ANTI-NOTCH3 ANTIBODIES AND ANTIBODY-DRUG CONJUGATES

RELATED APPLICATIONS

This application claims the benefits of U.S. Provisional Application No. 61/723,772 filed November 7, 2012 and U.S. Provisional Application No. 61/889,744 filed October 11, 2013, which are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

This application is being filed electronically via EFS-Web and includes an electronically submitted sequence listing in .txt format. The .txt file contains a sequence listing entitled "PC071980A_Sequence_Listing.txt" created on October 11, 2013, and having a size of 61 KB. The sequence listing contained in this .txt file is part of the specification and is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to anti-Notch3 antibodies and anti-Notch3 antibody-drug conjugates. The present invention further relates to methods of using such antibodies and antibody-drug conjugates for the treatment of cancer.

BACKGROUND

Notch signaling is triggered by extracellular receptor and ligand interactions. Notch receptors control normal cellular proliferation, differentiation, and death in multicellular organisms through a signaling cascade that is triggered by ligand-induced proteolysis. After furin-like protease cleavage at site S1, the mature Notch heterodimer is translocated into the cell membrane where it is held in an auto-inhibited state by a juxtamembrane Negative Regulatory Region (NRR) consisting of three Lin12/Notch repeats (LNR-A, B, C) and the heterodimerization (HD) domain. The HD domain is divided into N-terminal (HD1) and C-terminal (HD2) halves after cleavage at site S1. Through an uncertain mechanism, binding of ligands of the Delta/Serrate/Lag-2 (DSL) family to the extracellular EGF-repeat region relieves this inhibition and induces two

additional cleavage events. First, ADAM-type metalloproteinase mediate cleavage at site S2 near the C-terminal region of the HD-2 domain, thereby releasing the extracellular domain (ECD) from the cell surface which then undergoes trans-endocytosis into the ligand-expressing cell. Next, gamma-secretase mediates cleavage at site S3 within the transmembrane domain which releases the intracellular domain of Notch (Notch-ICD) from the membrane, permitting it to translocate to the nucleus and activate the transcription of target genes (Bray, S., Nature Reviews Molecular Cell Biology, 2006, volume 7, 678-689).

The X-ray crystal structure of the human Notch2-NRR domain in an auto-inhibited conformation revealed extensive interactions between the LNR repeats and heterdimerization domains within the NRR burying the metalloprotease S2 site, suggesting that a substantial conformational movement is necessary to expose the site during activation by ligand (Gordon, W.R., et.al, Nature Structural & Molecular Biology, 2007, volume 14, 295-300). Studies suggest that stabilization of the interactions within the NRR may prevent ligand-induced Notch activation. The availability of structural information on the Notch auto-inhibited conformation provided new opportunities for the development of therapeutics, particularly antibodies that target Notch signaling. (Li, K., et. al, Journal of Biological Chemistry, 2008, volume 283, 8046-8054; Aste-Amezaga, M, et. al, PLOS ONE, 2010, volume 5, e9094; Wu, Y., et. al, Nature, 2010, volume 464, 1052-1057;).

In mammalian cells, there are four known Notch receptors. Notch-1, -2, -3 and -4 have broad, overlapping patterns of expression in embryonic and adult tissues, and fulfill non-redundant roles during hematopoietic stem cell specification, T cell development, intestinal crypt cell specification and vascular development. Notch3 is expressed primarily in vascular smooth muscle cells (vSMC), various thymocyte subpopulations and the developing nervous system. Consistent with its restricted tissue distribution, targeted deletion of murine Notch3 does not lead to embryonic lethality like Notch1 and Notch2 deletion. Instead, Notch3-null mice are viable, but have defects in the maturation and differentiation of vSMCs (Domenga, V., et. al, Genes and Development, 2004, volume 18, 2730-2735).

Notch activation is oncogenic in many contexts; constitutively active, intracellular forms of all four Notch homologues function as oncogenes *in vitro* and in transgenic mouse models. Recent studies indicate that Notch3 is often amplified and over-expressed in various human solid tumors and the over-expression of developmental signaling pathways, such as Notch3, in human cancers implicates them as key mediators of tumorigenesis. Several strategies are in development to block Notch signaling for therapeutic purposes in cancer; however there is still a need in the art for more potent and efficacious anti-Notch targeted therapies for the treatment of cancer.

Antibody-drug conjugates (ADCs) combine the specificity and targeting of high affinity antibodies with the cytotoxicity of a therapeutic agent, such as cytotoxic agents, biological response modifiers, enzymes, apoptosis-inducing agents, and radioisotopes. Release of therapeutic agents from the antibody can require trafficking and localization of the antibody-drug conjugate to lysosomes and both Notch3-ECD and Notch3-ICD undergo lysosomal degradation, thus antibodies that bind Notch3 are expected to traffic to the lysosome (Jia L, et. al, International Journal of Biochemistry and Cell Biology, 2009, volume 41, 2594-2598). The present invention provides novel anti-Notch3 antibodies and antibody-drug conjugates that fulfill an unmet clinical need in the diagnosis and therapeutic use in the treatment of cancer.

SUMMARY

The present invention provides for anti-Notch3 antibodies and antibody-drug conjugates (ADCs). The present invention also provides for methods of using such anti-Notch3 antibodies and antibody-drug conjugates for the treatment of cancer.

