 9**, variable domain (Fv) fragments, fragment antigen binding (Fab) fragments, F(ab)₂ fragments, peptides, or proteolytic fragments containing an epitope binding region.

11. The antibody population according to any one of claims 8 to 10, wherein the first anti-glypican-1 (anti-GPC1) antibodies comprise or consist of a heavy chain sequence as defined by positions 20-461 of **SEQ ID NO: 3** and a light chain sequence as defined by positions 21-234 of **SEQ ID NO: 4**.

12. The antibody population according to any one of claims 8 to 11, wherein the cytotoxic agent is selected from the group consisting of:

(a) an adrenocortical suppressant, an alkylating agent, an alkyl sulfonate, an anthracycline, an anti-angiogenic agent, an antibiotic, an antimetabolite, an antimitotic, an auristatin, a calicheamycin, a camptothecin, a COX-2 inhibitor, an enzyme inhibitor, an epipodophyllotoxin, an ethylenimine derivative, a folic acid analog, an HDAC inhibitor, a heat shock protein (HSP90) inhibitor, a hormone antagonist, a maytansinoid, a methyl hydrazine derivative, an mTOR inhibitor, a nitrogen mustard, a nitrosourea, a platinum coordination complex, a pro-apoptotic agent, a proteasome inhibitor, a purine analog, a pyrimidine analog, a radioisotope, a substituted urea, a taxane, triazene, a tubulin inhibitor, a tyrosine kinase inhibitor, and a vinca alkaloid;

(b) afatinib, aplidin, anastrozole, anthracyclines, AVL-101, AVL-291, axitinib, azaribine, bendamustine, bleomycin, bortezomib, bosutinib, bryostatin-1, busulfan, camptothecans, carboplatin, calicheamycin, camptothecin, carboplatin, carmustine, celecoxib, chlorambucil, cisplatin, cladribine, COX-2 inhibitors, crizotinib, cyano-morpholino doxorubicin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, dasatinib, daunorubicin, dinaciclib, 3',5'-O-dioleoyl-FudR (FUDR-d0), DM1, DM3, DM4, docetaxel, doxorubicin, doxorubicin glucuronide, duocarmycin, endostatin, entinostat, epidophyllotoxin, epirubicin glucuronide, erlotinib, estramustine, estrogen receptor binding agents, etoposide glucuronide, etoposide phosphate, etoposide (VP16), exemestane, farnesyl-protein transferase inhibitors, fingolimod, flavopiridol, floxuridine (FUDR), fludarabine, 5-fluorouracil, flutamide, fostamatinib, ganetespib, GDC-0834, gefitinib, gemcitabine, GS-1101, 10-

hydroxycamptothecin, hydroxyurea, ibrutinib, idarubicin, idelalisib, ifosfamide, imatinib, irinotecan (CPT-11), lapatinib, lenolidamide, leucovorin, LFM-A13, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mithramycin, mitomycin, mitotane, mitoxantrone, monomethylauristatin D (MMAD), monomethylauristatin E (MMAE), monomethylauristatin F (MMAF), navelbine, neratinib, nilotinib, nitrosurea, olaparib, paclitaxel, PCI-32765, pentostatin, plicomycin, a 2-PDox pro-drug (pro-2-PDox), procarbazine, PSI-341, 2-pyrrolinodoxorubicine (2-PDox), raloxifene, semustine, SN-38, sorafenib, streptozocin, SU1 1248, sunitinib, tamoxifen, temazolomide, teniposide, thalidomide, thioguanine, thiotepa, topotecan, transplatinum, uracil mustard, vatalanib, vinblastine, vinca alkaloids, vincristine, vinorelbine, and ZD 1839.

13. The antibody population according to any one of claims 8 to 12, wherein the cytotoxic agent is:

- (a) a radioisotope;
- (b) a radioisotope selected from the group consisting of: ^{90}Y , ^{188}Re , ^{166}Ho , ^{165}Dy , ^{109}Pd , ^{111}Ag , ^{186}Re , ^{198}Au , ^{153}Sm , ^{64}Cu , ^{177}Lu , ^{131}I , ^{125}I , ^{67}Cu , ^{175}Yb , ^{166}Dy , ^{169}Er , ^{212}Bi , ^{213}Bi , ^{225}Ac , ^{212}Pb , ^{66}Ga , ^{67}Ga , ^{68}Ga , ^{64}Cu , ^{86}Y , $^{94\text{m}}\text{Tc}$, and ^{89}Zr .