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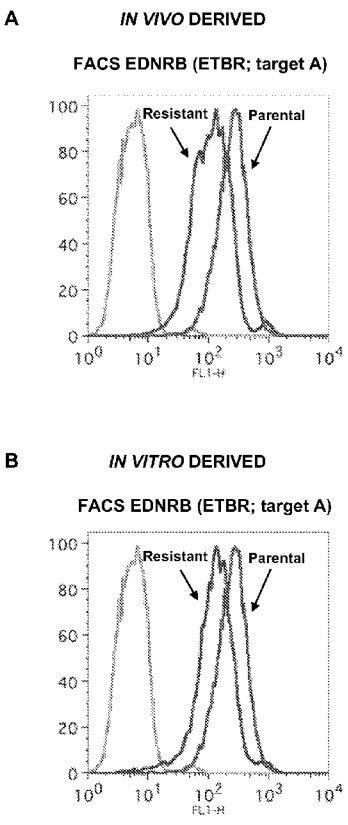

FIG. 9

(57) Abstract: The invention provides anti-ETBR antibodies and immunoconjugates and methods of using the same. In some embodiments, an immunoconjugate comprising an antibody that binds ETBR covalently attached to a cytotoxic agent is provided, wherein the antibody binds an epitope within amino acids 64 to 101 of SEQ ID NO: 10. In some embodiments, the cytotoxic agent is a nemorubicin derivative.

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ANTI-ETBR ANTIBODIES AND IMMUNOCONJUGATES

FIELD OF THE INVENTION

[001] The present invention relates to immunoconjugates comprising anti-ETBR antibodies and methods of using the same.

BACKGROUND

[002] Melanoma is an aggressive form of skin cancer that has recently undergone an alarming increase in incidence. Although cures can be achieved with surgical resection of localized lesions, the advanced stages of melanoma are only poorly responsive to currently approved therapies. The 5-year survival rate for stage IV metastatic melanoma is approximately 10%. New therapeutic approaches, including antisense to Bcl2, antibodies to CTLA4, small molecule RAF kinase inhibitors, and adoptive immunotherapy, are currently in clinical testing for metastatic melanoma. The results from some of these recent studies seem to be encouraging, but a durable impact on overall survival will likely require therapeutic combinations including additional new agents.

[003] More than 20 years ago, endothelin-1 (ET-1) was isolated from aortic endothelial cells and found to have potent vasoconstrictive activity. The receptors for endothelins were cloned shortly thereafter and their expression in various cell types, including melanocytes and melanoma cells, pointed to functions independent of their role in endothelium. It is now well recognized that the endothelin B receptor (ETBR, EDNBR) is critical for the faithful derivation of melanocytic cells emanating from the neural crest during embryonic development. Melanocyte precursors rely on ETBR activity to proliferate and migrate from the neural tube to their final destinations. Mice with defective genes coding for either ETBR or endothelin-3 (ET-3) exhibit a pigmentation deficit in their coats and a shortage of enteric ganglion cells, also derived from the neural crest. These characteristics strongly resemble those associated with the WS4 variant of Waardenburg syndrome in humans, which has been attributed to germline mutations in either ET-3 or ETBR. An additional variant of this syndrome, WS2, has been mapped to heritable mutations in the microphthalmia-associated transcription factor (MITF), a key regulator of melanocyte development and a melanoma proto-oncogene.

[004] The strong genetic evidence linking ETBR activity to the fate of melanoblasts underscores a potential role for this receptor in the progression of melanoma. The expression of ETBR mRNA and protein was reported to increase during disease progression from

dysplastic nevi to metastatic melanoma. Blockade of ETBR activity by 2 independent small molecule inhibitors interfered with growth and survival of melanoma cells and tumor xenografts. These preclinical studies implicate ETBR as a potential driver of melanoma progression.

[005] There is a need in the art for agents that target ETBR for the diagnosis and treatment of ETBR-associated conditions, such as cancer. The invention fulfills that need and provides other benefits.

SUMMARY

[006] The invention provides anti-ETBR antibodies and immunoconjugates and methods of using the same.

[007] In some embodiments, an immunoconjugate comprising an antibody that binds ETBR covalently attached to a cytotoxic agent is provided, wherein the antibody binds an epitope within amino acids 64 to 101 of SEQ ID NO: 10. In some embodiments, the cytotoxic agent is a nemorubicin derivative.

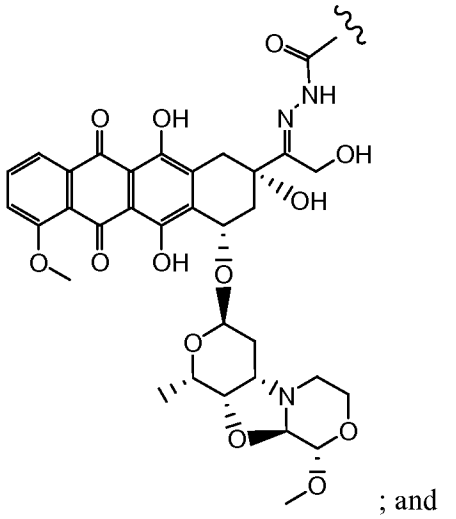
[008] In some embodiments, the antibody comprises (i) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14, (ii) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17, and (iii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13. In some embodiments, the antibody comprises (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (iii) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14. In some embodiments, the antibody comprises (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, (iii) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14, (iv) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (v) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (vi) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In some embodiments, the antibody comprises (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (iii) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In some embodiments, the antibody comprises: a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 8; or b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 7; or c) a VH sequence as in (a) and a VL sequence as in (b). In some embodiments, the antibody comprises a VH sequence having the amino acid sequence of SEQ ID NO: 8 or SEQ ID NO: 9. In some embodiments, the antibody comprises

a VL sequence having the amino acid sequence of SEQ ID NO: 7. In some embodiments, the antibody is an IgG1, IgG2a or IgG2b antibody.

[009] In some embodiments, an immunoconjugate comprising an antibody that binds ETBR covalently attached to a cytotoxic agent is provided, wherein the antibody comprises (a) a VH sequence having the amino acid sequence of SEQ ID NO: 8 and a VL sequence having the amino acid sequence of SEQ ID NO: 7, and wherein the cytotoxic agent is a nemorubicin derivative.

[010] In some embodiments, the immunoconjugate has the formula Ab-(L-D)p, wherein: (a) Ab is the antibody; (b) L is a linker; (c) D is the cytotoxic agent; and (d) p ranges from 1-8.

[011] In some embodiments, D is a nemorubicin derivative. In some such embodiments, D has a structure selected from:



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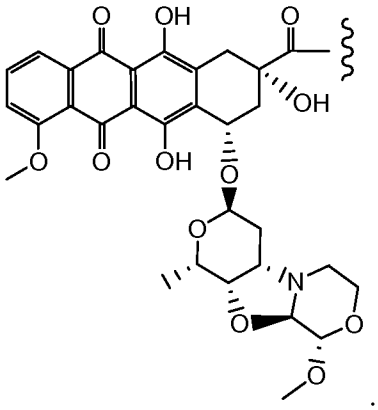

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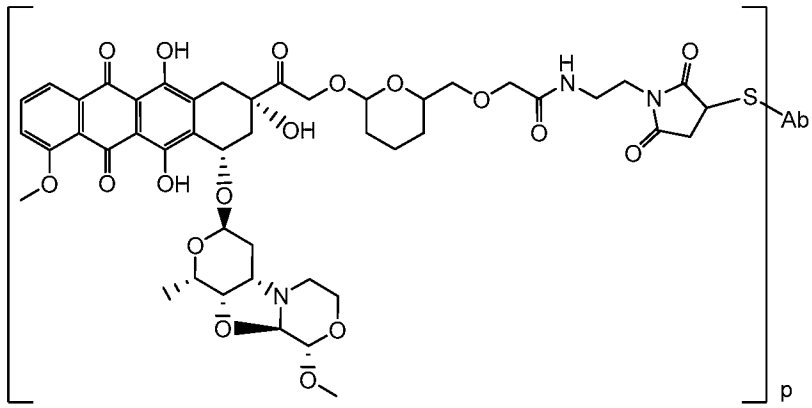
[012] In some embodiments, the immunoconjugate comprises a linker that is cleavable by a protease. In some such embodiments, the linker comprises a val-cit dipeptide or a Phe-homoLys dipeptide. In some embodiments, the immunoconjugate comprises a linker that is acid-labile.

[013] In some embodiments, the immunoconjugate has a formula selected from:



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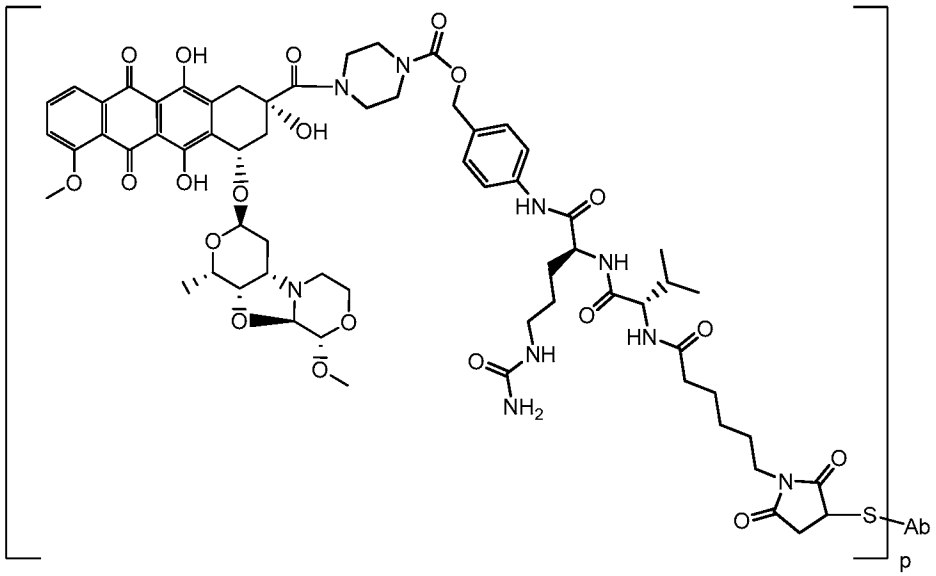

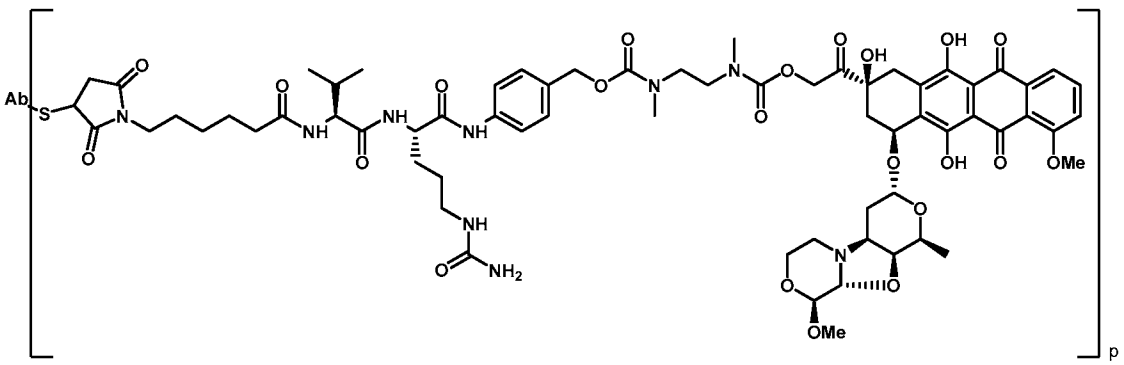

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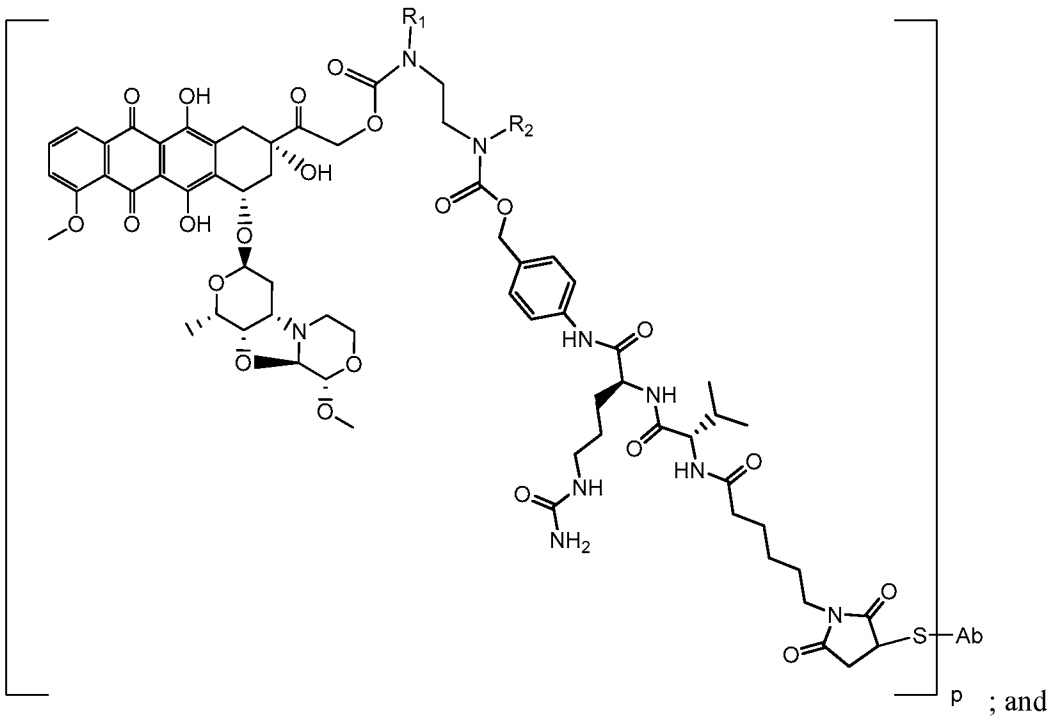

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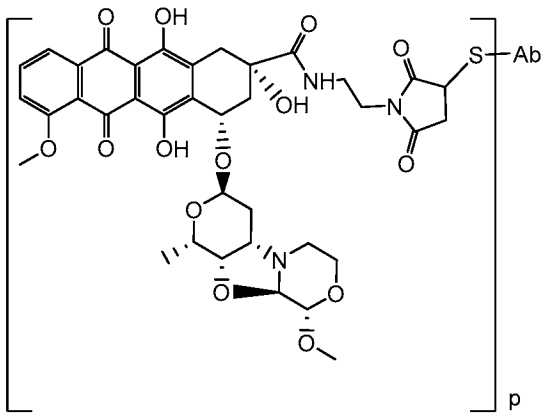

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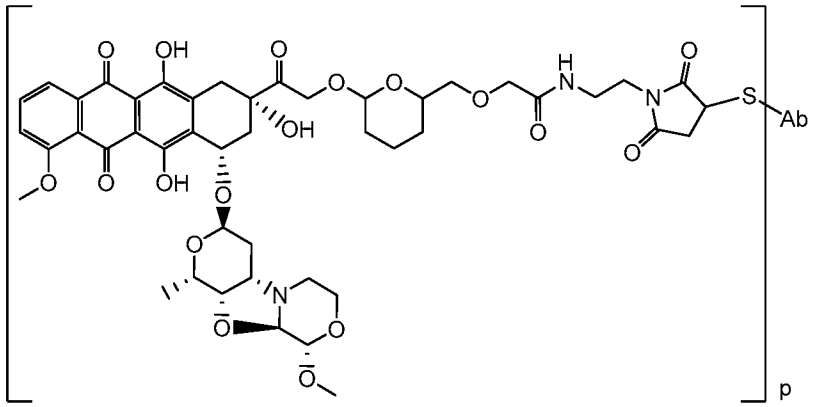
wherein R_1 and R_2 are independently selected from H and C_1-C_6 alkyl. In some embodiments, p ranges from 1-3.

[014] In some embodiments, an immunoconjugate is provided, wherein the immunoconjugate has a formula selected from:



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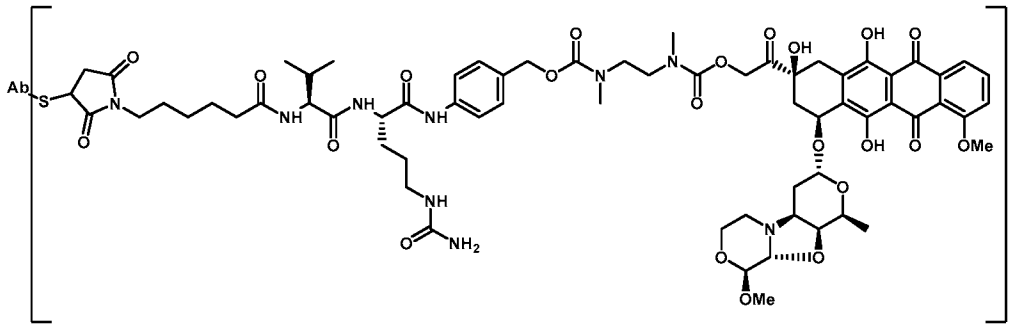

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wherein Ab is an antibody comprising (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, (iii) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14, (iv) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (v) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (vi) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17; and wherein p ranges from 1to 8, or 1 to 7, or 1 to 6, or 1 to 5, or 1 to 4, or 1 to 3. In some such embodiments, the antibody comprises a VH sequence of SEQ ID NO: 8 and a VL sequence of SEQ ID NO: 7. In some embodiments, the antibody comprises a heavy chain of SEQ ID NO: 6 and a light chain of SEQ ID NO: 5.

[015] In any of the embodiments discussed herein, the antibody may be a monoclonal antibody. In some embodiments, the antibody may be a human, humanized, or chimeric antibody. In some embodiments, the antibody is an antibody fragment that binds ETBR. In some embodiments, the antibody binds human ETBR. In some such embodiments, human ETBR has the sequence of SEQ ID NO: 10 or SEQ ID NO: 11.

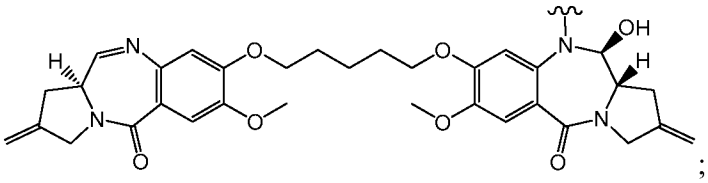
[016] In some embodiments, pharmaceutical formulations are provided, wherein the formulation comprises an immunoconjugate described herein and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical formulation comprises an additional therapeutic agent.

[017] In some embodiments, methods of treating an individual with an ETBR-positive cancer are provided. In some embodiments, a method comprises administering to the individual an effective amount of an immunoconjugate described herein. In some embodiments, the ETBR-positive cancer is selected from melanoma and multiple myeloma. In some embodiments, the method further comprises administering an additional therapeutic agent to the individual. In some such embodiments, the additional therapeutic agent comprises an antibody that binds PMEL17. In some embodiments, the additional therapeutic agent is an immunoconjugate comprising an antibody that binds PMEL17 covalently attached to a cytotoxic agent.

[018] In some embodiments, a method of treating an individual having an ETBR-positive cancer, wherein the ETBR-positive cancer is resistant to a first therapeutic is provided. In some embodiments, the method comprises administering to the individual an effective amount of an immunoconjugate described herein. In some embodiments, the ETBR-positive cancer is melanoma. In some embodiments, the first therapeutic comprises a first antibody that binds an antigen other than ETBR. In some embodiments, the first therapeutic is a first immunoconjugate comprising a first antibody that binds an antigen other than ETBR and a first cytotoxic agent. In some embodiments, the first antibody binds an antigen selected from melanocyte protein PMEL17, tyrosinase-related protein 1 (TYRP1), cytotoxic T lymphocyte antigen 4 (CTLA-4), and glycoprotein NMB (GPNMB). In some embodiments, the first therapeutic comprises a first antibody that binds ETBR. In some embodiments, the first therapeutic is a first immunoconjugate comprising a first antibody that binds ETBR and a first cytotoxic agent. In some embodiments, the first cytotoxic agent and the cytotoxic agent of the immunoconjugate described herein are different. In some embodiments, the first cytotoxic agent is MMAE.

[019] In some embodiments, a method of treating an individual with ETBR-positive cancer is provided, wherein the method comprises administering to the individual an effective amount of a first immunoconjugate described herein in combination with a second immunoconjugate comprising an antibody that binds PMEL17. In some embodiments, the antibody that binds PMEL17 comprises an HVR H1 comprising a sequence of SEQ ID NO: 21, an HVR H2 comprising a sequence of SEQ ID NO: 22, an HVR H3 comprising a sequence of SEQ ID NO: 23, an HVR L1 comprising a sequence of SEQ ID NO: 24, an HVR L2 comprising a sequence of SEQ ID NO: 25, and an HVR L3 comprising a sequence of SEQ ID NO: 26. In some embodiments, the second immunoconjugate comprises a cytotoxic agent selected from an auristatin, a pyrrolobenzodiazepine, and a nemorubicin derivative. In some embodiments, the second immunoconjugate comprises an auristatin or a

pyrrolobenzodiazepine. In some embodiments, the second immunoconjugate comprises MMAE. In some such embodiments, the second immunoconjugate comprises a linker-drug portion comprising MC-val-cit-PAB-MMAE. In some embodiments, the second immunoconjugate comprises a PBD dimer having the structure:



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wherein the wavy line indicates attachment to a linker. In some embodiments, the second immunoconjugate comprises a linker comprising MC-val-cit-PAB. In any of the foregoing embodiments, the ETBR-positive cancer may be melanoma. In some embodiments, the ETBR-positive cancer is also PMEL17-positive.

[020] In some embodiments, a method of inhibiting proliferation of an ETBR-positive cell is provided. In some such embodiments, the method comprises exposing the cell to the immunoconjugate described herein under conditions permissive for binding of the immunoconjugate to ETBR on the surface of the cell, thereby inhibiting proliferation of the cell. In some embodiments, the cell is a melanoma cell.

BRIEF DESCRIPTION OF THE FIGURES

[021] **Figure 1** shows the amino acid sequences of the full-length light chain of the murine 5E9 monoclonal antibody (SEQ ID NO: 1) and the variable light chain of the murine 5E9 monoclonal antibody (SEQ ID NO: 3).

[022] **Figure 2** shows the amino acid sequences of the full-length heavy chain of the murine 5E9 monoclonal antibody (SEQ ID NO: 2) and the variable heavy chain of the murine 5E9 monoclonal antibody (SEQ ID NO: 4).

[023] **Figure 3** shows the amino acid sequences of the full-length light chain of the humanized hu5E9.v1 antibody (SEQ ID NO: 5) and the variable light chain of the humanized hu5E9.v1 antibody (SEQ ID NO: 7).

[024] **Figure 4** shows the amino acid sequences of the full-length heavy chain of the humanized hu5E9.v1 antibody (SEQ ID NO: 6) and the variable heavy chain of the humanized hu5E9.v1 antibody (SEQ ID NO: 8).

[025] **Figure 5** shows the amino acid sequence of the variable heavy chain of the humanized hu5E9.v2 antibody (SEQ ID NO: 9).

[026] **Figure 6** shows the amino acid sequence of an exemplary human ETBR protein (SEQ ID NO:10) and various characteristics of the protein.

[027] **Figure 7** shows the structures of various antibody-drug conjugates, including (A) Ab-MC-val-cit-PAB-MMAE; (B) Ab-MC-acetal-PNU-159682; (C) Ab-MC-val-cit-PAB-PNU-159682; and (D) Ab-PNU-159682.

[028] **Figure 8** shows (A) tumor volume over time in mice inoculated with UACC-257X2.2 melanoma cells and administered varying doses of anti-ETBR-vc-MMAE ("5E9v1-vcE"), and (B) parental and resistant UACC-257X2.2 cells grown *in vitro* in the presence of increasing concentrations of anti-ETBR-vc-MMAE ADC, as described in Example B.

[029] **Figure 9** shows expression of ETBR (also referred to as "EDNRB") in parental and resistant UACC-257X2.2 cells derived *in vivo* (A) and *in vitro* (B), as described in Example B.

DETAILED DESCRIPTION

I. DEFINITIONS

[030] An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

[031] "Affinity" refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

[032] An "affinity matured" antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

[033] The terms "anti-ETBR antibody" and "an antibody that binds to ETBR" refer to an antibody that is capable of binding ETBR with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting ETBR. In one embodiment, the extent of binding of an anti-ETBR antibody to an unrelated, non-ETBR protein is less than about 10% of the binding of the antibody to ETBR as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to ETBR has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, , $\leq$ 5 Nm, , $\leq$ 4 nM, , $\leq$ 3 nM, , $\leq$ 2 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (*e.g.*, 10^{-8} M or less, *e.g.* from 10^{-8} M to 10^{-13} M, *e.g.*, from 10^{-9} M to 10^{-13} M). In certain embodiments, an anti-ETBR antibody binds to an epitope of ETBR that is conserved among ETBR from different species.

[034] The term "antibody" is used herein in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

[035] An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody and that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

[036] An "antibody that binds to the same epitope" as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more. An exemplary competition assay is provided herein.

[037] The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include, but are not limited to, melanoma, carcinoma, lymphoma (e.g., Hodgkin's and non-Hodgkin's lymphoma), blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancer.

[038] The term "chimeric" antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

[039] The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

[040] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. Cytotoxic agents include, but are not limited to, radioactive isotopes (e.g., At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu); chemotherapeutic agents or drugs (e.g., methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents); growth inhibitory agents; enzymes and fragments thereof such as nucleolytic enzymes; antibiotics; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; and the various antitumor or anticancer agents disclosed below.

[041] A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin,

phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, e.g., paclitaxel (TAXOL®; Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and docetaxel (TAXOTERE®; Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine

(NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®); pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone; CVP, an abbreviation for a combined therapy of cyclophosphamide, vincristine, and prednisolone; and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovorin.

[042] "Effector functions" refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

[043] An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

[044] The term "epitope" refers to the particular site on an antigen molecule to which an antibody binds.

[045] The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

[046] "Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) or complementarity determining region (CDR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

[047] The terms "full length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

[048] The term "glycosylated forms of ETBR" refers to naturally occurring forms of ETBR that are post-translationally modified by the addition of carbohydrate residues.

[049] The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

[050] A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

[051] A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., *supra.* In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., *supra.*

[052] A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

[053] The term "hypervariable region" or "HVR," as used herein, refers to each of the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops ("hypervariable loops"). Generally, native four-chain antibodies

comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the "complementarity determining regions" (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition. Exemplary hypervariable loops occur at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3). (Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987).) Exemplary CDRs (CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3) occur at amino acid residues 24-34 of L1, 50-56 of L2, 89-97 of L3, 31-35B of H1, 50-65 of H2, and 95-102 of H3. (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991).) With the exception of CDR1 in VH, CDRs generally comprise the amino acid residues that form the hypervariable loops. CDRs also comprise "specificity determining residues," or "SDRs," which are residues that contact antigen. SDRs are contained within regions of the CDRs called abbreviated-CDRs, or a-CDRs. Exemplary a-CDRs (a-CDR-L1, a-CDR-L2, a-CDR-L3, a-CDR-H1, a-CDR-H2, and a-CDR-H3) occur at amino acid residues 31-34 of L1, 50-55 of L2, 89-96 of L3, 31-35B of H1, 50-58 of H2, and 95-102 of H3. (See Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008).) Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*.

[054] An "immunoconjugate" is an antibody conjugated to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

[055] An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

[056] An "isolated antibody" is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman et al., *J. Chromatogr. B* 848:79-87 (2007).

[057] An "isolated nucleic acid" refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

[058] "Isolated nucleic acid encoding an anti-ETBR antibody" refers to one or more nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

[059] The term "ETBR," as used herein, refers to any native ETBR from any vertebrate source, including mammals such as primates (e.g. humans, cynomolgus monkey (cyno)) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed ETBR as well as any form of ETBR that results from processing in the cell. The term also encompasses naturally occurring variants of ETBR, e.g., splice variants, allelic variants, and isoforms. The amino acid sequence of an exemplary human ETBR precursor (with signal sequence) is shown in SEQ ID NO: 10. The amino acid sequence of an exemplary human mature ETBR (without signal sequence) is shown in SEQ ID NO: 11.

[060] The term "ETBR-positive cancer" refers to a cancer comprising cells that express ETBR on their surface.

[061] The term "ETBR-positive cell" refers to a cell that expresses ETBR on its surface.

[062] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

[063] A "naked antibody" refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

[064] "Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

[065] The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

[066] "Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

[067] In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

[068] The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

[069] A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

[070] As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, immunoconjugates of the invention are used to delay development of a disease or to slow the progression of a disease.

[071] The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody

generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). (See, e.g., Kindt et al. *Kuby Immunology*, 6th ed., W.H. Freeman and Co., page 91 (2007).) A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano et al., *J. Immunol.* 150:880-887 (1993); Clarkson et al., *Nature* 352:624-628 (1991).

[072] The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors."

[073] "Alkyl" is C_1-C_{18} hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms. Examples are methyl (Me, -CH_3), ethyl (Et, -CH_2CH_3), 1-propyl (n-Pr, n-propyl, -$CH_2CH_2CH_3$), 2-propyl (i-Pr, i-propyl, -$CH(CH_3)_2$), 1-butyl (n-Bu, n-butyl, -$CH_2CH_2CH_2CH_3$), 2-methyl-1-propyl (i-Bu, i-butyl, -$CH_2CH(CH_3)_2$), 2-butyl (s-Bu, s-butyl, -$CH(CH_3)CH_2CH_3$), 2-methyl-2-propyl (t-Bu, t-butyl, -$C(CH_3)_3$), 1-pentyl (n-pentyl, -$CH_2CH_2CH_2CH_2CH_3$), 2-pentyl (-$CH(CH_3)CH_2CH_2CH_3$), 3-pentyl (-$CH(CH_2CH_3)_2$), 2-methyl-2-butyl (-$C(CH_3)_2CH_2CH_3$), 3-methyl-2-butyl (-$CH(CH_3)CH(CH_3)_2$), 3-methyl-1-butyl (-$CH_2CH_2CH(CH_3)_2$), 2-methyl-1-butyl (-$CH_2CH(CH_3)CH_2CH_3$), 1-hexyl (-$CH_2CH_2CH_2CH_2CH_2CH_3$), 2-hexyl (-$CH(CH_3)CH_2CH_2CH_2CH_3$), 3-hexyl (-$CH(CH_2CH_3)(CH_2CH_2CH_3)$), 2-methyl-2-pentyl (-$C(CH_3)_2CH_2CH_2CH_3$), 3-methyl-2-pentyl (-$CH(CH_3)CH(CH_3)CH_2CH_3$), 4-methyl-2-pentyl (-$CH(CH_3)CH_2CH(CH_3)_2$), 3-methyl-3-pentyl (-$C(CH_3)(CH_2CH_3)_2$), 2-methyl-3-pentyl (-$CH(CH_2CH_3)CH(CH_3)_2$), 2,3-dimethyl-2-butyl (-$C(CH_3)_2CH(CH_3)_2$), 3,3-dimethyl-2-butyl (-$CH(CH_3)C(CH_3)_3$.

[074] The term "C_1-C_8 alkyl," as used herein refers to a straight chain or branched, saturated or unsaturated hydrocarbon having from 1 to 8 carbon atoms. Representative "C_1-C_8 alkyl" groups include, but are not limited to, -methyl, -ethyl, -n-propyl, -n-butyl, -n-pentyl, -n-hexyl, -n-heptyl, -n-octyl, -n-nonyl and -n-decyl; while branched C_1-C_8 alkyls include, but are not limited to, -isopropyl, -*sec*-butyl, -isobutyl, -*tert*-butyl, -isopentyl, 2-methylbutyl, unsaturated C_1-C_8 alkyls include, but are not limited to, -vinyl, -allyl, -1-butenyl, -2-butenyl, -isobutylenyl, -1-pentenyl, -2-pentenyl, -3-methyl-1-butenyl, -2-methyl-2-butenyl, -

2,3-dimethyl-2-butenyl, 1-hexyl, 2-hexyl, 3-hexyl,-acetylenyl, -propynyl, -1-butynyl, -2-butynyl, -1-pentynyl, -2-pentynyl, -3-methyl-1 butynyl. A C_1-C_8 alkyl group can be unsubstituted or substituted with one or more groups including, but not limited to, -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2 , -C(O)NHR', -C(O)N(R')$_2$ -NHC(O)R', -SO_3R', -S(O)$_2$R', -S(O)R', -OH, -halogen, -N_3 , -NH_2, -NH(R'), -N(R')$_2$ and -CN; where each R' is independently selected from H, -C_1-C_8 alkyl and aryl.

[075] The term "C_1-C_6 alkyl," as used herein refers to a straight chain or branched, saturated or unsaturated hydrocarbon having from 1 to 6 carbon atoms. Representative "C_1-C_6 alkyl" groups include, but are not limited to, -methyl, -ethyl, -n-propyl, -n-butyl, -n-pentyl, - and n-hexyl; while branched C_1-C_6 alkyls include, but are not limited to, -isopropyl, -*sec*-butyl, -isobutyl, -*tert*-butyl, -isopentyl, and 2-methylbutyl; unsaturated C_1-C_6 alkyls include, but are not limited to, -vinyl, -allyl, -1-butenyl, -2-butenyl, and -isobutylenyl, -1-pentenyl, -2-pentenyl, -3-methyl-1-butenyl, -2-methyl-2-butenyl, -2,3-dimethyl-2-butenyl, 1-hexyl, 2-hexyl, and 3-hexyl. A C_1-C_6 alkyl group can be unsubstituted or substituted with one or more groups, as described above for C_1-C_8 alkyl group.

[076] The term "C_1-C_4 alkyl," as used herein refers to a straight chain or branched, saturated or unsaturated hydrocarbon having from 1 to 4 carbon atoms. Representative "C_1-C_4 alkyl" groups include, but are not limited to, -methyl, -ethyl, -n-propyl, -n-butyl; while branched C_1-C_4 alkyls include, but are not limited to, -isopropyl, -*sec*-butyl, -isobutyl, -*tert*-butyl; unsaturated C_1-C_4 alkyls include, but are not limited to, -vinyl, -allyl, -1-butenyl, -2-butenyl, and -isobutylenyl. A C_1-C_4 alkyl group can be unsubstituted or substituted with one or more groups, as described above for C_1-C_8 alkyl group.

[077] "Alkoxy" is an alkyl group singly bonded to an oxygen. Exemplary alkoxy groups include, but are not limited to, methoxy (-OCH_3) and ethoxy (-OCH_2CH_3). A "C_1-C_5 alkoxy" is an alkoxy group with 1 to 5 carbon atoms. Alkoxy groups may can be unsubstituted or substituted with one or more groups, as described above for alkyl groups.

[078] "Alkenyl" is C_2-C_{18} hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms with at least one site of unsaturation, i.e. a carbon-carbon, sp^2 double bond. Examples include, but are not limited to: ethylene or vinyl (-$CH=CH_2$), allyl (-$CH_2CH=CH_2$), cyclopentenyl (-C_5H_7), and 5-hexenyl (-CH_2 $CH_2CH_2CH_2CH=CH_2$). A "C_2-C_8 alkenyl" is a hydrocarbon containing 2 to 8 normal, secondary, tertiary or cyclic carbon atoms with at least one site of unsaturation, i.e. a carbon-carbon, sp^2 double bond.

[079] "Alkynyl" is C_2-C_{18} hydrocarbon containing normal, secondary, tertiary or cyclic carbon atoms with at least one site of unsaturation, i.e. a carbon-carbon, *sp* triple bond.

Examples include, but are not limited to: acetylenic (-C≡CH) and propargyl (-CH_2C≡CH). A "C_2-C_8 alkynyl" is a hydrocarbon containing 2 to 8 normal, secondary, tertiary or cyclic carbon atoms with at least one site of unsaturation, i.e. a carbon-carbon, *sp* triple bond.

[080] "Alkylene" refers to a saturated, branched or straight chain or cyclic hydrocarbon radical of 1-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkane. Typical alkylene radicals include, but are not limited to: methylene (-CH_2-) 1,2-ethyl (-CH_2CH_2-), 1,3-propyl (-$CH_2CH_2CH_2$-), 1,4-butyl (-$CH_2CH_2CH_2CH_2$-), and the like.

[081] A "C_1-C_{10} alkylene" is a straight chain, saturated hydrocarbon group of the formula -$(CH_2)_{1-10}$-. Examples of a C_1-C_{10} alkylene include methylene, ethylene, propylene, butylene, pentylene, hexylene, heptylene, ocytylene, nonylene and decalene.

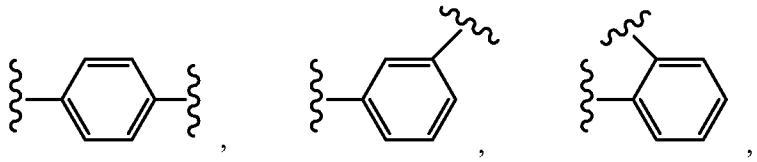
[082] "Alkenylene" refers to an unsaturated, branched or straight chain or cyclic hydrocarbon radical of 2-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkene. Typical alkenylene radicals include, but are not limited to: 1,2-ethylene (-CH=CH-).

[083] "Alkynylene" refers to an unsaturated, branched or straight chain or cyclic hydrocarbon radical of 2-18 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkyne. Typical alkynylene radicals include, but are not limited to: acetylene (-C≡C-), propargyl (-CH_2C≡C-), and 4-pentynyl (-$CH_2CH_2CH_2$C≡C-).

[084] "Aryl" refers to a carbocyclic aromatic group. Examples of aryl groups include, but are not limited to, phenyl, naphthyl and anthracenyl. A carbocyclic aromatic group or a heterocyclic aromatic group can be unsubstituted or substituted with one or more groups including, but not limited to, -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2 , -C(O)NHR', -C(O)N(R')$_2$ -NHC(O)R', -S(O)$_2$R', -S(O)R', -OH, -halogen, -N_3 , -NH_2, -NH(R'), -N(R')$_2$ and -CN; wherein each R' is independently selected from H, -C_1-C_8 alkyl and aryl.

[085] A "C_5-C_{20} aryl" is an aryl group with 5 to 20 carbon atoms in the carbocyclic aromatic rings. Examples of C_5-C_{20} aryl groups include, but are not limited to, phenyl, naphthyl and anthracenyl. A C_5-C_{20} aryl group can be substituted or unsubstituted as described above for aryl groups. A "C_5-C_{14} aryl" is an aryl group with 5 to 14 carbon atoms in the carbocyclic aromatic rings. Examples of C_5-C_{14} aryl groups include, but are not limited to, phenyl, naphthyl and anthracenyl. A C_5-C_{14} aryl group can be substituted or unsubstituted as described above for aryl groups.

[086] An "arylene" is an aryl group which has two covalent bonds and can be in the ortho, meta, or para configurations as shown in the following structures:



in which the phenyl group can be unsubstituted or substituted with up to four groups including, but not limited to, -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -$C(O)NH_2$, -C(O)NHR', -$C(O)N(R')_2$ -NHC(O)R', -$S(O)_2$R', -S(O)R', -OH, -halogen, -N_3, -NH_2, -NH(R'), -$N(R')_2$ and -CN; wherein each R' is independently selected from H, -C_1-C_8 alkyl and aryl.

[087] "Arylalkyl" refers to an acyclic alkyl radical in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or sp^3 carbon atom, is replaced with an aryl radical. Typical arylalkyl groups include, but are not limited to, benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl, 2-naphthylethan-1-yl, 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like. The arylalkyl group comprises 6 to 20 carbon atoms, e.g. the alkyl moiety, including alkanyl, alkenyl or alkynyl groups, of the arylalkyl group is 1 to 6 carbon atoms and the aryl moiety is 5 to 14 carbon atoms.

[088] "Heteroarylalkyl" refers to an acyclic alkyl radical in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or sp^3 carbon atom, is replaced with a heteroaryl radical. Typical heteroarylalkyl groups include, but are not limited to, 2-benzimidazolylmethyl, 2-furylethyl, and the like. The heteroarylalkyl group comprises 6 to 20 carbon atoms, e.g. the alkyl moiety, including alkanyl, alkenyl or alkynyl groups, of the heteroarylalkyl group is 1 to 6 carbon atoms and the heteroaryl moiety is 5 to 14 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S. The heteroaryl moiety of the heteroarylalkyl group may be a monocycle having 3 to 7 ring members (2 to 6 carbon atoms or a bicycle having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system.

[089] "Substituted alkyl," "substituted aryl," and "substituted arylalkyl" mean alkyl, aryl, and arylalkyl respectively, in which one or more hydrogen atoms are each independently replaced with a substituent. Typical substituents include, but are not limited to, -X, -R, -O^-, -OR, -SR, -S^-, -NR_2, -NR_3, =NR, -CX_3, -CN, -OCN, -SCN, -N=C=O, -NCS, -NO, -NO_2, =N_2, -N_3, NC(=O)R, -C(=O)R, -$C(=O)NR_2$, -SO_3^-, -SO_3H, -$S(=O)_2$R, -$OS(=O)_2$OR, -$S(=O)_2$NR, -S(=O)R, -$OP(=O)(OR)_2$, -$P(=O)(OR)_2$, -PO^-

$_3$, $-PO_3H_2$, -C(=O)R, -C(=O)X, -C(=S)R, $-CO_2R$, $-CO_2^-$
, -C(=S)OR, -C(=O)SR, -C(=S)SR, $-C(=O)NR_2$, $-C(=S)NR_2$, $-C(=NR)NR_2$, where each X is independently a halogen: F, Cl, Br, or I; and each R is independently -H, C_2-C_{18} alkyl, C_6-C_{20} aryl, C_3-C_{14} heterocycle, protecting group or prodrug moiety. Alkylene, alkenylene, and alkynylene groups as described above may also be similarly substituted.

[090] "Heteroaryl" and "heterocycle" refer to a ring system in which one or more ring atoms is a heteroatom, e.g. nitrogen, oxygen, and sulfur. The heterocycle radical comprises 3 to 20 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S. A heterocycle may be a monocycle having 3 to 7 ring members (2 to 6 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S) or a bicycle having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 3 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system.

[091] Exemplary heterocycles are described, e.g., in Paquette, Leo A., "Principles of Modern Heterocyclic Chemistry" (W.A. Benjamin, New York, 1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28; and *J. Am. Chem. Soc.* (1960) 82:5566.

[092] Examples of heterocycles include by way of example and not limitation pyridyl, dihydroypyridyl, tetrahydropyridyl (piperidyl), thiazolyl, tetrahydrothiophenyl, sulfur oxidized tetrahydrothiophenyl, pyrimidinyl, furanyl, thienyl, pyrrolyl, pyrazolyl, imidazolyl, tetrazolyl, benzofuranyl, thianaphthalenyl, indolyl, indolenyl, quinolinyl, isoquinolinyl, benzimidazolyl, piperidinyl, 4-piperidonyl, pyrrolidinyl, 2-pyrrolidonyl, pyrrolinyl, tetrahydrofuranyl, bis-tetrahydrofuranyl, tetrahydropyranyl, bis-tetrahydropyranyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, decahydroquinolinyl, octahydroisoquinolinyl, azocinyl, triazinyl, 6H-1,2,5-thiadiazinyl, 2H,6H-1,5,2-dithiazinyl, thienyl, thianthrenyl, pyranyl, isobenzofuranyl, chromenyl, xanthenyl, phenoxathinyl, 2H-pyrrolyl, isothiazolyl, isoxazolyl, pyrazinyl, pyridazinyl, indolizinyl, isoindolyl, 3H-indolyl, 1H-indazolyl, purinyl, 4H-quinolizinyl, phthalazinyl, naphthyridinyl, quinoxalinyl, quinazolinyl, cinnolinyl, pteridinyl, 4aH-carbazolyl, carbazolyl, β-carbolinyl, phenanthridinyl, acridinyl, pyrimidinyl, phenanthrolinyl, phenazinyl, phenothiazinyl, furazanyl, phenoxazinyl, isochromanyl, chromanyl, imidazolidinyl, imidazolinyl, pyrazolidinyl, pyrazolinyl, piperazinyl, indolinyl, isoindolinyl, quinuclidinyl, morpholinyl, oxazolidinyl, benzotriazolyl, benzisoxazolyl, oxindolyl, benzoxazolinyl, and isatinoyl.

[093] By way of example and not limitation, carbon bonded heterocycles are bonded at position 2, 3, 4, 5, or 6 of a pyridine, position 3, 4, 5, or 6 of a pyridazine, position 2, 4, 5, or

6 of a pyrimidine, position 2, 3, 5, or 6 of a pyrazine, position 2, 3, 4, or 5 of a furan, tetrahydrofuran, thiofuran, thiophene, pyrrole or tetrahydropyrrole, position 2, 4, or 5 of an oxazole, imidazole or thiazole, position 3, 4, or 5 of an isoxazole, pyrazole, or isothiazole, position 2 or 3 of an aziridine, position 2, 3, or 4 of an azetidine, position 2, 3, 4, 5, 6, 7, or 8 of a quinoline or position 1, 3, 4, 5, 6, 7, or 8 of an isoquinoline. Still more typically, carbon bonded heterocycles include 2-pyridyl, 3-pyridyl, 4-pyridyl, 5-pyridyl, 6-pyridyl, 3-pyridazinyl, 4-pyridazinyl, 5-pyridazinyl, 6-pyridazinyl, 2-pyrimidinyl, 4-pyrimidinyl, 5-pyrimidinyl, 6-pyrimidinyl, 2-pyrazinyl, 3-pyrazinyl, 5-pyrazinyl, 6-pyrazinyl, 2-thiazolyl, 4-thiazolyl, or 5-thiazolyl.

[094] By way of example and not limitation, nitrogen bonded heterocycles are bonded at position 1 of an aziridine, azetidine, pyrrole, pyrrolidine, 2-pyrroline, 3-pyrroline, imidazole, imidazolidine, 2-imidazoline, 3-imidazoline, pyrazole, pyrazoline, 2-pyrazoline, 3-pyrazoline, piperidine, piperazine, indole, indoline, 1H-indazole, position 2 of a isoindole, or isoindoline, position 4 of a morpholine, and position 9 of a carbazole, or β-carboline. Still more typically, nitrogen bonded heterocycles include 1-aziridyl, 1-azetedyl, 1-pyrrolyl, 1-imidazolyl, 1-pyrazolyl, and 1-piperidinyl.

[095] A "C_3-C_8 heterocycle" refers to an aromatic or non-aromatic C_3-C_8 carbocycle in which one to four of the ring carbon atoms are independently replaced with a heteroatom from the group consisting of O, S and N. Representative examples of a C_3-C_8 heterocycle include, but are not limited to, benzofuranyl, benzothiophene, indolyl, benzopyrazolyl, coumarinyl, isoquinolinyl, pyrrolyl, thiophenyl, furanyl, thiazolyl, imidazolyl, pyrazolyl, triazolyl, quinolinyl, pyrimidinyl, pyridinyl, pyridonyl, pyrazinyl, pyridazinyl, isothiazolyl, isoxazolyl and tetrazolyl. A C_3-C_8 heterocycle can be unsubstituted or substituted with up to seven groups including, but not limited to, -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2 , -C(O)NHR', -C(O)$N(R')_2$ -NHC(O)R', -$S(O)_2$R', -S(O)R', -OH, -halogen, -N_3 , -NH_2, -NH(R'), -$N(R')_2$ and -CN; wherein each R' is independently selected from H, -C_1-C_8 alkyl and aryl.

[096] "C_3-C_8 heterocyclo" refers to a C_3-C_8 heterocycle group defined above wherein one of the heterocycle group's hydrogen atoms is replaced with a bond. A C_3-C_8 heterocyclo can be unsubstituted or substituted with up to six groups including, but not limited to, -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2 , -C(O)NHR', -C(O)$N(R')_2$ -NHC(O)R', -$S(O)_2$R', -S(O)R', -OH, -halogen, -N_3 , -NH_2, -NH(R'), -$N(R')_2$ and -CN; wherein each R' is independently selected from H, -C_1-C_8 alkyl and aryl.

[097] "Carbocycle" means a saturated or unsaturated ring having 3 to 7 carbon atoms as a monocycle or 7 to 12 carbon atoms as a bicycle. Monocyclic carbocycles have 3 to 6 ring

atoms, still more typically 5 or 6 ring atoms. Bicyclic carbocycles have 7 to 12 ring atoms, e.g. arranged as a bicyclo [4,5], [5,5], [5,6] or [6,6] system, or 9 or 10 ring atoms arranged as a bicyclo [5,6] or [6,6] system. Examples of monocyclic carbocycles include cyclopropyl, cyclobutyl, cyclopentyl, 1-cyclopent-1-enyl, 1-cyclopent-2-enyl, 1-cyclopent-3-enyl, cyclohexyl, 1-cyclohex-1-enyl, 1-cyclohex-2-enyl, 1-cyclohex-3-enyl, cycloheptyl, and cyclooctyl.

[098] A "C_3-C_8 carbocycle" is a 3-, 4-, 5-, 6-, 7- or 8-membered saturated or unsaturated non-aromatic carbocyclic ring. Representative C_3-C_8 carbocycles include, but are not limited to, -cyclopropyl, -cyclobutyl, -cyclopentyl, -cyclopentadienyl, -cyclohexyl, -cyclohexenyl, -1,3-cyclohexadienyl, -1,4-cyclohexadienyl, -cycloheptyl, -1,3-cycloheptadienyl, -1,3,5-cycloheptatrienyl, -cyclooctyl, and -cyclooctadienyl. A C_3-C_8 carbocycle group can be unsubstituted or substituted with one or more groups including, but not limited to, -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2, -C(O)NHR', -C(O)N(R')$_2$ -NHC(O)R', -S(O)$_2$R', -S(O)R', -OH, -halogen, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and -CN; where each R' is independently selected from H, -C_1-C_8 alkyl and aryl.

[099] A "C_3-C_8 carbocyclo" refers to a C_3-C_8 carbocycle group defined above wherein one of the carbocycle groups' hydrogen atoms is replaced with a bond.

[0100] "Linker" refers to a chemical moiety comprising a covalent bond or a chain of atoms that covalently attaches an antibody to a drug moiety. In various embodiments, linkers include a divalent radical such as an alkyldiyl, an aryldiyl, a heteroaryldiyl, moieties such as: $-(CR_2)_nO(CR_2)_n-$, repeating units of alkyloxy (e.g. polyethylenoxy, PEG, polymethyleneoxy) and alkylamino (e.g. polyethyleneamino, Jeffamine™); and diacid ester and amides including succinate, succinamide, diglycolate, malonate, and caproamide. In various embodiments, linkers can comprise one or more amino acid residues, such as valine, phenylalanine, lysine, and homolysine.

[0101] The term "chiral" refers to molecules which have the property of non-superimposability of the mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner.

[0102] The term "stereoisomers" refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

[0103] "Diastereomer" refers to a stereoisomer with two or more centers of chirality and whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities.

Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.

[0104] "Enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another.

[0105] Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., *McGraw-Hill Dictionary of Chemical Terms* (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., *Stereochemistry of Organic Compounds* (1994) John Wiley & Sons, Inc., New York. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L, or *R* and *S,* are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (-) are employed to designate the sign of rotation of plane-polarized light by the compound, with (-) or l meaning that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these stereoisomers are identical except that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate, which may occur where there has been no stereoselection or stereospecificity in a chemical reaction or process. The terms "racemic mixture" and "racemate" refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

[0106] "Leaving group" refers to a functional group that can be substituted by another functional group. Certain leaving groups are well known in the art, and examples include, but are not limited to, a halide (*e.g.*, chloride, bromide, iodide), methanesulfonyl (mesyl), p-toluenesulfonyl (tosyl), trifluoromethylsulfonyl (triflate), and trifluoromethylsulfonate.

[0107] The term "protecting group" refers to a substituent that is commonly employed to block or protect a particular functionality while reacting other functional groups on the compound. For example, an "amino-protecting group" is a substituent attached to an amino group that blocks or protects the amino functionality in the compound. Suitable amino-protecting groups include, but are not limited to, acetyl, trifluoroacetyl, t-butoxycarbonyl (BOC), benzyloxycarbonyl (CBZ) and 9-fluorenylmethylenoxycarbonyl (Fmoc). For a general description of protecting groups and their use, see T. W. Greene, Protective Groups in Organic Synthesis, John Wiley & Sons, New York, 1991, or a later edition.

II. COMPOSITIONS AND METHODS

[0108] In one aspect, the invention is based, in part, on antibodies that bind to ETBR and immunoconjugates comprising such antibodies. Antibodies and immunoconjugates of the invention are useful, e.g., for the diagnosis or treatment of ETBR-positive cancers.

A. Exemplary Anti-ETBR Antibodies

[0109] In some embodiments, isolated antibodies that bind to ETBR are provided. ETBR is a G-protein coupled receptor expressed in melanocytes.

[0110] An exemplary naturally occurring human ETBR precursor sequence, with signal sequence (amino acids 1 to 26) is provided in SEQ ID NO: 10, and the corresponding mature ETBR sequence is shown in SEQ ID NO: 11 (corresponding to amino acids 27 to 442 of SEQ ID NO: 10).

[0111] In certain embodiments, an anti-ETBR antibody binds an epitope within amino acids 64 to 101 of SEQ ID NO: 10. Nonlimiting exemplary such antibodies include 5E9 and humanized versions thereof. In some embodiments, an anti-ETBR antibody binds human ETBR. In some embodiments, an anti-ETBR antibody binds human ETBR and cynomolgus monkey ETBR.

[0112] In some embodiments, an anti-ETBR antibody binds human ETBR with an affinity of $\leq$ 10 nM, or $\leq$ 7 nM, or $\leq$ 6 nM, or $\leq$ 5 nM, or $\leq$ 4 nM, and optionally $\geq$ 0.0001 nM, or $\geq$ 0.001 nM, or $\geq$ 0.01 nM. Nonlimiting exemplary such antibodies include mu5E9 and hu5E9.v1, which bind to human ETBR with an affinity of 5 nM and 3.7 nM, respectively.

Assays

[0113] To determine whether an anti-ETBR antibody "binds to an epitope within amino acids 64 to 101 of SEQ ID NO: 10," ETBR polypeptides with N- and C-terminal deletions are expressed in mammalian cells (such as CHO cells or 293 cells) and binding of the antibody to the truncated polypeptides is tested by FACS. A substantial reduction ($\geq$ 70% reduction) or elimination of binding of the antibody to a truncated polypeptide relative to binding to full-length ETBR expressed in cells indicates that the antibody does not bind to that truncated polypeptide. Alternatively, in some embodiments, whether an anti-ETBR antibody "binds to an epitope within amino acids 64 to 101 of SEQ ID NO: 10" is determined using an ELISA assay. A substantial reduction ($\geq$ 70% reduction) or elimination of binding of the antibody to a truncated polypeptide relative to binding to a longer portion of ETBR, such as the extracellular domain, indicates that the antibody does not bind to that truncated polypeptide.

[0114] Whether an anti-ETBR antibody "binds with an affinity of" $\leq$ 10 nM, or $\leq$ 7 nM, or $\leq$ 6 nM, or $\leq$ 5 nM, or $\leq$ 4 nM, may be determined using mammalian cells (such as

CHO cells or 293 cells) expressing ETBR on the surface in a competition assay using serially diluted, unlabeled anti-ETBR antibody. Binding affinity, K_D, of the antibodies may be determined in accordance with standard Scatchard analysis performed utilizing a non-linear curve fitting program (see, for example, Munson et al., Anal Biochem, 107: 220-239, 1980). In some embodiments, whether an anti-ETBR antibody "binds with an affinity of" ≤ 10 nM, or ≤ 7 nM, or ≤ 6 nM, or ≤ 5 nM, or ≤ 4 nM, may be determined using surface plasmon resonance, such as a Biacore™ assay, or kinetic exclusion assay (KinExA®, Sapidyne Instruments, Boise, ID).

Antibody 5E9 and other embodiments

[0115] In some embodiments, the invention provides an anti-ETBR antibody or immunoconjugate comprising at least one, two, three, four, five, or six HVRs selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

[0116] In one aspect, the invention provides an antibody or immunoconjugate comprising at least one, at least two, or all three VH HVR sequences selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14. In one embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14. In another embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14 and HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In a further embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14, HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17, and HVR-H2 comprising the amino acid sequence of SEQ ID NO: 15. In a further embodiment, the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14.

[0117] In another aspect, the invention provides an antibody or immunoconjugate comprising at least one, at least two, or all three VL HVR sequences selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In another aspect, the invention provides an antibody or immunoconjugate comprising at least one, at least two, or all three VL HVR sequences selected from (a) HVR-L1

comprising the amino acid sequence of SEQ ID NO: 15; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In one embodiment, the antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In one embodiment, the antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

[0118] In another aspect, an antibody of the invention or immunoconjugate comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences selected from (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (iii) HVR-H3 comprising an amino acid sequence selected from SEQ ID NO: 14; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences selected from (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In another aspect, an antibody or immunoconjugate of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences selected from (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (iii) HVR-H3 comprising an amino acid sequence selected from SEQ ID NO: 14; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences selected from (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

[0119] In another aspect, the invention provides an antibody or immunoconjugate comprising (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In another aspect, the invention provides an antibody or immunoconjugate comprising (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (e) HVR-L2 comprising the amino

acid sequence of SEQ ID NO: 16; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

[0120] In any of the above embodiments, an anti-ETBR antibody is humanized. In one embodiment, an anti-ETBR antibody comprises HVRs as in any of the above embodiments, and further comprises a human acceptor framework, e.g. a human immunoglobulin framework or a human consensus framework. In certain embodiments, the human acceptor framework is the human VL kappa 1 (VL_{KI}) framework and/or the VH framework VH_{III}. In some embodiments, a humanized anti-ETBR antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17. In some embodiments, a humanized anti-ETBR antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

[0121] In another aspect, an anti-ETBR antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 8. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence of SEQ ID NO: 8 contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-ETBR antibody comprising that sequence retains the ability to bind to ETBR. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 8. In certain embodiments, a total of 1 to 5 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 8. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs).

[0122] Optionally, the anti-ETBR antibody comprises a VH sequence selected from SEQ ID NOs: 4, 8, and 9, including post-translational modifications of that sequence. In a particular embodiment, the VH comprises one, two or three HVRs selected from: (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14.

[0123] In some embodiments, an anti-ETBR antibody is provided, wherein the antibody comprises a light chain variable domain (VL) having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 7. In certain embodiments, a VL sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence of SEQ ID NO: 7 contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-ETBR antibody comprising that sequence retains the ability to bind to ETBR. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 7. In certain embodiments, a total of 1 to 5 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 7. In certain embodiments, the substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-ETBR antibody comprises the VL sequence of SEQ ID NO: 3 or SEQ ID NO: 7, including post-translational modifications of that sequence. In a particular embodiment, the VL comprises one, two or three HVRs selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

[0124] In another aspect, an anti-ETBR antibody is provided, wherein the antibody comprises a VH as in any of the embodiments provided above, and a VL as in any of the embodiments provided above. In some embodiments, the antibody comprises the VH and VL sequences in SEQ ID NO: 8 and SEQ ID NO: 7, respectively, including post-translational modifications of those sequences. In some embodiments, the antibody comprises the VH and VL sequences in SEQ ID NO: 9 and SEQ ID NO: 7, respectively, including post-translational modifications of those sequences. In some embodiments, the antibody comprises the heavy chain and light chain sequences in SEQ ID NO: 6 and SEQ ID NO: 5, respectively, including post-translational modifications of those sequences. In some embodiments, the antibody comprises the heavy chain and light chain sequences in SEQ ID NO: 6 and SEQ ID NO: 18, respectively, including post-translational modifications of those sequences. In some embodiments, the antibody comprises the heavy chain and light chain sequences in SEQ ID NO: 19 and SEQ ID NO: 5, respectively, including post-translational modifications of those sequences. In some embodiments, the antibody comprises the heavy chain and light chain sequences in SEQ ID NO: 20 and SEQ ID NO: 5, respectively, including post-translational modifications of those sequences.

[0125] In a further aspect, the invention provides an antibody or immunoconjugate that binds to the same epitope as an anti-ETBR antibody provided herein. For example, in certain embodiments, an antibody or immunoconjugate is provided that binds to the same epitope as

an anti-ETBR antibody comprising a VH sequence of SEQ ID NO: 8 and a VL sequence of SEQ ID NO: 7. In certain embodiments, an antibody is provided that binds to an epitope of SEQ ID NO: 10 from, within, or overlapping amino acids 64 to 101.

[0126] In a further aspect of the invention, an anti-ETBR antibody according to any of the above embodiments is a monoclonal antibody, including a chimeric, humanized or human antibody. In one embodiment, an anti-ETBR antibody is an antibody fragment, e.g., a Fv, Fab, Fab', scFv, diabody, or $F(ab')_2$ fragment. In another embodiment, the antibody is a substantially full length antibody, e.g., an IgG1 antibody or other antibody class or isotype as defined herein.

[0127] In any of the immunoconjugates described above, the antibody may be conjugated to a drug moiety. In some embodiments, the antibody is conjugated to a cytotoxic agent. In some such embodiments, the cytotoxic agent is a nemorubicin derivative, such as PNU-159682. Various nonlimiting exemplary nemorubicin derivatives are discussed herein.

[0128] In a further aspect, an anti-ETBR antibody or immunoconjugate according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-7 below.

1. Antibody Affinity

[0129] In certain embodiments, an antibody provided herein has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM, and optionally is $\geq 10^{-13}$ M. (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M).

[0130] In one embodiment, Kd is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay. Solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, e.g., Chen et al., *J. Mol. Biol.* 293:865-881(1999)). To establish conditions for the assay, MICROTITER® multi-well plates (Thermo Scientific) are coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta et al., *Cancer Res.* 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may

continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% polysorbate 20 (TWEEN-20®) in PBS. When the plates have dried, 150 µl/well of scintillant (MICROSCINT-20™; Packard) is added, and the plates are counted on a TOPCOUNT™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

[0131] According to another embodiment, Kd is measured using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 µg/ml (~0.2 µM) before injection at a flow rate of 5 µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of approximately 25 µl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, e.g., Chen et al., *J. Mol. Biol.* 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

2. Antibody Fragments

[0132] In certain embodiments, an antibody provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, $F(ab')_2$, Fv, and scFv fragments, and other fragments described below. For a review of certain antibody fragments,

see Hudson et al. *Nat. Med.* 9:129-134 (2003). For a review of scFv fragments, see, e.g., Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and $F(ab')_2$ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

[0133] Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; Hudson et al., *Nat. Med.* 9:129-134 (2003); and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., *Nat. Med.* 9:129-134 (2003).

[0134] Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; *see,* e.g., U.S. Patent No. 6,248,516 B1).

[0135] Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. *E. coli* or phage), as described herein.

3. Chimeric and Humanized Antibodies

[0136] In certain embodiments, an antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

[0137] In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody

(e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

[0138] Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008), and are further described, e.g., in Riechmann et al., *Nature* 332:323-329 (1988); Queen et al., *Proc. Nat'l Acad. Sci. USA* 86:10029-10033 (1989); US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri *et al.*, *Methods* 36:25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol. Immunol.* 28:489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36:43-60 (2005) (describing "FR shuffling"); and Osbourn et al., *Methods* 36:61-68 (2005) and Klimka et al., *Br. J. Cancer*, 83:252-260 (2000) (describing the "guided selection" approach to FR shuffling).

[0139] Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims et al. *J. Immunol.* 151:2296 (1993)); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter et al. *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); and Presta et al. *J. Immunol.*, 151:2623 (1993)); human mature (somatically mutated) framework regions or human germline framework regions (see, e.g., Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008)); and framework regions derived from screening FR libraries (see, e.g., Baca et al., *J. Biol. Chem.* 272:10678-10684 (1997) and Rosok et al., *J. Biol. Chem.* 271:22611-22618 (1996)).

4. Human Antibodies

[0140] In certain embodiments, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr. Opin. Pharmacol.* 5: 368-74 (2001) and Lonberg, *Curr. Opin. Immunol.* 20:450-459 (2008).

[0141] Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, *Nat. Biotech.* 23:1117-1125 (2005). See also, e.g., U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSE™

technology; U.S. Patent No. 5,770,429 describing HUMAB® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

[0142] Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (See, e.g., Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., *J. Immunol.*, 147: 86 (1991).) Human antibodies generated via human B-cell hybridoma technology are also described in Li et al., *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

[0143] Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described below.

5. Library-Derived Antibodies

[0144] Antibodies may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, e.g., in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and further described, e.g., in the McCafferty et al., *Nature* 348:552-554; Clackson et al., *Nature* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Marks and Bradbury, in *Methods in Molecular Biology* 248:161-175 (Lo, ed., Human Press, Totowa, NJ, 2003); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132(2004).

[0145] In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-455 (1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., *EMBO J,* 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent No. 5,750,373, and US Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

[0146] Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

6. Multispecific Antibodies

[0147] In certain embodiments, an antibody provided herein is a multispecific antibody, e.g. a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for ETBR and the other is for any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of ETBR. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ETBR. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

[0148] Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983)), WO 93/08829, and Traunecker et al., *EMBO J.* 10: 3655 (1991)), and "knob-in-hole" engineering (see, e.g., U.S. Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al., *Science*, 229: 81 (1985)); using leucine zippers to produce

bi-specific antibodies (see, e.g., Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992)); using "diabody" technology for making bispecific antibody fragments (see, e.g., Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (see,e.g. Gruber et al., *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, e.g., in Tutt et al. *J. Immunol.* 147: 60 (1991).

[0149] Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (see, e.g. US 2006/0025576A1).

[0150] The antibody or fragment herein also includes a "Dual Acting FAb" or "DAF" comprising an antigen binding site that binds to ETBR as well as another, different antigen (see, US 2008/0069820, for example).

7. Antibody Variants

[0151] In certain embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

a) Substitution, Insertion, and Deletion Variants

[0152] In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions." More substantial changes are provided in Table 1 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys

Original Residue	Exemplary Substitutions	Preferred Substitutions
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

[0153] Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0154] One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain

biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

[0155] Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve antibody affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, *Methods Mol. Biol.* 207:179-196 (2008)), and/or SDRs (a-CDRs), with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

[0156] In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

[0157] A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an

antigen-antibody complex is used to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

[0158] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

b) Glycosylation variants

[0159] In certain embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

[0160] Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody may be made in order to create antibody variants with certain improved properties.

[0161] In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or

downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. See, e.g., US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al. *J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8,* knockout CHO cells (see, e.g., Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004); Kanda, Y. et al., *Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

[0162] Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/011878 (Jean-Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana *et al.*). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

c) Fc region variants

[0163] In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions.

[0164] In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in*

vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, e.g. Hellstrom, I. et al. *Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I et al., *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in a animal model such as that disclosed in Clynes et al. *Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. See, e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro *et al., J. Immunol. Methods* 202:163 (1996); Cragg, M.S. et al., *Blood* 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., *Int'l. Immunol.* 18(12):1759-1769 (2006)).

[0165] Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

[0166] Certain antibody variants with improved or diminished binding to FcRs are described. (See, e.g., U.S. Patent No. 6,737,056; WO 2004/056312, and Shields et al., *J. Biol. Chem.* 9(2): 6591-6604 (2001).)

[0167] In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

[0168] In some embodiments, alterations are made in the Fc region that result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

[0169] Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826).

[0170] See also Duncan & Winter, *Nature* 322:738-40 (1988); U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

d) Cysteine engineered antibody variants

[0171] In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Nonlimiting exemplary cysteine engineered heavy chains and light chains of anti-ETBR antibodies are shown in SEQ ID NOs: 18, 19, and 20. Cysteine engineered antibodies may be generated as described, e.g., in U.S. Patent No. 7,521,541.

e) Antibody Derivatives

[0172] In certain embodiments, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available.

The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

[0173] In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam et al., *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

B. Recombinant Methods and Compositions

[0174] Antibodies may be produced using recombinant methods and compositions, e.g., as described in U.S. Patent No. 4,816,567. In one embodiment, isolated nucleic acid encoding an anti-ETBR antibody described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or (2) a first vector comprising a

nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of making an anti-ETBR antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

[0175] For recombinant production of an anti-ETBR antibody, nucleic acid encoding an antibody, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody).

[0176] Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523. (See also Charlton, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*.) After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

[0177] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern. See Gerngross, *Nat. Biotech.* 22:1409-1414 (2004), and Li et al., *Nat. Biotech.* 24:210-215 (2006).

[0178] Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells.

[0179] Plant cell cultures can also be utilized as hosts. *See,* e.g., US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

[0180] Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982); MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including $DHFR^-$ CHO cells (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki and Wu, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003).

C. Assays

[0181] Anti-ETBR antibodies provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

[0182] In one aspect, an antibody is tested for its antigen binding activity, e.g., by known methods such as ELISA, BIACore®, FACS, or Western blot.

[0183] In another aspect, competition assays may be used to identify an antibody that competes with any of the antibodies described herein for binding to ETBR. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by an antibody described herein. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in *Methods in Molecular Biology* vol. 66 (Humana Press, Totowa, NJ).

[0184] In an exemplary competition assay, immobilized ETBR is incubated in a solution comprising a first labeled antibody that binds to ETBR (e.g., any of the antibodies described herein) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to ETBR. The second antibody may be present in a hybridoma supernatant. As a control, immobilized ETBR is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under

conditions permissive for binding of the first antibody to ETBR, excess unbound antibody is removed, and the amount of label associated with immobilized ETBR is measured. If the amount of label associated with immobilized ETBR is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to ETBR. See Harlow and Lane (1988) *Antibodies: A Laboratory Manual* ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

D. Immunoconjugates

[0185] The invention also provides immunoconjugates comprising an anti-ETBR antibody herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (e.g., protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes (i.e., a radioconjugate).

[0186] Immunoconjugates allow for the targeted delivery of a drug moiety to a tumor, and, in some embodiments intracellular accumulation therein, where systemic administration of unconjugated drugs may result in unacceptable levels of toxicity to normal cells (Polakis P. (2005) *Current Opinion in Pharmacology* 5:382-387).

[0187] Antibody-drug conjugates (ADC) are targeted chemotherapeutic molecules which combine properties of both antibodies and cytotoxic drugs by targeting potent cytotoxic drugs to antigen-expressing tumor cells (Teicher, B.A. (2009) *Current Cancer Drug Targets* 9:982-1004), thereby enhancing the therapeutic index by maximizing efficacy and minimizing off-target toxicity (Carter, P.J. and Senter P.D. (2008) *The Cancer Jour.* 14(3):154-169; Chari, R.V. (2008) *Acc. Chem. Res.* 41:98-107 .

[0188] The ADC compounds of the invention include those with anticancer activity. In some embodiments, the ADC compounds include an antibody conjugated, i.e. covalently attached, to the drug moiety. In some embodiments, the antibody is covalently attached to the drug moiety through a linker. The antibody-drug conjugates (ADC) of the invention selectively deliver an effective dose of a drug to tumor tissue whereby greater selectivity, i.e. a lower efficacious dose, may be achieved while increasing the therapeutic index ("therapeutic window").

[0189] The drug moiety (D) of the antibody-drug conjugates (ADC) may include any compound, moiety or group that has a cytotoxic or cytostatic effect. Exemplary drug moieties include, but are not limited to, nemorubicin and its derivatives, such as PNU-159682, that have cytotoxic activity. Nonlimiting examples of such immunoconjugates are discussed in further detail below.

1. Exemplary Antibody-drug Conjugates

[0190] An exemplary embodiment of an antibody-drug conjugate (ADC) compound comprises an antibody (Ab) which targets a tumor cell, a drug moiety (D), and a linker moiety (L) that attaches Ab to D. In some embodiments, the antibody is attached to the linker moiety (L) through one or more amino acid residues, such as lysine and/or cysteine.

[0191] An exemplary ADC has Formula I:

$$Ab–(L–D)_p \qquad \mathbf{I}$$

where p is 1 to about 20. In some embodiments, the number of drug moieties that can be conjugated to an antibody is limited by the number of free cysteine residues. In some embodiments, free cysteine residues are introduced into the antibody amino acid sequence by the methods described herein. Exemplary ADC of Formula I include, but are not limited to, antibodies that have 1, 2, 3, or 4 engineered cysteine amino acids (Lyon, R. et al (2012) *Methods in Enzym.* 502:123-138). In some embodiments, one or more free cysteine residues are already present in an antibody, without the use of engineering, in which case the existing free cysteine residues may be used to conjugate the antibody to a drug. In some embodiments, an antibody is exposed to reducing conditions prior to conjugation of the antibody in order to generate one or more free cysteine residues.

a) Exemplary Linkers

[0192] A "Linker" (L) is a bifunctional or multifunctional moiety that can be used to link one or more drug moieties (D) to an antibody (Ab) to form an antibody-drug conjugate (ADC) of Formula I. In some embodiments, antibody-drug conjugates (ADC) can be prepared using a Linker having reactive functionalities for covalently attaching to the drug and to the antibody. For example, in some embodiments, a cysteine thiol of an antibody (Ab) can form a bond with a reactive functional group of a linker or a drug-linker intermediate to make an ADC.

[0193] In one aspect, a linker has a functionality that is capable of reacting with a free cysteine present on an antibody to form a covalent bond. Nonlimiting exemplary such reactive functionalities include maleimide, haloacetamides, α-haloacetyl, activated esters such as succinimide esters, 4-nitrophenyl esters, pentafluorophenyl esters, tetrafluorophenyl esters, anhydrides, acid chlorides, sulfonyl chlorides, isocyanates, and isothiocyanates. *See, e.g.*, the conjugation method at page 766 of Klussman, et al (2004), *Bioconjugate Chemistry* 15(4):765-773, and the Examples herein.

[0194] In some embodiments, a linker has a functionality that is capable of reacting with an electrophilic group present on an antibody. Exemplary such electrophilic groups

include, but are not limited to, aldehyde and ketone carbonyl groups. In some embodiments, a heteroatom of the reactive functionality of the linker can react with an electrophilic group on an antibody and form a covalent bond to an antibody unit. Nonlimiting exemplary such reactive functionalities include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide.

[0195] A linker may comprise one or more linker components. Exemplary linker components include 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit" or "vc"), alanine-phenylalanine ("ala-phe"), p-aminobenzyloxycarbonyl (a "PAB"), N-Succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), and 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("MCC"). Various linker components are known in the art, some of which are described below.

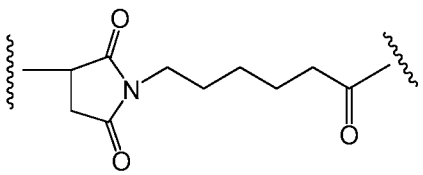
[0196] A linker may be a "cleavable linker," facilitating release of a drug. Nonlimiting exemplary cleavable linkers include acid-labile linkers (e.g., comprising hydrazone), protease-sensitive (e.g., peptidase-sensitive) linkers, photolabile linkers, or disulfide-containing linkers (Chari et al., Cancer Research 52:127-131 (1992); US 5208020).

[0197] In certain embodiments, a linker has the following Formula II:

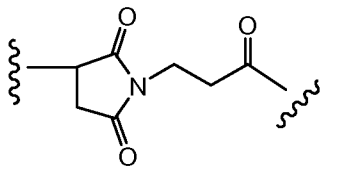
$$—A_a—W_w—Y_y— \qquad \textbf{II}$$

wherein A is a "stretcher unit", and a is an integer from 0 to 1; W is an "amino acid unit", and w is an integer from 0 to 12; Y is a "spacer unit", and y is 0, 1, or 2. An ADC comprising the linker of Formula II has the Formula I(A): Ab-(A_a-W_w-Y_y-D)p, wherein Ab, D, and p are defined as above for Formula I. Exemplary embodiments of such linkers are described in U.S. Patent No. 7,498,298, which is expressly incorporated herein by reference.

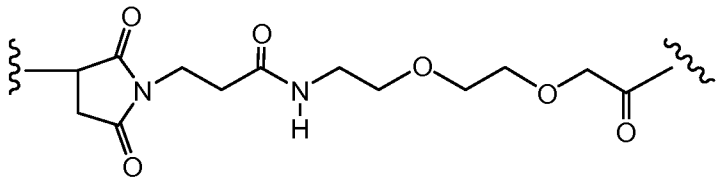
[0198] In some embodiments, a linker component comprises a "stretcher unit" (A) that links an antibody to another linker component or to a drug moiety. Nonlimiting exemplary stretcher units are shown below (wherein the wavy line indicates sites of covalent attachment to an antibody, drug, or additional linker components):



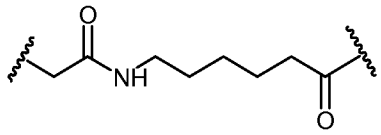
MC



MP



mPEG



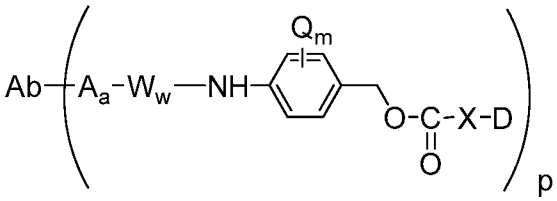
[0199] In some embodiments, a linker component comprises an "amino acid unit" (W). In some such embodiments, the amino acid unit allows for cleavage of the linker by a protease, thereby facilitating release of the drug from the immunoconjugate upon exposure to intracellular proteases, such as lysosomal enzymes (Doronina et al. (2003) *Nat. Biotechnol.* 21:778-784). Exemplary amino acid units include, but are not limited to, dipeptides, tripeptides, tetrapeptides, and pentapeptides. Exemplary dipeptides include, but are not limited to, valine-citrulline (vc or val-cit), alanine-phenylalanine (af or ala-phe); phenylalanine-lysine (fk or phe-lys); phenylalanine-homolysine (phe-homolys); and N-methyl-valine-citrulline (Me-val-cit). Exemplary tripeptides include, but are not limited to, glycine-valine-citrulline (gly-val-cit) and glycine-glycine-glycine (gly-gly-gly). An amino acid unit may comprise amino acid residues that occur naturally and/or minor amino acids and/or non-naturally occurring amino acid analogs, such as citrulline. Amino acid units can be designed and optimized for enzymatic cleavage by a particular enzyme, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

[0200] Typically, peptide-type linkers can be prepared by forming a peptide bond between two or more amino acids and/or peptide fragments. Such peptide bonds can be prepared, for example, according to a liquid phase synthesis method (e.g., E. Schröder and K. Lübke (1965) "The Peptides", volume 1, pp 76-136, Academic Press).

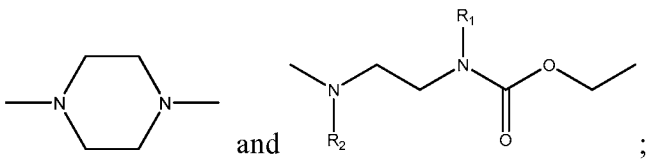
[0201] In some embodiments, a linker component comprises a "spacer unit" (Y) that links the antibody to a drug moiety, either directly or through a stretcher unit and/or an amino acid unit. A spacer unit may be "self-immolative" or a "non-self-immolative." A "non-self-

immolative" spacer unit is one in which part or all of the spacer unit remains bound to the drug moiety upon cleavage of the ADC. Examples of non-self-immolative spacer units include, but are not limited to, a glycine spacer unit and a glycine-glycine spacer unit. In some embodiments, enzymatic cleavage of an ADC containing a glycine-glycine spacer unit by a tumor-cell associated protease results in release of a glycine-glycine-drug moiety from the remainder of the ADC. In some such embodiments, the glycine-glycine-drug moiety is subjected to a hydrolysis step in the tumor cell, thus cleaving the glycine-glycine spacer unit from the drug moiety.

[0202] A "self-immolative" spacer unit allows for release of the drug moiety. In certain embodiments, a spacer unit of a linker comprises a p-aminobenzyl unit. In some such embodiments, a p-aminobenzyl alcohol is attached to an amino acid unit via an amide bond, and a carbamate, methylcarbamate, or carbonate is made between the benzyl alcohol and the drug (Hamann et al. (2005) *Expert Opin. Ther. Patents* (2005) 15:1087-1103). In some embodiments, the spacer unit comprises p-aminobenzyloxycarbonyl (PAB). In some embodiments, an ADC comprising a self-immolative linker has the structure:



wherein Q is -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -halogen, -nitro, or -cyano; m is an integer ranging from 0 to 4; X may be one or more additional spacer units or may be absent; and p ranges from 1 to about 20. In some embodiments, p ranges from 1 to 10, 1 to 7, 1 to 5, or 1 to 4. Nonlimiting exemplary X spacer units include:



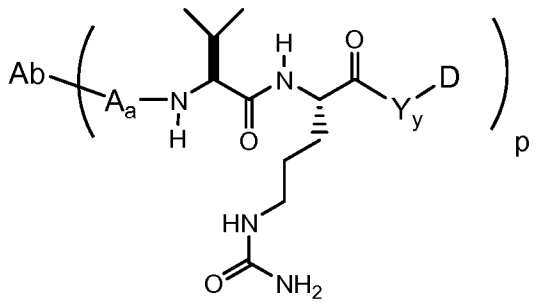
; wherein R_1 and R_2 are independently selected from H and C_1-C_6 alkyl. In some embodiments, R1 and R2 are each –CH_3.

[0203] Other examples of self-immolative spacers include, but are not limited to, aromatic compounds that are electronically similar to the PAB group, such as 2-aminoimidazol-5-methanol derivatives (U.S. Patent No. 7,375,078; Hay et al. (1999) *Bioorg. Med. Chem. Lett.* 9:2237) and ortho- or para-aminobenzylacetals. In some embodiments, spacers can be used that undergo cyclization upon amide bond hydrolysis, such as substituted

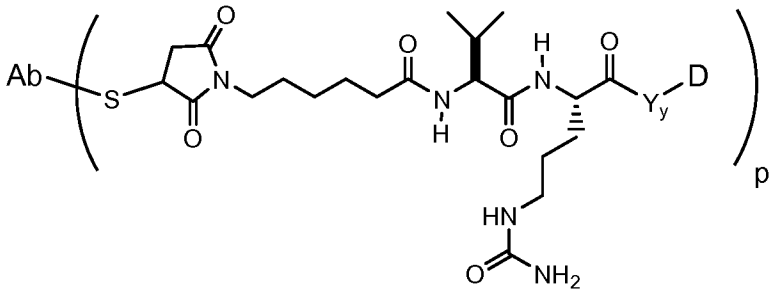
and unsubstituted 4-aminobutyric acid amides (Rodrigues et al (1995) *Chemistry Biology* 2:223), appropriately substituted bicyclo[2.2.1] and bicyclo[2.2.2] ring systems (Storm et al (1972) *J. Amer. Chem. Soc.* 94:5815) and 2-aminophenylpropionic acid amides (Amsberry, et al (1990) *J. Org. Chem.* 55:5867). Linkage of a drug to the α-carbon of a glycine residue is another example of a self-immolative spacer that may be useful in ADC (Kingsbury et al (1984) *J. Med. Chem.* 27:1447).

[0204] In some embodiments, linker L may be a dendritic type linker for covalent attachment of more than one drug moiety to an antibody through a branching, multifunctional linker moiety (Sun et al (2002) *Bioorganic & Medicinal Chemistry Letters* 12:2213-2215; Sun et al (2003) *Bioorganic & Medicinal Chemistry* 11:1761-1768). Dendritic linkers can increase the molar ratio of drug to antibody, i.e. loading, which is related to the potency of the ADC. Thus, where an antibody bears only one reactive cysteine thiol group, a multitude of drug moieties may be attached through a dendritic linker.

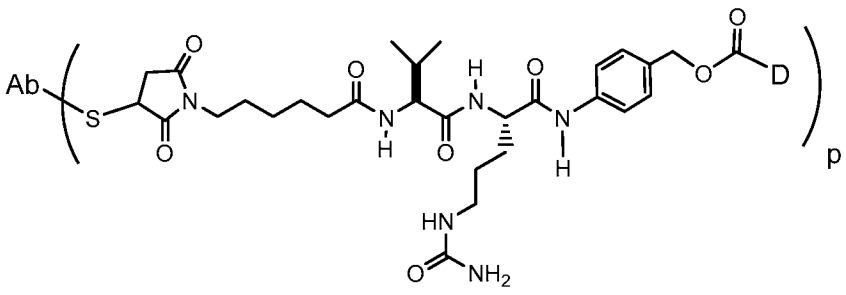
[0205] Nonlimiting exemplary linkers are shown below in the context of an ADC of Formula I:



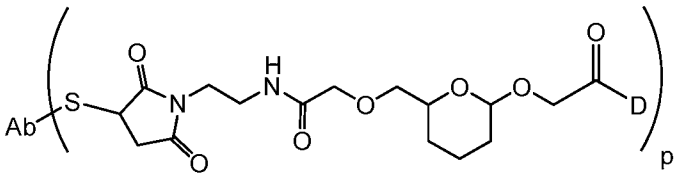
val-cit

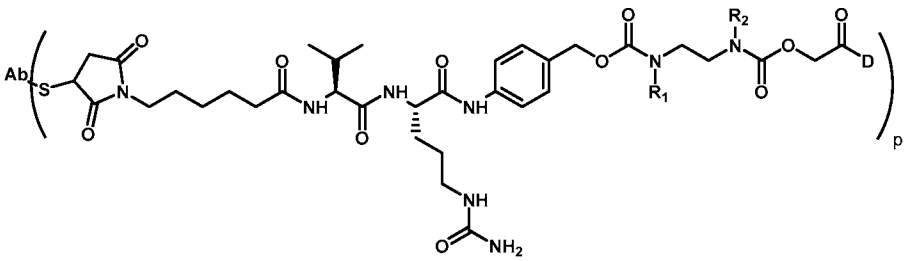


MC-val-cit

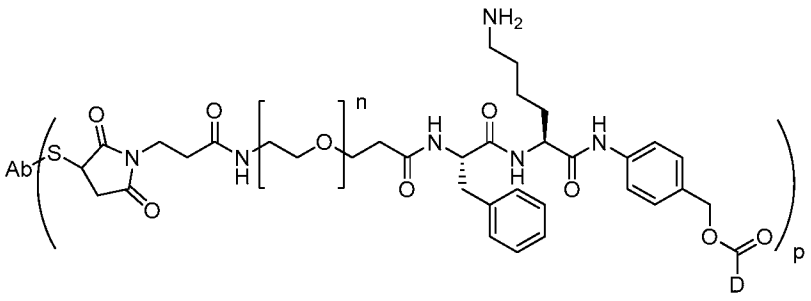


MC-val-cit-PAB



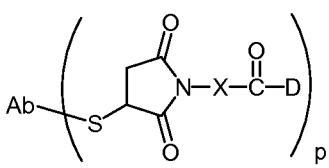


; wherein R_1 and R_2 are independently selected from H and C_1-C_6 alkyl. In some embodiments, R1 and R2 are each $-CH_3$.

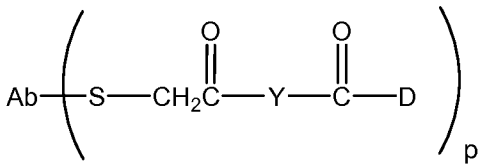


Phe-homoLys-PAB-Ab; wherein n is 0 to 12. In some embodiments, n is 2 to 10. In some embodiments, n is 4 to 8.

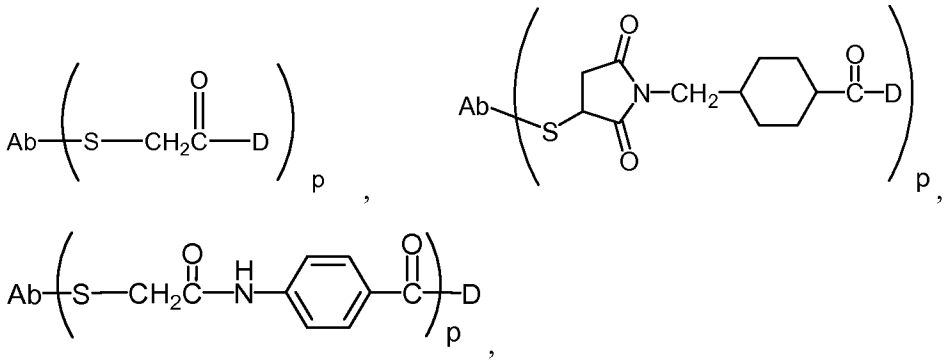
[0206] Further nonlimiting exemplary ADCs include the structures:



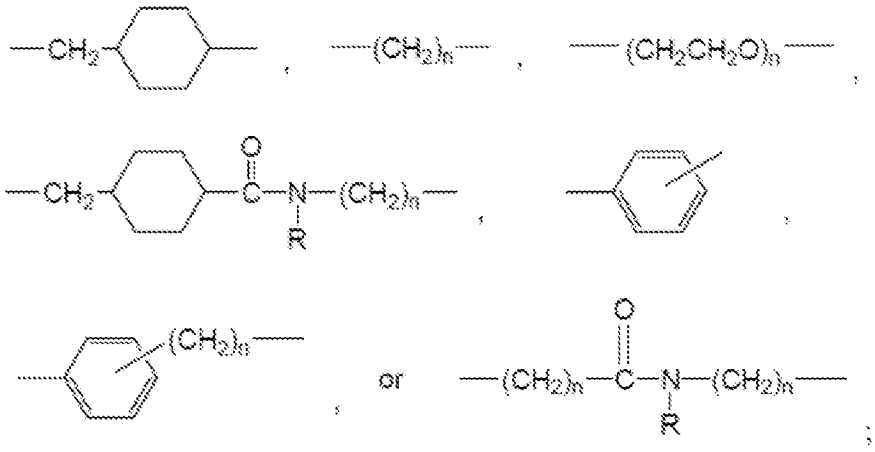
,



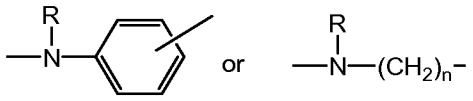
,



where X is:



Y is:

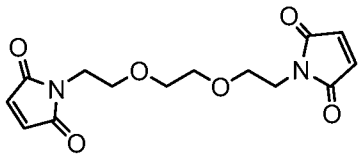


each R is independently H or C_1–C_6 alkyl; and n is 1 to 12.

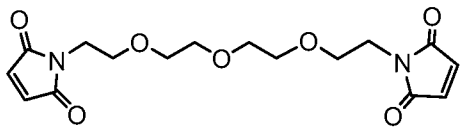
[0207] In some embodiments, a linker is substituted with groups that modulate solubility and/or reactivity. As a nonlimiting example, a charged substituent such as sulfonate ($-SO_3^-$) or ammonium may increase water solubility of the linker reagent and facilitate the coupling reaction of the linker reagent with the antibody and/or the drug moiety, or facilitate the coupling reaction of Ab-L (antibody-linker intermediate) with D, or D-L (drug-linker intermediate) with Ab, depending on the synthetic route employed to prepare the ADC. In some embodiments, a portion of the linker is coupled to the antibody and a portion of the linker is coupled to the drug, and then the Ab-(linker portion)^a is coupled to drug-(linker portion)^b to form the ADC of Formula I.

[0208] The compounds of the invention expressly contemplate, but are not limited to, ADC prepared with the following linker reagents: bis-maleimido-trioxyethylene glycol (BMPEO), N-(β-maleimidopropyloxy)-N-hydroxy succinimide ester (BMPS), N-(ε-maleimidocaproyloxy) succinimide ester (EMCS), N-[γ-maleimidobutyryloxy]succinimide

ester (GMBS), 1,6-hexane-bis-vinylsulfone (HBVS), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), 4-(4-N-Maleimidophenyl)butyric acid hydrazide (MPBH), succinimidyl 3-(bromoacetamido)propionate (SBAP), succinimidyl iodoacetate (SIA), succinimidyl (4-iodoacetyl)aminobenzoate (SIAB), N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), succinimidyl 6-[(beta-maleimidopropionamido)hexanoate] (SMPH), iminothiolane (IT), sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and succinimidyl-(4-vinylsulfone)benzoate (SVSB), and including bis-maleimide reagents: dithiobismaleimidoethane (DTME), 1,4-Bismaleimidobutane (BMB), 1,4 Bismaleimidyl-2,3-dihydroxybutane (BMDB), bismaleimidohexane (BMH), bismaleimidoethane (BMOE), $BM(PEG)_2$ (shown below), and $BM(PEG)_3$ (shown below); bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). In some embodiments, bis-maleimide reagents allow the attachment of the thiol group of a cysteine in the antibody to a thiol-containing drug moiety, linker, or linker-drug intermediate. Other functional groups that are reactive with thiol groups include, but are not limited to, iodoacetamide, bromoacetamide, vinyl pyridine, disulfide, pyridyl disulfide, isocyanate, and isothiocyanate.



$BM(PEG)_2$



$BM(PEG)_3$

[0209] Certain useful linker reagents can be obtained from various commercial sources, such as Pierce Biotechnology, Inc. (Rockford, IL), Molecular Biosciences Inc.(Boulder, CO), or synthesized in accordance with procedures described in the art; for example, in Toki et al (2002) *J. Org. Chem.* 67:1866-1872; Dubowchik, et al. (1997) *Tetrahedron Letters*, 38:5257-60; Walker, M.A. (1995) *J. Org. Chem.* 60:5352-5355; Frisch et al (1996) *Bioconjugate Chem.* 7:180-186; US 6214345; WO 02/088172; US 2003130189; US2003096743; WO 03/026577; WO 03/043583; and WO 04/032828.

[0210] Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See, e.g., WO94/11026.

b) Exemplary Drug Moieties

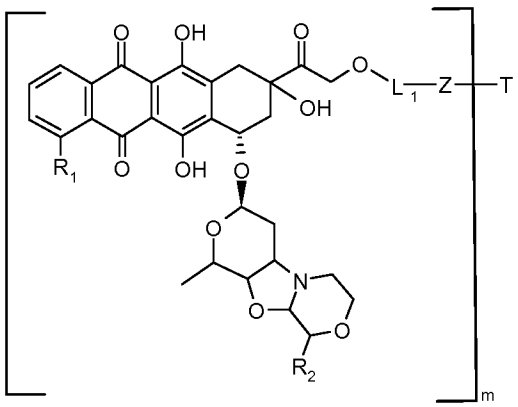
[0211] In some embodiments, an ADC comprises an anthracycline. Anthracyclines are antibiotic compounds that exhibit cytotoxic activity. While not intending to be bound by any particular theory, studies have indicated that anthracyclines may operate to kill cells by a number of different mechanisms, including: 1) intercalation of the drug molecules into the DNA of the cell thereby inhibiting DNA-dependent nucleic acid synthesis; 2) production by the drug of free radicals which then react with cellular macromolecules to cause damage to the cells, and/or 3) interactions of the drug molecules with the cell membrane (see, e.g., C. Peterson et al., "Transport And Storage Of Anthracycline In Experimental Systems And Human Leukemia" in Anthracycline Antibiotics In Cancer Therapy; N.R. Bachur, "Free Radical Damage" id. at pp.97-102). Because of their cytotoxic potential anthracyclines have been used in the treatment of numerous cancers such as leukemia, breast carcinoma, lung carcinoma, ovarian adenocarcinoma and sarcomas (see e.g., P.H- Wiernik, in Anthracycline: Current Status And New Developments p 11).

[0212] Nonlimiting exemplary anthracyclines include doxorubicin, epirubicin, idarubicin, daunomycin, nemorubicin, and derivatives thereof. Immunoconjugates and prodrugs of daunorubicin and doxorubicin have been prepared and studied (Kratz et al (2006) *Current Med. Chem.* 13:477-523; Jeffrey et al (2006) *Bioorganic & Med. Chem. Letters* 16:358-362; Torgov et al (2005) *Bioconj. Chem.* 16:717-721; Nagy et al (2000) *Proc. Natl. Acad. Sci. USA* 97:829-834; Dubowchik et al (2002) *Bioorg. & Med. Chem. Letters* 12:1529-1532; King et al (2002) *J. Med. Chem.* 45:4336-4343; EP 0328147; US 6630579). The antibody-drug conjugate BR96-doxorubicin reacts specifically with the tumor-associated antigen Lewis-Y and has been evaluated in phase I and II studies (Saleh et al (2000) *J. Clin. Oncology* 18:2282-2292; Ajani et al (2000) *Cancer Jour.* 6:78-81; Tolcher et al (1999) *J. Clin. Oncology* 17:478-484).

[0213] PNU-159682 is a potent metabolite (or derivative) of nemorubicin (Quintieri, et al. (2005) *Clinical Cancer Research* 11(4):1608-1617). Nemorubicin is a semisynthetic analog of doxorubicin with a 2-methoxymorpholino group on the glycoside amino of doxorubicin and has been under clinical evaluation (Grandi et al (1990) *Cancer Treat. Rev.* 17:133; Ripamonti et al (1992) *Brit. J. Cancer* 65:703;), including phase II/III trials for hepatocellular carcinoma (Sun et al (2003) *Proceedings of the American Society for Clinical Oncology* 22, Abs1448;

Quintieri (2003) *Proceedings of the American Association of Cancer Research*, 44:1st Ed, Abs 4649; Pacciarini et al (2006) *Jour. Clin. Oncology* 24:14116).

[0214] A nonlimiting exemplary ADC comprising nemorubicin or nemorubicin derivatives is shown in Formula Ia:



(Ia)

wherein R_1 is hydrogen atom, hydroxy or methoxy group and R_2 is a C_1-C_5 alkoxy group; or a pharmaceutically acceptable salt thereof;

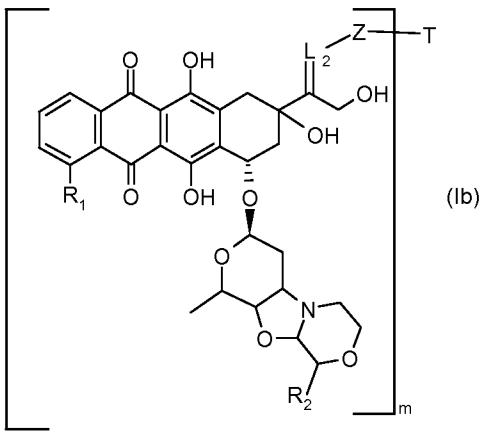
L_1 and Z together are a linker (L) as described herein;

T is an antibody (Ab) as described herein; and

m is 1 to about 20. In some embodiments, m is 1 to 10, 1 to 7, 1 to 5, or 1 to 4.

[0215] In some embodiments, R_1 and R_2 are both methoxy (-OMe).

[0216] A further nonlimiting exemplary ADC comprising nemorubicin or nemorubicin derivatives is shown in Formula Ib:



(Ib)

wherein R_1 is hydrogen atom, hydroxy or methoxy group and R_2 is a C_1-C_5 alkoxy group; or a pharmaceutically acceptable salt thereof;

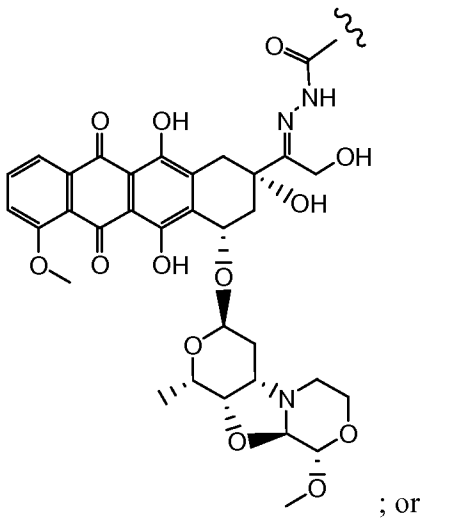
L_2 and Z together are a linker (L) as described herein;

T is an antibody (Ab) as described herein; and

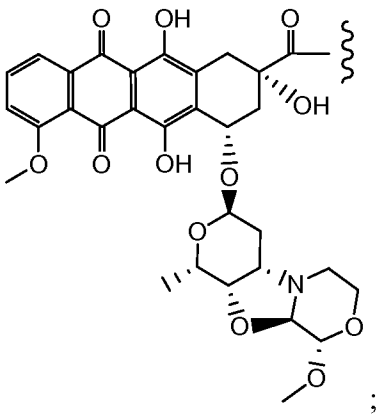
m is 1 to about 20. In some embodiments, m is 1 to 10, 1 to 7, 1 to 5, or 1 to 4.

[0217] In some embodiments, R_1 and R_2 are both methoxy (-OMe).

[0218] In some embodiments, the nemorubicin component of a nemorubicin-containing ADC is PNU-159682. In some such embodiments, the drug portion of the ADC may have one of the following structures:



; or

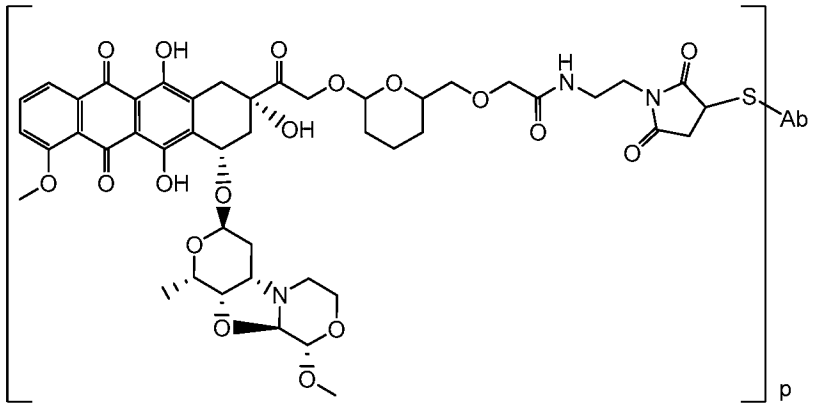


;

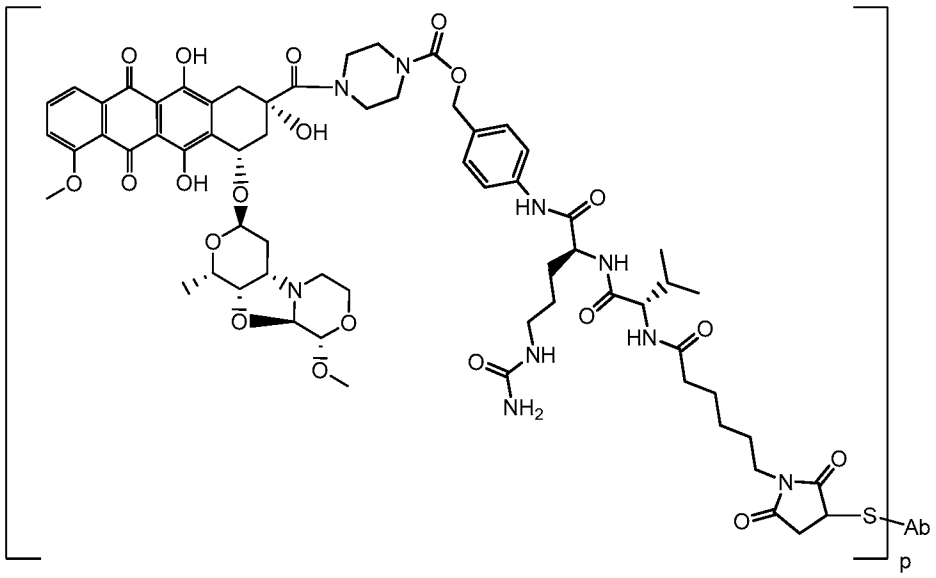
wherein the wavy line indicates the attachment to the linker (L).

[0219] Anthracyclines, including PNU-159682, may be conjugated to antibodies through several linkage sites and a variety of linkers (US 2011/0076287; WO2009/099741; US 2010/0034837; WO 2010/009124) , including the linkers described herein.

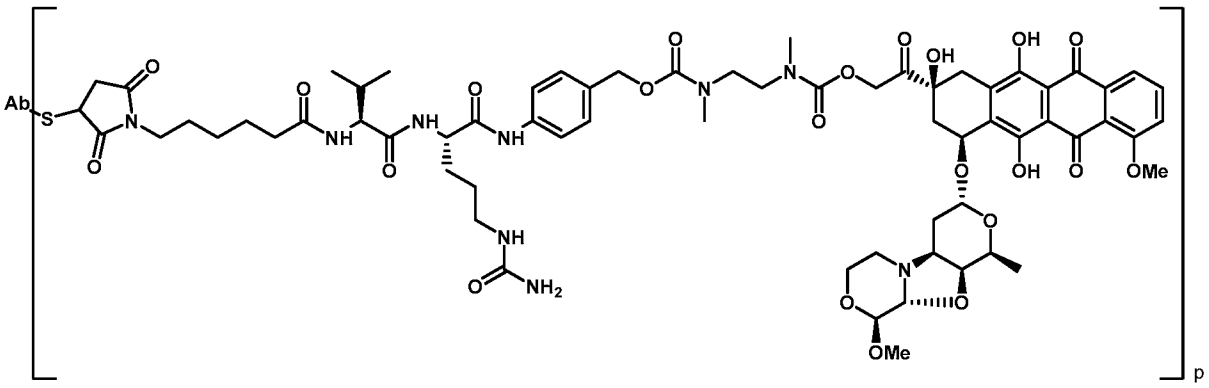
[0220] Exemplary ADCs comprising a nemorubicin and linker include, but are not limited to:



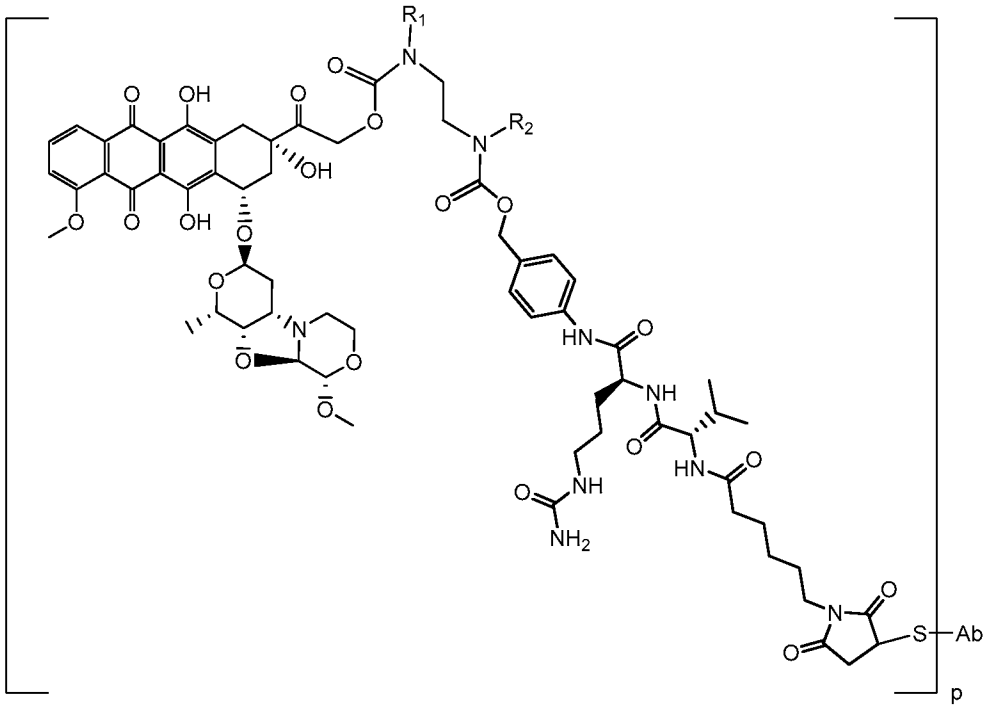
PNU-159682 maleimide acetal-Ab;



PNU-159682-val-cit-PAB-Ab;

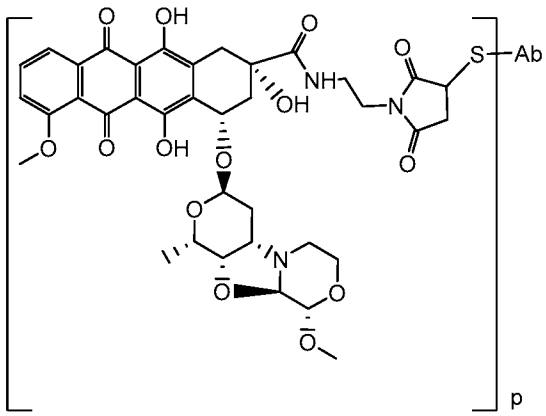


PNU-159682-val-cit-PAB-spacer-Ab;



PNU-159682-val-cit-PAB-spacer(R^1R^2)-Ab, wherein:

R_1 and R_2 are independently selected from H and C_1-C_6 alkyl; and



PNU-159682-maleimide-Ab.

[0221] The linker of PNU-159682 maleimide acetal-Ab is acid-labile, while the linkers of PNU-159682-val-cit-PAB-Ab, PNU-159682-val-cit-PAB-spacer-Ab, and PNU-159682-val-cit-PAB-spacer(R^1R^2)-Ab are protease cleavable.

c) Drug Loading

[0222] Drug loading is represented by p, the average number of drug moieties per antibody in a molecule of Formula I. Drug loading may range from 1 to 20 drug moieties (D) per antibody. ADCs of Formula I include collections of antibodies conjugated with a range of drug moieties, from 1 to 20. The average number of drug moieties per antibody in preparations

of ADC from conjugation reactions may be characterized by conventional means such as mass spectroscopy, ELISA assay, and HPLC. The quantitative distribution of ADC in terms of p may also be determined. In some instances, separation, purification, and characterization of homogeneous ADC where p is a certain value from ADC with other drug loadings may be achieved by means such as reverse phase HPLC or electrophoresis.

[0223] For some antibody-drug conjugates, p may be limited by the number of attachment sites on the antibody. For example, where the attachment is a cysteine thiol, as in certain exemplary embodiments above, an antibody may have only one or several cysteine thiol groups, or may have only one or several sufficiently reactive thiol groups through which a linker may be attached. In certain embodiments, higher drug loading, e.g. p >5, may cause aggregation, insolubility, toxicity, or loss of cellular permeability of certain antibody-drug conjugates. In certain embodiments, the average drug loading for an ADC ranges from 1 to about 8; from about 2 to about 6; or from about 3 to about 5. Indeed, it has been shown that for certain ADCs, the optimal ratio of drug moieties per antibody may be less than 8, and may be about 2 to about 5 (US 7498298).

[0224] In certain embodiments, fewer than the theoretical maximum of drug moieties are conjugated to an antibody during a conjugation reaction. An antibody may contain, for example, lysine residues that do not react with the drug-linker intermediate or linker reagent, as discussed below. Generally, antibodies do not contain many free and reactive cysteine thiol groups which may be linked to a drug moiety; indeed most cysteine thiol residues in antibodies exist as disulfide bridges. In certain embodiments, an antibody may be reduced with a reducing agent such as dithiothreitol (DTT) or tricarbonylethylphosphine (TCEP), under partial or total reducing conditions, to generate reactive cysteine thiol groups. In certain embodiments, an antibody is subjected to denaturing conditions to reveal reactive nucleophilic groups such as lysine or cysteine.

[0225] The loading (drug/antibody ratio) of an ADC may be controlled in different ways, and for example, by: (i) limiting the molar excess of drug-linker intermediate or linker reagent relative to antibody, (ii) limiting the conjugation reaction time or temperature, and (iii) partial or limiting reductive conditions for cysteine thiol modification.

[0226] It is to be understood that where more than one nucleophilic group reacts with a drug-linker intermediate or linker reagent, then the resulting product is a mixture of ADC compounds with a distribution of one or more drug moieties attached to an antibody. The average number of drugs per antibody may be calculated from the mixture by a dual ELISA antibody assay, which is specific for antibody and specific for the drug. Individual ADC molecules may be identified in the mixture by mass spectroscopy and separated by HPLC, e.g.

hydrophobic interaction chromatography (*see, e.g.*, McDonagh et al (2006) Prot. Engr. Design & Selection 19(7):299-307; Hamblett et al (2004) Clin. Cancer Res. 10:7063-7070; Hamblett, K.J., et al. "Effect of drug loading on the pharmacology, pharmacokinetics, and toxicity of an anti-CD30 antibody-drug conjugate," Abstract No. 624, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004; Alley, S.C., et al. "Controlling the location of drug attachment in antibody-drug conjugates," Abstract No. 627, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004). In certain embodiments, a homogeneous ADC with a single loading value may be isolated from the conjugation mixture by electrophoresis or chromatography.

d) Certain Methods of Preparing Immunoconjugates

[0227] An ADC of Formula I may be prepared by several routes employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent to form Ab-L via a covalent bond, followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with a nucleophilic group of an antibody. Exemplary methods for preparing an ADC of Formula I via the latter route are described in US 7498298, which is expressly incorporated herein by reference.

[0228] Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; and (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol) or tricarbonylethylphosphine (TCEP), such that the antibody is fully or partially reduced. Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through modification of lysine residues, e.g., by reacting lysine residues with 2-iminothiolane (Traut's reagent), resulting in conversion of an amine into a thiol. Reactive thiol groups may also be introduced

into an antibody by introducing one, two, three, four, or more cysteine residues (e.g., by preparing variant antibodies comprising one or more non-native cysteine amino acid residues).

[0229] Antibody-drug conjugates of the invention may also be produced by reaction between an electrophilic group on an antibody, such as an aldehyde or ketone carbonyl group, with a nucleophilic group on a linker reagent or drug. Useful nucleophilic groups on a linker reagent include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. In one embodiment, an antibody is modified to introduce electrophilic moieties that are capable of reacting with nucleophilic substituents on the linker reagent or drug. In another embodiment, the sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either galactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the antibody that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, antibodies containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) *Bioconjugate Chem.* 3:138-146; US 5362852). Such an aldehyde can be reacted with a drug moiety or linker nucleophile.

[0230] Exemplary nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

[0231] Nonlimiting exemplary cross-linker reagents that may be used to prepare ADC are described herein in the section titled "Exemplary Linkers." Methods of using such cross-linker reagents to link two moieties, including a proteinaceous moiety and a chemical moiety, are known in the art. In some embodiments, a fusion protein comprising an antibody and a cytotoxic agent may be made, e.g., by recombinant techniques or peptide synthesis. A recombinant DNA molecule may comprise regions encoding the antibody and cytotoxic portions of the conjugate either adjacent to one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

[0232] In yet another embodiment, an antibody may be conjugated to a "receptor" (such as streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) which is conjugated to a cytotoxic agent (e.g., a drug or radionucleotide).

E. Methods and Compositions for Diagnostics and Detection

[0233] In certain embodiments, any of the anti-ETBR antibodies provided herein is useful for detecting the presence of ETBR in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. A "biological sample" comprises, e.g., a cell or tissue (e.g., biopsy material, including cancerous or potentially cancerous skin tissue, including tissue from subjects having or suspected of having melanoma).

[0234] In one embodiment, an anti-ETBR antibody for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of ETBR in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with an anti-ETBR antibody as described herein under conditions permissive for binding of the anti-ETBR antibody to ETBR, and detecting whether a complex is formed between the anti-ETBR antibody and ETBR in the biological sample. Such method may be an *in vitro* or *in vivo* method. In one embodiment, an anti-ETBR antibody is used to select subjects eligible for therapy with an anti-ETBR antibody, e.g. where ETBR is a biomarker for selection of patients. In a further embodiment, the biological sample is a cell or tissue (e.g., cancerous or potentially cancerous skin tissue, including tissue of subjects having or suspected of having melanoma).

[0235] In a further embodiment, an anti-ETBR antibody is used in vivo to detect, e.g., by in vivo imaging, an ETBR-positive cancer in a subject, e.g., for the purposes of diagnosing, prognosing, or staging cancer, determining the appropriate course of therapy, or monitoring response of a cancer to therapy. One method known in the art for in vivo detection is immuno-positron emission tomography (immuno-PET), as described, e.g., in van Dongen et al., *The Oncologist* 12:1379-1389 (2007) and Verel et al., *J. Nucl. Med.* 44:1271-1281 (2003). In such embodiments, a method is provided for detecting an ETBR-positive cancer in a subject, the method comprising administering a labeled anti-ETBR antibody to a subject having or suspected of having an ETBR-positive cancer, and detecting the labeled anti-ETBR antibody in the subject, wherein detection of the labeled anti-ETBR antibody indicates an ETBR-positive cancer in the subject. In certain of such embodiments, the labeled anti-ETBR antibody

comprises an anti-ETBR antibody conjugated to a positron emitter, such as ^{68}Ga, ^{18}F, ^{64}Cu, ^{86}Y, ^{76}Br, ^{89}Zr, and ^{124}I. In a particular embodiment, the positron emitter is ^{89}Zr.

[0236] In further embodiments, a method of diagnosis or detection comprises contacting a first anti-ETBR antibody immobilized to a substrate with a biological sample to be tested for the presence of ETBR, exposing the substrate to a second anti-ETBR antibody, and detecting whether the second anti-ETBR is bound to a complex between the first anti-ETBR antibody and ETBR in the biological sample. A substrate may be any supportive medium, e.g., glass, metal, ceramic, polymeric beads, slides, chips, and other substrates. In certain embodiments, a biological sample comprises a cell or tissue (e.g., biopsy material, including cancerous or potentially cancerous skin tissue, including tissue from subjects having or suspected of having melanoma). In certain embodiments, the first or second anti-ETBR antibody is any of the antibodies described herein.

[0237] Exemplary disorders that may be diagnosed or detected according to any of the above embodiments include ETBR-positive cancers, such as ETBR-positive melanoma. In some embodiments, an ETBR-positive cancer is a cancer that receives an anti-ETBR immunohistochemistry (IHC) score greater than "0," which corresponds to very weak or no staining in >90% of tumor cells. In some embodiments, an ETBR-positive cancer expresses ETBR at a 1+, 2+ or 3+ level, wherein 1+ corresponds to weak staining in >50% of neoplastic cells, 2+ corresponds to moderate staining in >50% neoplastic cells, and 3+ corresponds to strong staining in >50% of neoplastic cells. In some embodiments, an ETBR-positive cancer is a cancer that expresses ETBR according to an in situ hybridization (ISH) assay. In some such embodiments, a scoring system similar to that used for IHC is used. In some embodiments, an ETBR-positive cancer is a cancer that expresses ETBR according to a reverse-transcriptase PCR (RT-PCR) assay that detects ETBR mRNA. In some embodiments, the RT-PCR is quantitative RT-PCR.

[0238] In certain embodiments, labeled anti-ETBR antibodies are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate

dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like. In another embodiment, a label is a positron emitter. Positron emitters include but are not limited to ^{68}Ga, ^{18}F, ^{64}Cu, ^{86}Y, ^{76}Br, ^{89}Zr, and ^{124}I. In a particular embodiment, a positron emitter is ^{89}Zr.

F. Pharmaceutical Formulations

[0239] Pharmaceutical formulations of an anti-ETBR antibody or immunoconjugate as described herein are prepared by mixing such antibody or immunoconjugate having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include insterstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

[0240] Exemplary lyophilized antibody or immunoconjugate formulations are described in US Patent No. 6,267,958. Aqueous antibody or immunoconjugate formulations

include those described in US Patent No. 6,171,586 and WO2006/044908, the latter formulations including a histidine-acetate buffer.

[0241] The formulation herein may also contain more than one active ingredient as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other.

[0242] Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

[0243] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody or immunoconjugate, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules.

[0244] The formulations to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

G. Therapeutic Methods and Compositions

[0245] Any of the anti-ETBR antibodies or immunoconjugates provided herein may be used in methods, e.g., therapeutic methods.

[0246] In one aspect, an anti-ETBR antibody or immunoconjugate provided herein is used in a method of inhibiting proliferation of an ETBR-positive cell, the method comprising exposing the cell to the anti-ETBR antibody or immunoconjugate under conditions permissive for binding of the anti-ETBR antibody or immunoconjugate to ETBRon the surface of the cell, thereby inhibiting the proliferation of the cell. In certain embodiments, the method is an *in vitro* or an *in vivo* method. In some embodiments, the cell is a melanoma cell.

[0247] Inhibition of cell proliferation in vitro may be assayed using the CellTiter-Glo™ Luminescent Cell Viability Assay, which is commercially available from Promega (Madison, WI). That assay determines the number of viable cells in culture based on quantitation of ATP present, which is an indication of metabolically active cells. *See* Crouch et al. (1993) *J. Immunol. Meth.* 160:81-88, US Pat. No. 6602677. The assay may be conducted in 96- or 384-well format, making it amenable to automated high-throughput screening (HTS). *See* Cree et al. (1995) *AntiCancer Drugs* 6:398-404. The assay procedure involves adding a

single reagent (CellTiter-Glo® Reagent) directly to cultured cells. This results in cell lysis and generation of a luminescent signal produced by a luciferase reaction. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of viable cells present in culture. Data can be recorded by luminometer or CCD camera imaging device. The luminescence output is expressed as relative light units (RLU).

[0248] In another aspect, an anti-ETBR antibody or immunoconjugate for use as a medicament is provided. In further aspects, an anti-ETBR antibody or immunoconjugate for use in a method of treatment is provided. In certain embodiments, an anti-ETBR antibody or immunoconjugate for use in treating ETBR-positive cancer is provided. In certain embodiments, the invention provides an anti-ETBR antibody or immunoconjugate for use in a method of treating an individual having an ETBR-positive cancer, the method comprising administering to the individual an effective amount of the anti-ETBR antibody or immunoconjugate. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below.

[0249] In a further aspect, the invention provides for the use of an anti-ETBR antibody or immunoconjugate in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of ETBR-positive cancer. In a further embodiment, the medicament is for use in a method of treating ETBR-positive cancer, the method comprising administering to an individual having ETBR-positive cancer an effective amount of the medicament. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below.

[0250] In a further aspect, the invention provides a method for treating ETBR-positive cancer. In one embodiment, the method comprises administering to an individual having such ETBR-positive cancer an effective amount of an anti-ETBR antibody or immunoconjugate. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, as described below.

[0251] AN ETBR-positive cancer according to any of the above embodiments may be, e.g., melanoma. In some embodiments, an ETBR-positive cancer is a cancer that receives an anti-ETBR immunohistochemistry (IHC) or in situ hybridization (ISH) score greater than "0," which corresponds to very weak or no staining in >90% of tumor cells. In another embodiment, an ETBR-positive cancer expresses ETBR at a 1+, 2+ or 3+ level, wherein 1+ corresponds to weak staining in >50% of neoplastic cells, 2+ corresponds to moderate staining in >50% neoplastic cells, and 3+ corresponds to strong staining in >50% of neoplastic cells. In

some embodiments, an ETBR-positive cancer is a cancer that expresses ETBR according to a reverse-transcriptase PCR (RT-PCR) assay that detects ETBR mRNA. In some embodiments, the RT-PCR is quantitative RT-PCR.

[0252] In some embodiments, methods of treating an individual having an ETBR-positive cancer are provided, wherein the ETBR-positive cancer is resistant to a first therapeutic. In some embodiments, the method comprises administering to the individual an effective amount of an immunoconjugate comprising an antibody that binds to ETBR. In some embodiments, the ETBR-positive cancer is melanoma. In some embodiments, the first therapeutic comprises a first antibody that binds an antigen other than ETBR. In some embodiments, the first therapeutic is a first immunoconjugate comprising a first antibody that binds an antigen other than ETBR and a first cytotoxic agent. In some embodiments, the first antibody binds an antigen selected from melanocyte protein PMEL17, tyrosinase-related protein 1 (TYRP1), cytotoxic T lymphocyte antigen 4 (CTLA-4), and glycoprotein NMB (GPNMB). In some embodiments, the first antibody binds PMEL17. In some embodiments, the first cytotoxic agent and the cytotoxic agent of the immunoconjugate comprising an antibody that binds to ETBR are different. In some such embodiments, the first cytotoxic agent is selected from MMAE, a calicheamicin, and a pyrrolobenzodiazepine. In some such embodiments, the first cytotoxic agent is MMAE and the cytotoxic agent of the immunoconjugate comprising an antibody that binds to ETBR is a nemorubicin derivative.

[0253] In some embodiments, the first antibody binds ETBR. In some such embodiments, the first cytotoxic agent is selected from MMAE, a calicheamicin, and a pyrrolobenzodiazepine and the cytotoxic agent of the immunoconjugate described herein is a nemorubicin derivative. In some embodiments, the first cytotoxic agent is MMAE and the cytotoxic agent of the immunoconjugate described herein is a nemorubicin derivative.

[0254] In some embodiments, methods of treating an individual with cancer are provided, wherein the cancer is resistant to a first therapeutic. In some embodiments, the first therapeutic is a first immunoconjugate comprising a first antibody linked to a first cytotoxic agent through a first linker. In some embodiments, a method of treating an individual with a cancer that is resistant to a first therapeutic (such as a first immunoconjugate) comprises administering a second immunoconjugate comprising a second antibody linked to a second cytotoxic agent through a second linker. In some embodiments, the first antibody and the second antibody bind to different antigens and the first cytotoxic agent and the second cytotoxic agents are the same or different. In some embodiments, the first antibody and the second antibody bind to different antigens that are present on at least some of the same cells. In some embodiments, the first antibody and the second antibody bind to different antigens and

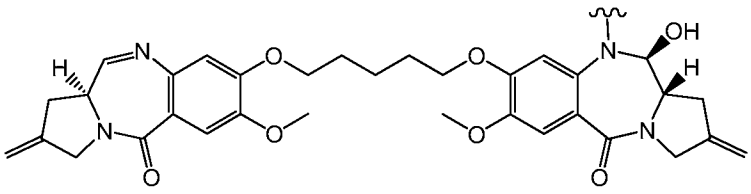
the first cytotoxic agent and the second cytotoxic agents are different. In some embodiments, the first antibody and the second antibody bind to the same antigens, and the first cytotoxic agent and the second cytotoxic agent are different. In any of the foregoing embodiments, the first linker and the second linker may be the same or different. In some embodiments, the first antibody and the second antibody bind to different antigens, the first and second linkers are different, and the first and second cytotoxic agents are different.

[0255] An "individual" according to any of the above embodiments may be a human.

[0256] In a further aspect, the invention provides pharmaceutical formulations comprising any of the anti-ETBR antibodies or immunoconjugate provided herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical formulation comprises any of the anti-ETBR antibodies or immunoconjugates provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical formulation comprises any of the anti-ETBR antibodies or immunoconjugates provided herein and at least one additional therapeutic agent, e.g., as described below.

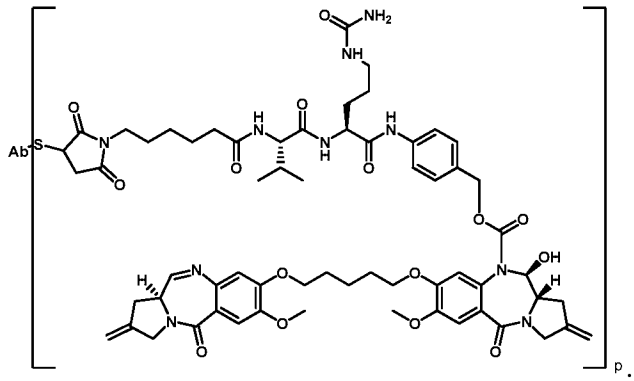
[0257] Antibodies or immunoconjugates of the invention can be used either alone or in combination with other agents in a therapy. For instance, an antibody or immunoconjugate of the invention may be co-administered with at least one additional therapeutic agent.

[0258] In some embodiments, methods of treating cancer comprise administering an immunoconjugate described herein in combination with a second immunoconjugate comprising an antibody that binds an antigen an antigen selected from melanocyte protein PMEL17, tyrosinase-related protein 1 (TYRP1), cytotoxic T lymphocyte antigen 4 (CTLA-4), and glycoprotein NMB (GPNMB). In some such embodiments, the cytotoxic agent of the second immunoconjugate is selected from MMAE, a calicheamicin, a nemorubicin derivative, and a pyrrolobenzodiazepine. In some such embodiments, the cytotoxic agent of the second immunoconjugate is selected from MMAE, a calicheamicin, and a pyrrolobenzodiazepine. In some embodiments, the cytotoxic agent of the second immunoconjugate is an MMAE. An exemplary MMAE structure is shown in Figure 7A. The linker portion of the MMAE-containing immunoconjugate may be the MC-val-cit-PAB linker shown in Figure 7A, or may be a different linker, such as any of those described herein. In some embodiments, the cytotoxic agent of the second immunoconjugate is a pyrrolobenzodiazepine. Nonlimiting exemplary pyrrolobenzodiazepines include a PBD dimer with the following structure:



PBD dimer;

wherein the wavy line indicates the attachment to the linker (L). *See, e.g.*, WO 2009/016516; US 2009/304710; US 2010/047257; US 2009/036431; US 2011/0256157; and WO 2011/130598. The linker portion of the PBD-containing immunoconjugate may, in some embodiments, be a linker described herein. In some embodiments, the linker comprises MC-val-cit-PAB, such as, for example, in the following structure:



[0259] In some embodiments, methods of treating cancer comprise administering an immunoconjugate described herein in combination with a second immunoconjugate comprising an antibody that binds PMEL17. In some such embodiments, the cancer is an ETBR-positive cancer and also a PMEL17-positive cancer. Determination of whether a cancer is also PMEL17-positive may be carried out by any method, including, but not limited to, the methods described herein for determining whether a cancer is ETBR-positive, and methods described in U.S. Publication No. US 2011/0206702. In some embodiments, the antibody that binds PMEL17 comprises an HVR H1 comprising a sequence of SEQ ID NO: 21, an HVR H2 comprising a sequence of SEQ ID NO: 22, an HVR H3 comprising a sequence of SEQ ID NO: 23, an HVR L1 comprising a sequence of SEQ ID NO: 24, an HVR L2 comprising a sequence of SEQ ID NO: 25, and an HVR L3 comprising a sequence of SEQ ID NO: 26.

[0260] Administration "in combination" encompasses combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody or immunoconjugate of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent (such as the anti-PMEL17 immunoconjugate) and/or adjuvant. In some embodiments, administration of the anti-ETBR immunoconjugate and administration of an anti-PMEL17 immunoconjugate occur within about one month, or within about one, two, or

three weeks, or within about one, two, three, four, five, or six days of one another. Antibodies or immunoconjugates of the invention can also be used in combination with radiation therapy.

[0261] An antibody or immunoconjugate of the invention (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

[0262] Antibodies or immunoconjugates of the invention would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody or immunoconjugate need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody or immunoconjugate present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

[0263] For the prevention or treatment of disease, the appropriate dosage of an antibody or immunoconjugate of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of antibody or immunoconjugate, the severity and course of the disease, whether the antibody or immunoconjugate is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody or immunoconjugate, and the discretion of the attending physician. The antibody or immunoconjugate is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1mg/kg-10mg/kg) of antibody or immunoconjugate can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors

mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody or immunoconjugate would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (*e.g.* such that the patient receives from about two to about twenty, or *e.g.* about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

[0264] It is understood that any of the above formulations or therapeutic methods may be carried out using both an immunoconjugate of the invention and an anti-ETBR antibody.

H. Articles of Manufacture

[0265] In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the disorder and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody or immunoconjugate of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody or immunoconjugate of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution or dextrose solution. It may further include other

materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

III. EXAMPLES

[0266] The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

A. Production of Anti-ETBR Antibody Drug Conjugates

[0267] Anti-ETBR antibody-drug conjugate (ADC) was produced by conjugating hu5E9.v1 (SEQ ID NOs: 5 and 6) or ch5E9 (SEQ ID NOs: 27 and 28) to the drug-linker moiety MC-vc-PAB-MMAE, which is depicted herein. For convenience, the drug-linker moiety MC-vc-PAB-MMAE is sometimes referred to in these Examples and in the Figures as "vcMMAE" or "VCE."

[0268] Prior to conjugation, the antibody was partially reduced with TCEP using standard methods in accordance with the methodology described in WO 2004/010957 A2. The partially reduced antibody was conjugated to the drug-linker moiety using standard methods in accordance with the methodology described, e.g., in Doronina et al. (2003) *Nat. Biotechnol.* 21:778-784 and US 2005/0238649 A1. Briefly, the partially reduced antibody was combined with the drug-linker moiety to allow conjugation of the drug-linker moiety to reduced cysteine residues of the antibody. The conjugation reaction was quenched by adding excess N-acetyl-cysteine to react with any free linker-drug moiety, and the ADC was purified. The structure of anti-ETBR-vc-MMAE (also referred to as anti-ETBR-MC-val-cit-PAB-MMAE) is shown in Figure 7A. *See also* U.S. Publication No. US 2011/0206702.

[0269] Anti-ETBR antibody-drug conjugate (ADC) comprising a protease-cleavable val-cit linker and the drug PNU-159682 was produced by conjugating ch5E9 to the drug-linker moiety MC-vc-PAB-PNU-159682, substantially as described above. The structure of anti-ETBR-MC-val-cit-PAB-PNU-159682 is shown in Figure 7C.

[0270] Anti-ETBR antibody-drug conjugate (ADC) comprising an acid-labile acetal linker and the drug PNU-159682 was produced by conjugating ch5E9 to the drug-linker moiety MC-acetal-PNU-159682, substantially as described above. The structure of anti-ETBR-MC-acetal-PNU-159682 is shown in Figure 7B.

[0271] Anti-ETBR antibody-drug conjugate (ADC) comprising non-cleavable linker and the drug PNU-159682 was produced by conjugating ch5E9 to the drug-linker moiety MC-

PNU-159682, substantially as described above. The structure of anti-ETBR- PNU-159682 is shown in Figure 7D.

B. Efficacy of Anti-ETBR Immunoconjugates Comprising a Nemorubicin Derivative in UACC-257X2.2 Melanoma Cells Resistant to anti-ETBR-vc-MMAE

[0272] To determine the efficacy of an anti-ETBR immunoconjugate comprising a nemorubicin derivative in melanoma that had developed resistance to anti-ETBR-vc-MMAE, UACC-257X2.2 melanoma cells that are resistant to anti-ETBR-vc-MMAE (also referred to as anti-EDNRB-vc-MMAE, anti-ETBR-MC-val-cit-PAB-MMAE, etc.; see, e.g., U.S. Publication No. US 2011/0206702) were developed *in vivo* and *in vitro*.

[0273] For resistance developed *in vivo*, NCr nude mice (Taconic, Hudson, NY) were inoculated subcutaneously in the dorsal right flank with 5 million UACC-257X2.2 cells in HBSS with Matrigel. UACC-257X2.2 cells are derived from UACC-257 cells (National Cancer Institute) and optimized for growth *in vivo* as follows. UACC-257 cells were injected subcutaneously in the right flank of female NCr nude mice to induce tumor growth. One tumor was harvested and grown *in vitro* (referred to as UACC-257X1.2 cell line). The UACC-257X1.2 line was injected again subcutaneously in the right flank of female NCr nude mice to improve the growth of the cell line *in vivo*. A tumor from the second injection round was collected and again adapted for *in vitro* growth to generate UACC-257X2.2. The UACC-257X2.2 cell line and tumors derived from this line express ETBR at levels comparable to the parental cell line UACC-257.

[0274] Ten mice inoculated with UACC-257X2.2 cells were dosed with 3 mg/kg hu5E9.v1-MC-vc-PAB-MMAE intravenously on day 0. To determine when the mice would be dosed again, and at what doses, the following was taken into consideration: whether or not tumors re-grew after the initial treatment (i.e., tumors that grew back to initial tumor volume size at day 0), and the rate of re-growth. Frequency of doses administered varied over time but did not exceed 2 doses/week. Intravenous doses did not exceed 300 μL. The range of doses administered were 3 mg/kg, 6 mg/kg, 8 mg/kg, and 10 mg/kg. Dosing was discontinued once a tumor no longer responded (i.e., it showed resistance to) a series of increasing doses.

[0275] As shown in Figure 8A, the UACC-257X2.2 tumor #359 developed resistance to anti-ETBR-vc-MMAE after about 120 days, whereas tumor #368 developed resistance more slowly. Tumor # 359 was harvested and the cells were dissociated for growth *in vitro* (referred to herein as *in vivo*-derived resistant UACC-257X2.2 cells).

[0276] For resistance developed *in vitro*, UACC-257X2.2 cells were adapted to increasing concentrations of anti-ETBR-vc-MMAE in culture dishes over the course of two

months. Figure 8B shows the resistant UACC-257X2.2 cell line derived *in vitro* (referred to herein as *in vitro*-derived resistant UACC-257X2.2 cells), which was relatively unaffected by concentrations of anti-ETBR-vc-MMAE up to at least 10 μg/ml. Figure 8B also shows the *in vitro*-derived resistant UACC-257X2.2 cells incubated with a control ADC, α-gD-vc-MMAE, and the parental UACC-257X2.2 cells incubated with α-ETBR-vc-MMAE.

[0277] Expression of ETBR on the surface of the *in vivo*- and *in vitro*- derived resistant UACC-257 cells and the parental UACC-257 cells was then determined by FACS. Cells were stained with anti-ETBR antibody (hu5E9.v1 or ch5E9), followed by an anti-human Alexa 488 antibody conjugate. As shown in Figures 9A and B, both the *in vivo*-derived and *in vitro*-derived resistant UACC-257X2.2 cells had decreased expression of ETBR on the surface of the cells relative to the parental cell line. The control peak in each figure shows staining with secondary antibody only.

[0278] *In vivo*- and *in vitro*-derived resistant UACC-257X2.2 cells were then assayed for sensitivity to increasing concentrations of anti-ETBR-vc-MMAE.

[0279] *In vitro* cell proliferation of the cell lines in the presence of the immunoconjugates was assessed. Cells were plated at 1,500 cells per well in 50 μL of normal growth medium in 96-well clear-bottom plates. Twenty-four hours later, an additional 50 μL of culture medium with serial dilutions of immunoconjugates (ch5E9-vc-MMAE or control anti-gD-vc-MMAE) were added to triplicate wells. Five days later, cell survival was determined using CellTiter-Glo Luminescent Cell Viability Reagent (G7572, Promega Corporation, Madison, WI) using an EnVision 2101 Mutilabel Reader (Perkin-Elmer, Waltham, MA).

[0280] Table 2 shows the EC50 observed for anti-ETBR-vc-MMAE and linker-drug (without antibody) for each of the cell lines.

Table 2: EC50s of anti-ETBR-vc-MMAE in sensitive and resistant UACC-257X2.2 cells

Cells	**EC50 (anti-ETBR-vc-MMAE)**	**EC50 (free MC-vc-PAB-MMAE)**
Parental UACC-257X2.2	45 ng/mL	0.28 nM
In vivo-derived resistant UACC-257X2.2	235 ng/mL	0.77 nM
In vitro-derived resistant UACC-257X2.2	n.d.*	3.5 nM

* Cell killing by anti-ETBR-vc-MMAE was comparable to killing by a control ADC, anti-gD-vc-MMAE.

[0281] The results of this experiment suggest that at least some of the resistance is developed to the drug portion of the ADC, suggesting that replacing the drug with a different drug may restore some sensitivity in resistant cells.

[0282] To determine if some sensitivity can be restored by changing the drug portion of the ADC, the *in vivo*- and *in vitro*-derived resistant UACC-257X2.2 cells were assayed for sensitivity to ADCs with different linkers and different drugs than were used to develop the resistant cells. The ADC tested in this experiment was anti-ETBR linked to PNU-159682 through a non-cleavable linker (ch5E9-PNU). *See* Figure 7D. Table 3 shows the EC50s observed for anti-ETBR-PNU and linker-drug (without antibody) for each of the cell lines.

Table 3: EC50s of anti-ETBR-PNU in sensitive and resistant UACC-257X2.2 cells

Cells	**EC50 (anti-ETBR-PNU)**	**EC50 (free MC-PNU-159682)**
Parental UACC-257X2.2	4.2 ng/mL	0.18 nM
In vivo-derived resistant UACC-257X2.2	14.3 ng/mL	0.133 nM
In vitro-derived resistant UACC-257X2.2	14.6 ng/mL	0.15 nM

[0283] The results of this experiment demonstrate that at least some sensitivity can be restored in the resistant cells by replacing the drug portion of the ADC.

[0284] The *in vivo*- and *in vitro*-derived resistant UACC-257X2.2 cells were assayed for sensitivity to anti-ETBR linked to PNU-159682 through an acid labile acetal linker (ch5E9-acetal-PNU). *See* Figure 7B. Table 4 shows the EC50s observed for anti-ETBR-acetal-PNU and linker-drug (without antibody) for each of the cell lines.

Table 4: EC50s of anti-ETBR-acetal-PNU in sensitive and resistant UACC-257X2.2 cells

Cells	**EC50 (anti-ETBR-acetal-PNU)**	**EC50 (free MC-acetal-PNU-159682)**
Parental UACC-257X2.2	4.4 ng/mL	7.2 nM
In vivo-derived resistant UACC-257X2.2	18.4 ng/mL	2.4 nM
In vitro-derived resistant UACC-257X2.2	15 ng/mL	15.8 nM

[0285] As above, the results of this experiment demonstrate that at least some sensitivity can be restored in the resistant cells by replacing the drug portion of the ADC.

[0286] The *in vivo*- and *in vitro*-derived resistant UACC-257X2.2 cells were assayed for sensitivity to anti-ETBR linked to PNU-159682 through the vc linker used in the MMAE immunoconjugate (ch5E9-vc-PNU). *See* Figure 7C. Table 5 shows the EC50s observed for anti-ETBR-vc-PNU and linker-drug (without antibody) for each of the cell lines.

Table 5: EC50s of anti-ETBR-vc-PNU in sensitive and resistant UACC-257X2.2 cells

Cells	**EC50 (anti-ETBR-vc-PNU)**	**EC50 (free MC-vc-PAB-PNU-159682)**
Parental UACC-257X2.2	3 ng/mL	2 nM
In vivo-derived resistant UACC-257X2.2	23 ng/mL	4.24 nM
In vitro-derived resistant UACC-257X2.2	41 ng/mL	6.6 nM

[0287] As above, the results of this experiment demonstrate that at least some sensitivity can be restored in the resistant cells by replacing the drug portion of the ADC. In addition, the results suggest that some resistance is also due to the linker portion of the ADC. *Compare* EC50s for the *in vitro*-derived resistant cells for anti-ETBR-PNU and anti-ETBR-acetal-PNU with anti-ETBR-vc-PNU.

[0288] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

Table of Sequences

SEQ ID NO	Description	Sequence
1	mu5E9 light chain	DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI TRVEAEDLGV YYCWQGTHFP YTFGGGTKLE IKRADAAPTV SIFPPSSEQL TSGGASVVCF LNNFYPKDIN VKWKIDGSER QNGVLNSWTD QDSKDSTYSM SSTLTLTKDE YERHNSYTCE ATHKTSTSPI VKSFNRNEC
2	mu5E9 heavy chain	QVQLLQSGAE LARPGASVKL SCKASGYTFT SYWMQWVKQR PGQGLEWIGT IYPGDGDTSY AQKFKGKATL TTDKYSSTAY MQLSSLASED SAVYYCARWG YAYDIDNWGQ GTTVTVSSAS TKGPSVYPLA PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGSLSSGVHT FPAVLQSDLY TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC PAPNLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLP APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH EGLHNHHTTK SFSRTPGK
3	mu5E9 light chain variable region	DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI TRVEAEDLGV YYCWQGTHFP YTFGGGTKLE IK
4	mu5E9 heavy chain variable region	QVQLLQSGAE LARPGASVKL SCKASGYTFT SYWMQWVKQR PGQGLEWIGT IYPGDGDTSY AQKFKGKATL TTDKYSSTAY MQLSSLASED SAVYYCARWG YAYDIDNWG
5	hu5E9.v1 light chain	DIQMTQSPSS LSASVGDRVT ITCKSSQSLL DSDGKTYLNW LQQKPGKAPK RLIYLVSKLD SGVPSRFSGS GSGTDFTLTI SSLQPEDFAT YYCWQGTHFP YTFGQGTKVE IKRTVAAPSV FIFPPSDEQL KSGTASVVCL LNNFYPREAK VQWKVDNALQ SGNSQESVTE QDSKDSTYSL SSTLTLSKAD YEKHKVYACE VTHQGLSSPV TKSFNRGEC
6	hu5E9.v1 heavy chain	EVQLVESGGG LVQPGGSLRL SCAASGYTFT SYWMQWVRQA PGKGLEWIGT IYPGDGDTSY AQKFKGRATL STDKSKNTAY LQMNSLRAED TAVYYCARWG YAYDIDNWGQ GTLVTVSSAS TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS CDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK
7	hu5E9.v1 light chain variable region	DIQMTQSPSS LSASVGDRVT ITCKSSQSLL DSDGKTYLNW LQQKPGKAPK RLIYLVSKLD SGVPSRFSGS GSGTDFTLTI SSLQPEDFAT YYCWQGTHFP YTFGQGTKVE IK
8	hu5E9.v1 heavy chain variable region	EVQLVESGGG LVQPGGSLRL SCAASGYTFT SYWMQWVRQA PGKGLEWIGT IYPGDGDTSY AQKFKGRATL STDKSKNTAY LQMNSLRAED TAVYYCARWG YAYDIDNWG
9	hu5E9.v2 heavy chain variable region	EVQLVQSGAE VKKPGASVKV SCKASGYTFT SYWMQWVRQA PGQGLEWIGT IYPGDGDTSY AQKFKGRVTI TRDTSTSTAY LELSSLRSED TAVYYCARWG YAYDIDNWG
10	Exemplary human endothelin B receptor protein	MQPPPSLCGR ALVALVLACG LSRIWGEERG FPPDRATPLL QTAEIMTPPT KTLWPKGSNA SLARSLAPAE VPKGDRTAGS PPRTISPPPC QGPIEIKETF KYINTVVSCL VFVLGIIGNS TLLRIIYKNK CMRNGPNILI ASLALGDLLH IVIDIPINVY KLLAEDWPFG AEMCKLVPFI QKASVGITVL SLCALSIDRY RAVASWSRIK GIGVPKWTAV EIVLIWVVSV VLAVPEAIGF DIITMDYKGS YLRICLLHPV QKTAFMQFYK TAKDWWLFSF YFCLPLAITA FFYTLMTCEM LRKKSGMQIA LNDHLKQRRE VAKTVFCLVL VFALCWLPLH LSRILKLTLY NQNDPNRCEL LSFLLVLDYI GINMASLNSC INPIALYLVS KRFKNCFKSC LCCWCQSFEE KQSLEEKQSC LKFKANDHGY DNFRSSNKYS SS
11	Exemplary human endothelin B receptor protein without	EERGFPPDRA TPLLQTAEIM TPPTKTLWPK GSNASLARSL APAEVPKGDR TAGSPPRTIS PPPCQGPIEI KETFKYINTV VSCLVFVLGI IGNSTLLRII YKNKCMRNGP NILIASLALG DLLHIVIDIP INVYKLLAED WPFGAEMCKL VPFIQKASVG ITVLSLCALS IDRYRAVASW SRIKGIGVPK WTAVEIVLIW VVSVVLAVPE AIGFDIITMD YKGSYLRICL LHPVQKTAFM QFYKTAKDWW

	signal sequence	LFSFYFCLPL AITAFFYTLM TCEMLRKKSG MQIALNDHLK QRREVAKTVF CLVLVFALCW LPLHLSRILK LTLYNQNDPN RCELLSFLLV LDYIGINMAS LNSCINPIAL YLVSKRFKNC FKSCLCCWCQ SFEEKQSLEE KQSCLKFKAN DHGYDNFRSS NKYSSS
12	5E9 HVR H1	GYTFTSYWMQ
13	5E9 HVR H2	TIYPGDGDTSYAQKFKG
14	5E9 HVR H3	WGYAYDIDN
15	5E9 HVR L1	KSSQSLLDSDGKTYLN
16	5E9 HVR L2	LVSKLDS
17	5E9 HVR L3	WQGTHFPYT
18	hu5E9.v1 V205C cysteine engineered light chain (Igκ)	DIQMTQSPSS LSASVGDRVT ITCKSSQSLL DSDGKTYLNW LQQKPGKAPK RLIYLVSKLD SGVPSRFSGS GSGTDFTLTI SSLQPEDFAT YYCWQGTHFP YTFGQGTKVE IKRTVAAPSV FIFPPSDEQL KSGTASVVCL LNNFYPREAK VQWKVDNALQ SGNSQESVTE QDSKDSTYSL SSTLTLSKAD YEKHKVYACE VTHQGLSSPC TKSFNRGEC
19	hu5E9.v1 A118C cysteine engineered heavy chain (IgG1)	EVQLVESGGG LVQPGGSLRL SCAASGYTFT SYWMQWVRQA PGKGLEWIGT IYPGDGDTSY AQKFKGRATL STDKSKNTAY LQMNSLRAED TAVYYCARWG YAYDIDNWG QGTLVTVSS CSTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
20	hu5E9.v1 S400C cysteine engineered heavy chain Fc region (IgG1)	EVQLVESGGG LVQPGGSLRL SCAASGYTFT SYWMQWVRQA PGKGLEWIGT IYPGDGDTSY AQKFKGRATL STDKSKNTAY LQMNSLRAED TAVYYCARWG YAYDIDNWG QGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDCDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
21	17A9 HVR H1	GYSFTRYTMN
22	17A9 HVR H2	VINPYNGGTVYNQKFKG
23	17A9 HVR H3	TDYDGYAMDY
24	17A9 HVR L2	SGSTLQS
25	17A9 HVR L3	QQHNEYPYT
26	17A9 HVR L2	SGSTLQS
27	ch5E9 light chain	DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI TRVEAEDLGV YYCWQGTHFP YTFGGGTKLE IKRTVAAPSV FIFPPSDEQL KSGTASVVCL LNNFYPREAK VQWKVDNALQ SGNSQESVTE QDSKDSTYSL SSTLTLSKAD YEKHKVYACE VTHQGLSSPV TKSFNRGEC
28	ch5E9 heavy chain	QVQLLQSGAE LARPGASVKL SCKASGYTFT SYWMQWVKQR PGQGLEWIGT IYPGDGDTSY AQKFKGKATL TTDKYSSTAY MQLSSLASED SAVYYCARWG YAYDIDNWGQ GTLVTVSSAS TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS CDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK

WHAT IS CLAIMED IS:

1. An immunoconjugate comprising an antibody that binds ETBR covalently attached to a cytotoxic agent, wherein the antibody binds an epitope within amino acids 64 to 101 of SEQ ID NO: 10, and wherein the cytotoxic agent is a nemorubicin derivative.

2. The immunoconjugate of claim 1, wherein the antibody comprises (i) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14, (ii) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17, and (iii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13.

3. The immunoconjugate of claim 1 or claim 2, wherein the antibody comprises (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, and (iii) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14.

4. The immunoconjugate of claim 1, wherein the antibody comprises (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, (iii) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14, (iv) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (v) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (vi) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

5. The immunoconjugate of any one of claims 1 to 3, wherein the antibody comprises (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (iii) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17.

6. The immunoconjugate of any one of claims 1 to 5, wherein the antibody comprises:

a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 8; or

b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 7; or

c) a VH sequence as in (a) and a VL sequence as in (b).

7. The immunoconjugate of claim 6, comprising a VH sequence having the amino acid sequence of SEQ ID NO: 8 or SEQ ID NO: 9.

8. The immunoconjugate of claim 6, comprising a VL sequence having the amino acid sequence of SEQ ID NO: 7.

9. An immunoconjugate comprising an antibody that binds ETBR covalently attached to a cytotoxic agent, wherein the antibody comprises (a) a VH sequence having the

amino acid sequence of SEQ ID NO: 8 and a VL sequence having the amino acid sequence of SEQ ID NO: 7, and wherein the cytotoxic agent is a nemorubicin derivative.

10. The immunoconjugate of any one of claims 1 to 9, wherein the antibody is an IgG1, IgG2a or IgG2b antibody.

11. An immunoconjugate of any one of claims 1 to 10, wherein the immunoconjugate has the formula Ab-(L-D)p, wherein:

(a) Ab is the antibody;

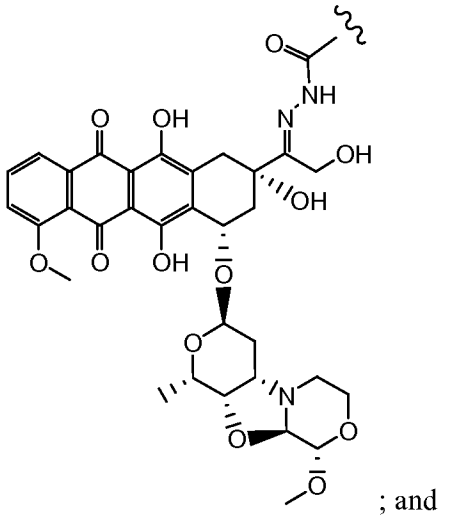
(b) L is a linker;

(c) D is the cytotoxic agent; and

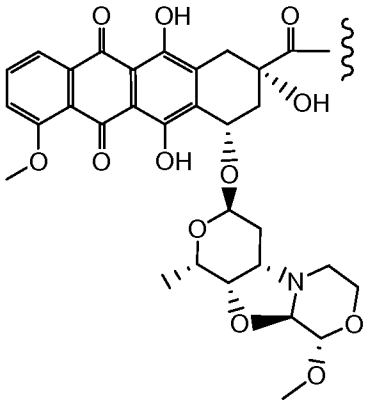
(d) p ranges from 1-8.

12. The immunoconjugate of claim 11, wherein D is a nemorubicin derivative.

13. The immunoconjugate of claim 12, wherein D has a structure selected from:



; and



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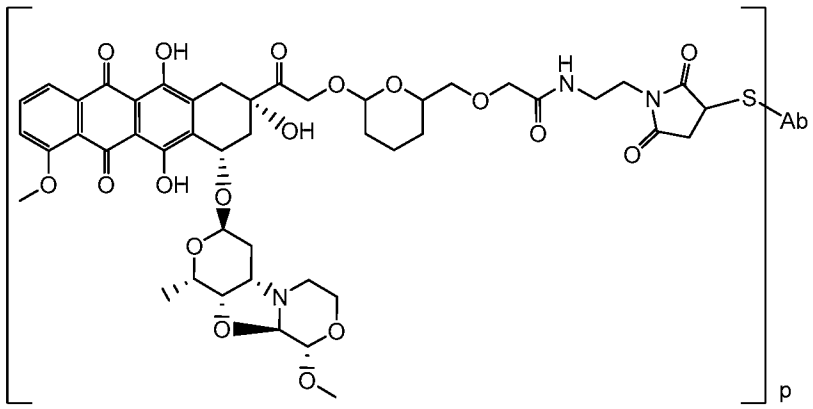
14. The immunoconjugate of any one of claims 11 to 13, wherein the linker is cleavable by a protease.

15. The immunoconjugate of claim 14, wherein the linker comprises a val-cit dipeptide or a Phe-homoLys dipeptide.

16. The immunoconjugate of any one of claims 11 to 13, wherein the linker is acid-labile.

17. The immunoconjugate of claim 16, wherein the linker comprises hydrazone.

18. The immunoconjugate of claim 12 having a formula selected from:



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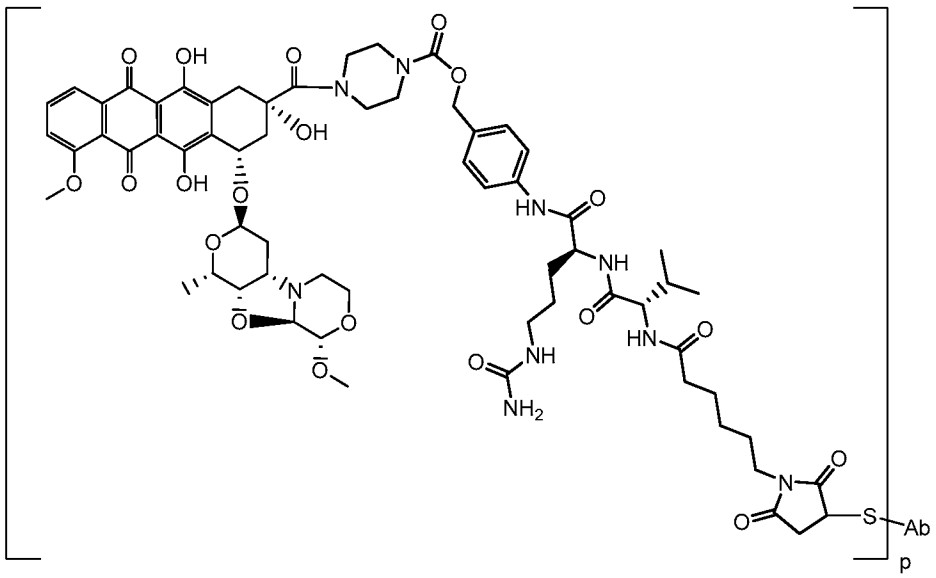

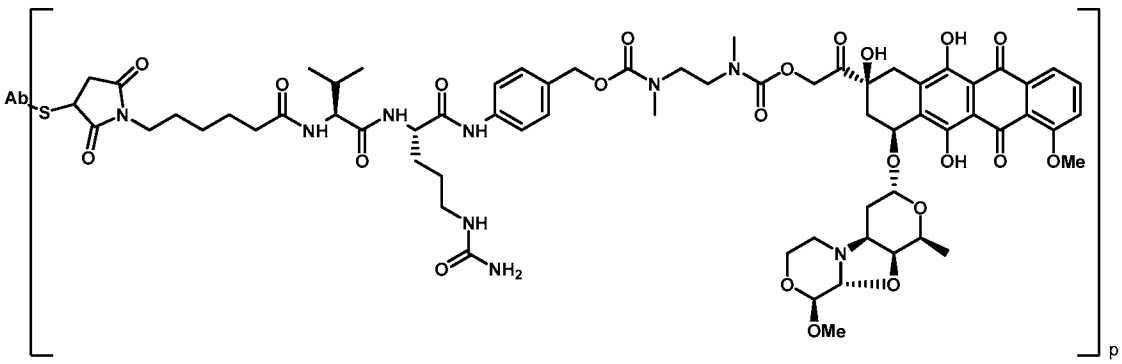

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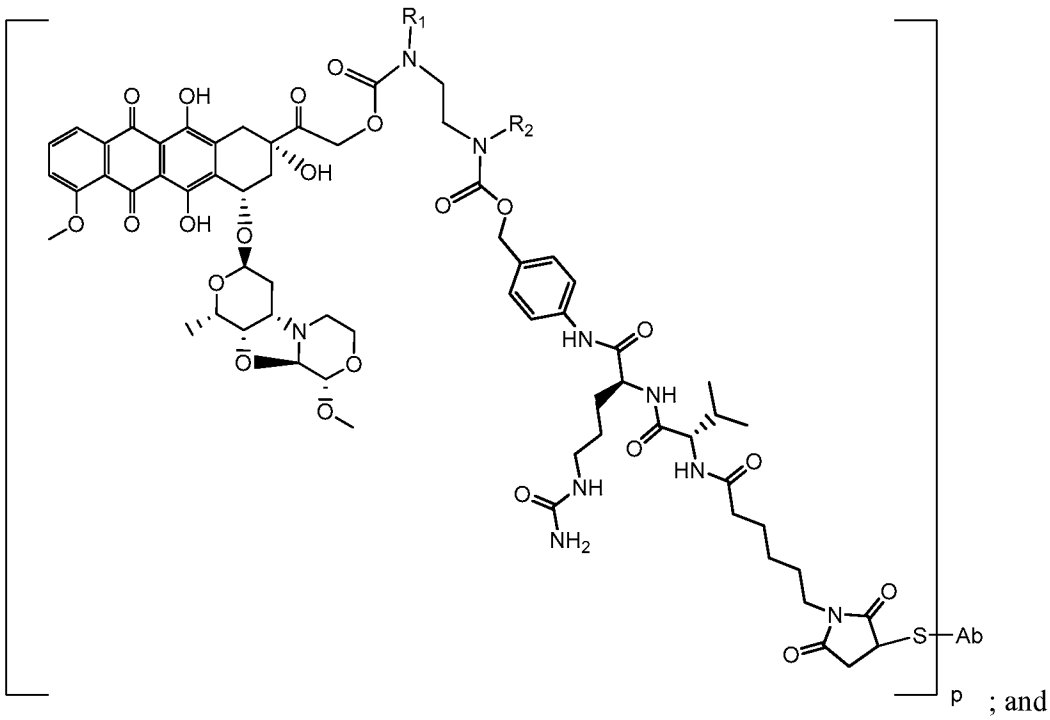
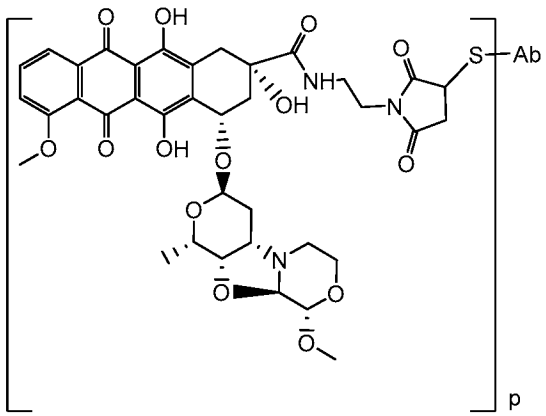


; and

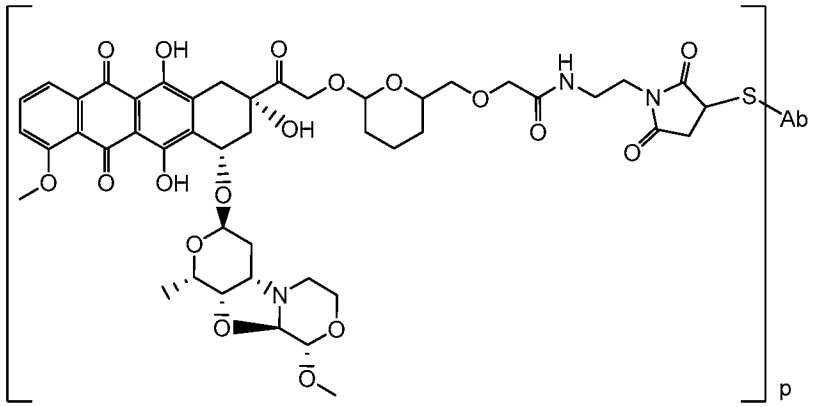


;

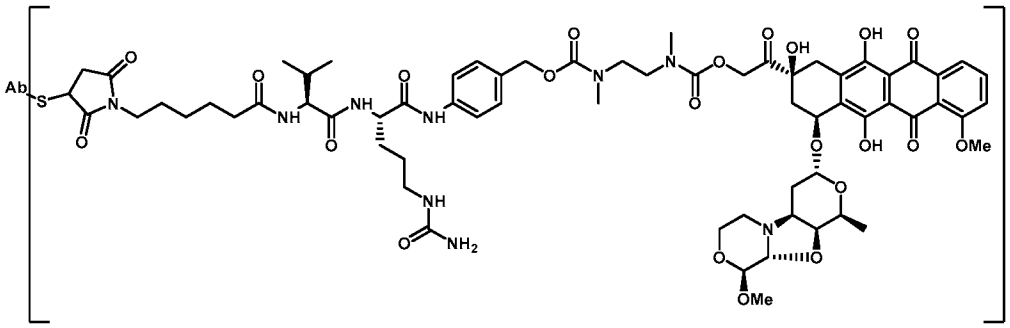
wherein R_1 and R_2 are independently selected from H and C_1-C_6 alkyl.

19. The immunoconjugate of any one of claims 11 to 18, wherein p ranges from 1-3.

20. An immunoconjugate having a formula selected from:



; and



;

wherein Ab is an antibody comprising (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO: 12, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO: 13, (iii) HVR-H3 comprising the amino acid sequence of SEQ ID NO: 14, (iv) HVR-L1 comprising the amino acid sequence of SEQ ID NO: 15, (v) HVR-L2 comprising the amino acid sequence of SEQ ID NO: 16, and (vi) HVR-L3 comprising the amino acid sequence of SEQ ID NO: 17; and wherein p ranges from 1 to 3.

21. The immunoconjugate of claim 20, wherein the antibody comprises a VH sequence of SEQ ID NO: 8 and a VL sequence of SEQ ID NO: 7.

22. The immunoconjugate of claim 21, wherein the antibody comprises a heavy chain of SEQ ID NO: 6 and a light chain of SEQ ID NO: 5.

23. The immunoconjugate of any one of the preceding claims, wherein the antibody is a monoclonal antibody.

24. The immunoconjugate of any one of the preceding claims, wherein the antibody is a human, humanized, or chimeric antibody.

25. The immunoconjugate of any one of the preceding claims, wherein the antibody is an antibody fragment that binds ETBR.

26. The immunoconjugate of claim any one of the preceding claims, wherein the antibody binds human ETBR.

27. The immunoconjugate of claim 26, wherein human ETBR has the sequence of SEQ ID NO: 10 or SEQ ID NO: 11.

28. A pharmaceutical formulation comprising the immunoconjugate of any one of the preceding claims and a pharmaceutically acceptable carrier.

29. The pharmaceutical formulation of claim 28, further comprising an additional therapeutic agent.

30. A method of treating an individual having an ETBR-positive cancer, the method comprising administering to the individual an effective amount of the immunoconjugate of any one of claims 1 to 27.

31. The method of claim 30, wherein the ETBR-positive cancer is melanoma.

32. The method of claim 31, further comprising administering an additional therapeutic agent to the individual.

33. The method of claim 32, wherein the additional therapeutic agent comprises an antibody that binds PMEL17.

34. The method of claim 33, wherein the additional therapeutic agent is an immunoconjugate comprising an antibody that binds PMEL17 covalently attached to a cytotoxic agent.

35. A method of inhibiting proliferation of an ETBR-positive cell, the method comprising exposing the cell to the immunoconjugate of any one of claims 1 to 27 under conditions permissive for binding of the immunoconjugate to ETBR on the surface of the cell, thereby inhibiting proliferation of the cell.

36. The method of claim 35, wherein the cell is a melanoma cell.

37. A method of treating an individual having an ETBR-positive cancer, wherein the ETBR-positive cancer is resistant to a first therapeutic, the method comprising administering to the individual an effective amount of the immunoconjugate of any one of claims 1 to 27.

38. The method of claim 37, wherein the ETBR-positive cancer is melanoma.

39. The method of claim 37 or claim 38, wherein the first therapeutic comprises a first antibody that binds an antigen other than ETBR.

40. The method of claim 39, wherein the first therapeutic is a first immunoconjugate comprising a first antibody that binds an antigen other than ETBR and a first cytotoxic agent.

41. The method of claim 39 or claim 40, wherein the first antibody binds an antigen selected from PMEL17, tyrosinase-related protein 1 (TYRP1), cytotoxic T lymphocyte antigen 4 (CTLA-4), and glycoprotein NMB (GPNMB).

42. The method of claim 41, wherein the first antibody binds PMEL17.

43. The method of claim 37 or claim 38, wherein the first therapeutic comprises a first antibody that binds ETBR.

44. The method of claim 43, wherein the first therapeutic is a first immunoconjugate comprising a first antibody that binds ETBR and a first cytotoxic agent.

45. The method of any one of claims 40 to 44, wherein the first cytotoxic agent and the cytotoxic agent of the immunoconjugate of any one of claims 1 to 27 are different.

46. The method of claim 45, wherein the first cytotoxic agent is MMAE.

47. A method of treating an individual with ETBR-positive cancer, comprising administering to the individual an effective amount of a first immunoconjugate of any one of claims 1 to 27 in combination with a second immunoconjugate comprising an antibody that binds PMEL17.

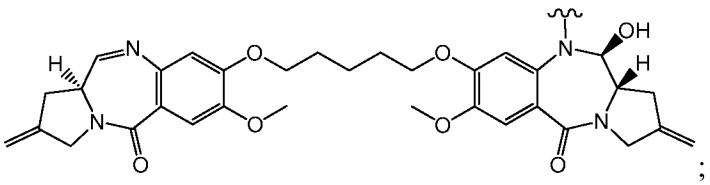
48. The method of claim 47, wherein the antibody that binds PMEL17 comprises an HVR H1 comprising a sequence of SEQ ID NO: 21, an HVR H2 comprising a sequence of SEQ ID NO: 22, an HVR H3 comprising a sequence of SEQ ID NO: 23, an HVR L1 comprising a sequence of SEQ ID NO: 24, an HVR L2 comprising a sequence of SEQ ID NO: 25, and an HVR L3 comprising a sequence of SEQ ID NO: 26.

49. The method of claim 47 or claim 48, wherein the second immunoconjugate comprises a cytotoxic agent selected from an auristatin, a pyrrolobenzodiazepine, and a nemorubicin derivative.

50. The method of any one of claims 47 to 49, wherein the second immunoconjugate comprises an auristatin or a pyrrolobenzodiazepine.

51. The method of any one of claims 47 to 50, wherein the second immunoconjugate comprises MMAE.

52. The method of any one of claims 47 to 50, wherein the second immunoconjugate comprises a PBD dimer having the structure:



;

wherein the wavy line indicates attachment to a linker.

53. The method of any one of claims 47 to 51, wherein the second immunoconjugate comprises a linker-drug portion comprising MC-val-cit-PAB-MMAE.

54. The method of any one of claims 47 to 50 and 52, wherein the second immunoconjugate comprises a linker comprising MC-val-cit-PAB.

55. The method of any one of claims 47 to 54, wherein the ETBR-positive cancer is melanoma.

56. The method of any one of claims 47 to 55, wherein the ETBR-positive cancer is also PMEL17-positive.

Full-Length Light Chain of Murine 5E9
DVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDF
TLKITRVEAEDLGVYYCWQGTHFPYTFGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVK
WKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID
NO:1)

VL of Murine 5E9
DVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDF
TLKITRVEAEDLGVYYCWQGTHFPYTFGGGTKLEIK (SEQ ID NO:3)

FIG. 1

Full-Length Heavy Chain of Murine 5E9
QVQLLQSGAELARPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLEWIGTIYPGDGDTSYAQKFKGKATLTTDKYS
STAYMQLSSLASEDSAVYYCARWGYAYDIDNWGQGTTVTVSSASTKGPSVYPLAPVCGDTTGSSVTLGCLVKGYFP
EPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPC
KCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNST
LRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPE
DIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK
(SEQ ID NO:2)

VH of Murine 5E9
QVQLLQSGAELARPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLEWIGTIYPGDGDTSYAQKFKGKATLTTDKYS
STAYMQLSSLASEDSAVYYCARWGYAYDIDNWG (SEQ ID NO:4)

FIG. 2

Full-Length Light Chain of hu5E9.v1
DIQMTQSPSSLSASVGDRVTITCKSSQSLLDSDGKTYLNWLQQKPGKAPKRLIYLVSKLDSGVPSRFSGSGSGTDF
TLTISSLQPEDFATYYCWQGTHFPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQ
WKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID
NO:5)

VL of hu5E9.v1
DIQMTQSPSSLSASVGDRVTITCKSSQSLLDSDGKTYLNWLQQKPGKAPKRLIYLVSKLDSGVPSRFSGSGSGTDF
TLTISSLQPEDFATYYCWQGTHFPYTFGQGTKVEIK (SEQ ID NO:7)

FIG. 3

Full-Length Heavy Chain of hu5E9.v1
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYWMQWVRQAPGKGLEWIGTIYPGDGDTSYAQKFKGRATLSTDKSK
NTAYLQMNSLRAEDTAVYYCARWGYAYDIDNWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP
EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST
YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO:6)

VH of hu5E9.v1
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYWMQWVRQAPGKGLEWIGTIYPGDGDTSYAQKFKGRATLSTDKSK
NTAYLQMNSLRAEDTAVYYCARWGYAYDIDNWG (SEQ ID NO:8)

FIG. 4

VH of hu5E9.v2
EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMQWVRQAPGQGLEWIGTIYPGDGDTSYAQKFKGRVTITRDTST
STAYLELSSLRSEDTAVYYCARWGYAYDIDNWG (SEQ ID NO:9)

FIG. 5

MQPPPSLCGRALVALVLACGLSRIWGEERGFPPDRATPLLQTAEIMTPPTKTLWPKGSNASLARSLAPAEVPKGDR
TAGSPPRTISPPPCQGPIEIKETFKYINTVVSCLVFVLGIIGNSTLLRIIYKNKCMRNGPNILIASLALGDLLHIV
IDIPINVYKLLAEDWPFGAEMCKLVPFIQKASVGITVLSLCALSIDRYRAVASWSRIKGIGVPKWTAVEIVLIWVV
SVVLAVPEAIGFDIITMDYKGSYLRICLLHPVQKTAFMQFYKTAKDWWLFSFYFCLPLAITAFFYTLMTCEMLRKK
SGMQIALNDHLKQRREVAKTVFCLVLVFALCWLPLHLSRILKLTLYNQNDPNRCELLSFLLVLDYIGINMASLNSC
INPIALYLVSKRFKNCFKSCLCCWCQSFEEKQSLEEKQSCLKFKANDHGYDNFRSSNKYSSS (SEQ ID
NO:10)

Signal sequence.

amino acids 1-26

Transmembrane domains.

amino acids 101-121, 137-157, 177-197, 216-236, 275-295, 323-343, 362-382

N-glycosylation sites.

amino acids 59-62, 119-122

cAMP- and cGMP-dependent protein kinase phosphorylation site.

amino acids 302-305

Tyrosine kinase phosphorylation site.

amino acids 424-430

N-myristoylation sites.

amino acids 57-62, 115-120, 170-175, 306-311, 371-376

7 transmembrane receptor homology.

amino acids 118-386

FIG. 6

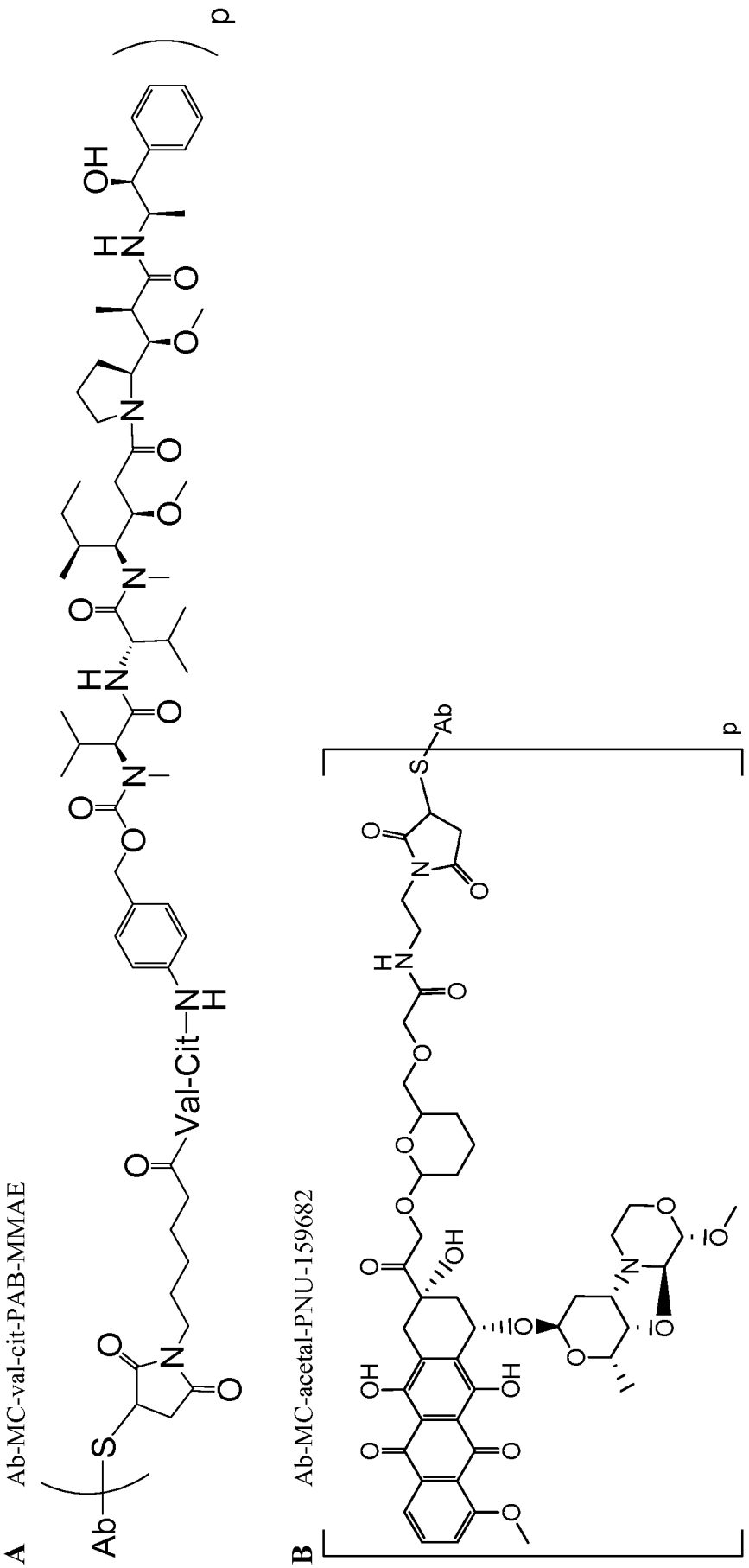

A
Ab-MC-val-cit-PAB-MMAE
Ab
S
Val-Cit
p
B
Ab-MC-acetal-PNU-159682
Ab
S
p

FIG. 7

C Ab-MC-val-cit-PAB-PNU-159682

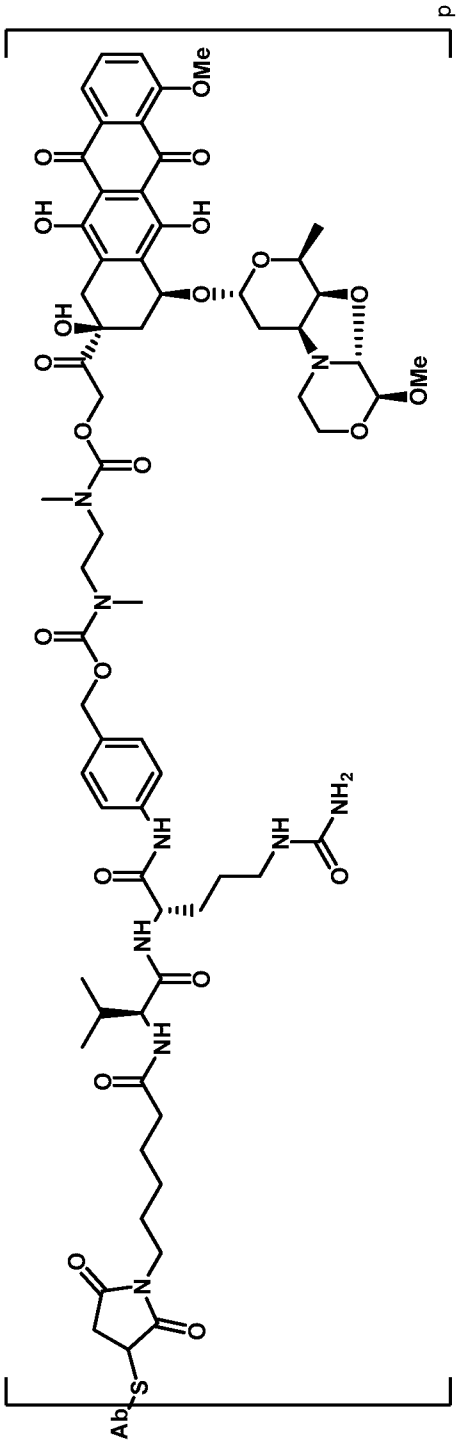


FIG. 7

D Ab-PNU-159682

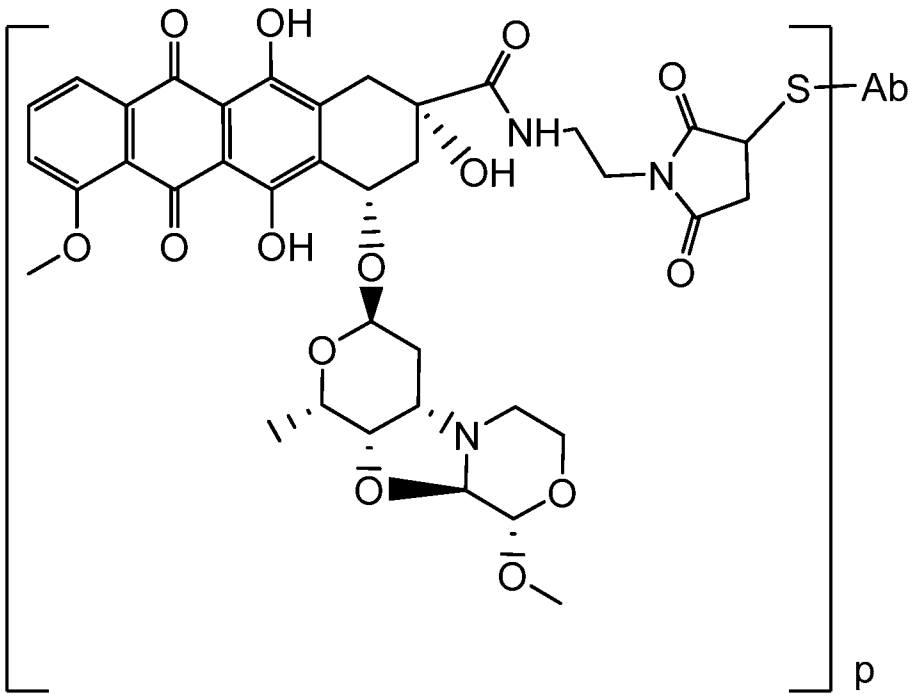
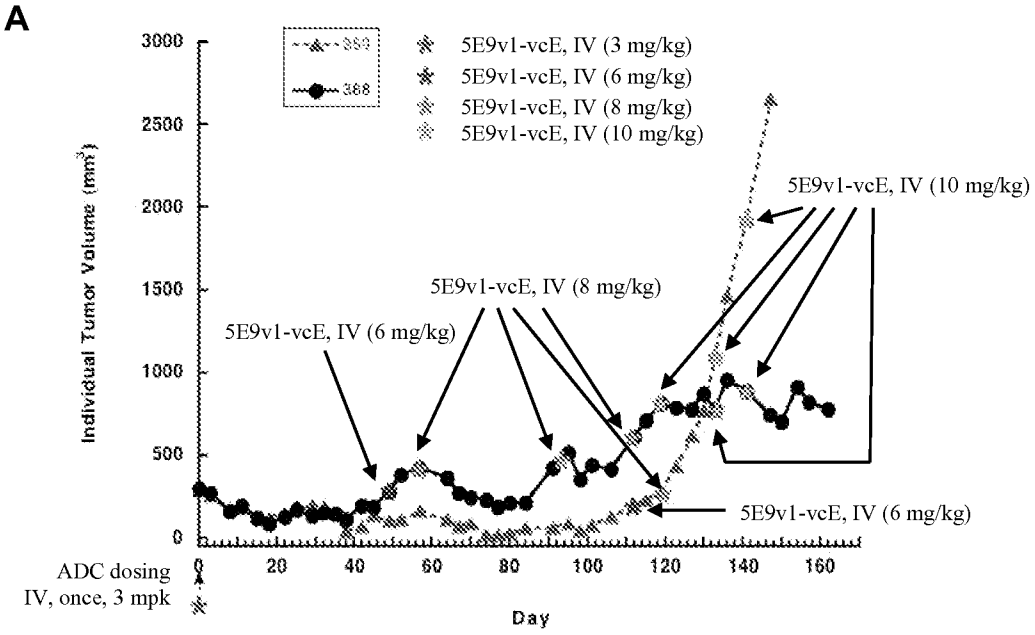


FIG. 7


A
3000
2500
2000
1500
1000
500
0
Individual Tumor Volume (mm³)
389
388
5E9v1-vcE, IV (3 mg/kg)
5E9v1-vcE, IV (6 mg/kg)
5E9v1-vcE, IV (8 mg/kg)
5E9v1-vcE, IV (10 mg/kg)
5E9v1-vcE, IV (10 mg/kg)
5E9v1-vcE, IV (8 mg/kg)
5E9v1-vcE, IV (6 mg/kg)
5E9v1-vcE, IV (6 mg/kg)
ADC dosing
IV, once, 3 mpk
0
20
40
60
80
100
120
140
160
Day

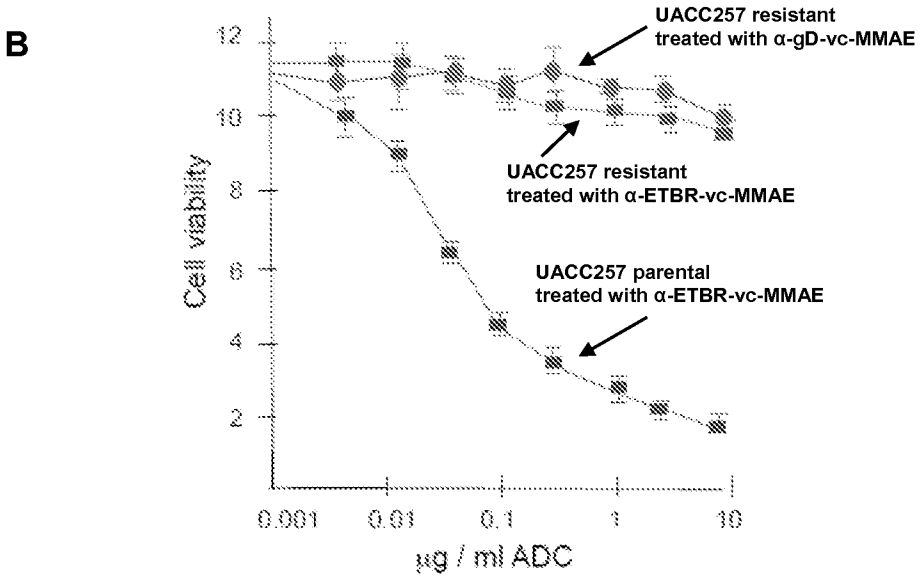
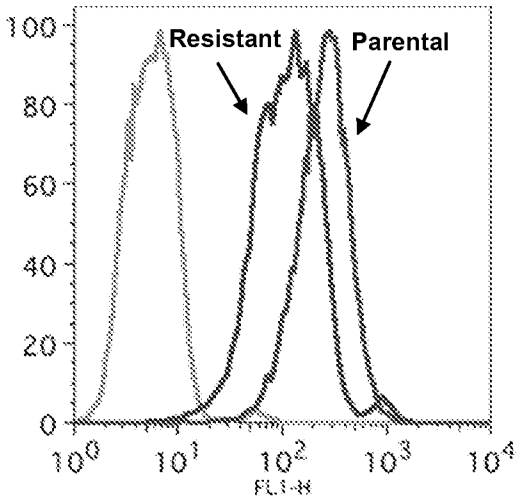

B
12
10
8
6
4
2
Cell viability
UACC257 resistant
treated with α-gD-vc-MMAE
UACC257 resistant
treated with α-ETBR-vc-MMAE
UACC257 parental
treated with α-ETBR-vc-MMAE
0.001
0.01
0.1
1
10
µg / ml ADC

FIG. 8

A ***IN VIVO*** **DERIVED**

FACS EDNRB (ETBR; target A)



B ***IN VITRO*** **DERIVED**

FACS EDNRB (ETBR; target A)

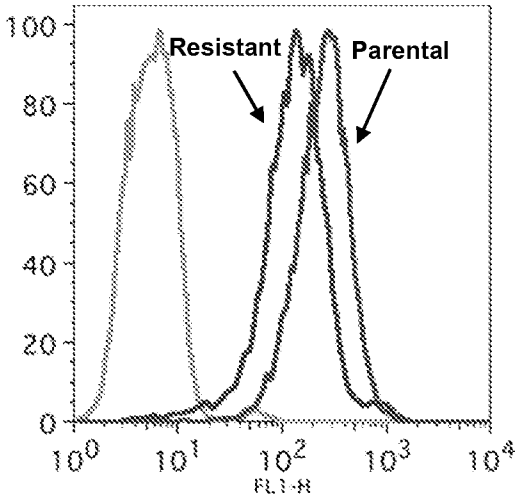


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053250

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/395 (2013.01)
USPC - 424/181.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 31/551, 31/5517, 38/05, 39/395, 47/42, 47/48; A61P 35/00; C07D 487/04, 519/00 (2013.01)
USPC - 424/178.1, 181.1; 530/391.7, 391.9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 47/48246, 47/48407, 47/48561, 47/48584, 47/48638 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, PubMed, Google, SureChem

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0206702 A1 (POLAKIS et al) 25 August 2011 (25.08.2011) entire document	1-4, 9, 20-22
Y	US 2012/0130059 A1 (BERIA et al) 24 May 2012 (24.05.2012) entire document	1-4, 9, 20-22
A	ASUNDI et al.,"An Antibody–Drug Conjugate Targeting the Endothelin B Receptor for the Treatment of Melanoma," Clinical Cancer Research, 18 January 2011 (18.01.2011), Vol. 17, Pgs. 965-976. entire document	1-4, 9, 20-22
T, X	US 2013/0295007 A1 (CHEN et al) 07 November 2013 (07.11.2013) entire document	1-4, 9, 20-22

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
18 December 2013	10 JAN 2014

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053250

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

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

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

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

3. Additional comments:

Specifically, SEQ ID NOs: 5-8, 10, and 12-17 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053250

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 5-8, 10-19, 23-56
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.