In one embodiment, the present invention provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, having a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 13 and, a light chain variable region having a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 25.

In another embodiment, the present invention provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, wherein the antibody or antigen-binding fragment: (a) internalizes into a cell, (b) does not inhibit Notch3 signaling, or (c)

does not activate Notch3 signaling. In a further embodiment, the present invention provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, wherein the antibody or antigen-binding fragment: (a) binds to the LNR-C and HD-1 domains of the Notch3 NRR, (b) does not maintain the Notch3 NRR in an auto-inhibitory conformation, or (c) does not inhibit S2-cleavage.

In a further embodiment, the present invention provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, having: (a) a heavy chain CDR1 comprising SEQ ID NO: 15 or 16; (b) a heavy chain CDR2 comprising SEQ ID NO: 19 or 20; (c) a heavy chain CDR3 comprising SEQ ID NO: 23; (d) a light chain CDR1 comprising SEQ ID NO: 27; (e) a light chain CDR2 comprising SEQ ID NO: 29; and, (f) a light chain CDR3 comprising SEQ ID NO: 31.

The present invention also provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, having a heavy chain variable region amino acid sequence that is at least 90% identical to SEQ ID NO: 13 or a light chain variable region amino acid sequence that is at least 90% identical to SEQ ID NO: 25.

The present invention also provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3 having a heavy chain variable region amino acid sequence of SEQ ID NO: 13 and isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3 having a light chain variable region amino acid sequence of SEQ ID NO: 25. The present invention also provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3 having a heavy chain amino acid sequence of SEQ ID NO: 33 and isolated antibodies, or antigen-binding fragment thereof, that bind Notch3 having a light chain amino acid sequence of SEQ ID NO: 35.

In another embodiment, the present invention provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, having a heavy chain variable region comprising a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 37 and, a light chain variable region having a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 49.

In further embodiment, the present invention provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, having: (a) a heavy chain CDR1 comprising SEQ ID NO: 39 or 40; (b) a heavy chain CDR2 comprising SEQ ID NO: 43 or 44; (c) a heavy chain CDR3 comprising SEQ ID NO: 47; (d) a light chain CDR1

comprising SEQ ID NO: 51; (e) a light chain CDR2 comprising SEQ ID NO: 53; and, (f) a light chain CDR3 comprising SEQ ID NO: 55.

The present invention also provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3, having a heavy chain variable region amino acid sequence that is at least 90% identical to SEQ ID NO: 37 or a light chain variable region amino acid sequence that is at least 90% identical to SEQ ID NO: 49.

The present invention also provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3 having a heavy chain variable region amino acid sequence of SEQ ID NO: 37 and isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3 having a light chain variable region amino acid sequence of SEQ ID NO: 49. The present invention also provides for isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3 having a heavy chain amino acid sequence of SEQ ID NO: 57 and isolated antibodies, or antigen-binding fragment thereof, that bind to Notch3 having a light chain amino acid sequence of SEQ ID NO: 59.

The invention further provides for isolated antibodies that compete with an antibody, or antigen-binding fragment thereof, of the present invention for specific binding to Notch3.

The present invention further provides for antibody-drug conjugates having a cytotoxic agent conjugated to any antibody, or antigen-binding fragment thereof, of the present invention.

In another embodiment, the present invention provides for antibody-drug conjugates of the formula: Ab-(L-D)*p*, or a pharmaceutically acceptable salt thereof wherein; Ab is an antibody, or antigen-binding fragment thereof, that binds to Notch3; L-D is a linker-drug moiety, wherein L is a linker, and D is a drug; and *p* is an integer from 1 to about 12.

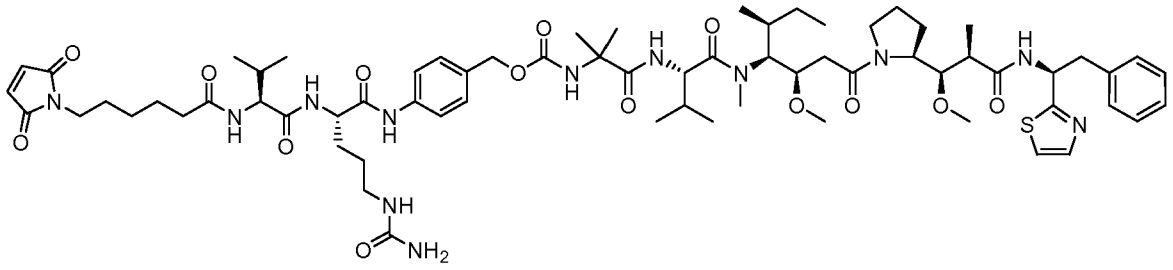
In a further embodiment, the present invention provides antibody-drug conjugates of the formula: Ab-(L-D)*p*, or a pharmaceutically acceptable salt thereof wherein; Ab is any antibody, or antigen-binding fragment thereof, of the present invention; L-D is a linker-drug moiety, wherein L is a linker, and D is a drug; and *p* is an integer from 1 to about 12.

In another embodiment, the present invention provides antibody-drug conjugates wherein L is selected from the group consisting of vc, mc, me and MalPeg6C2.

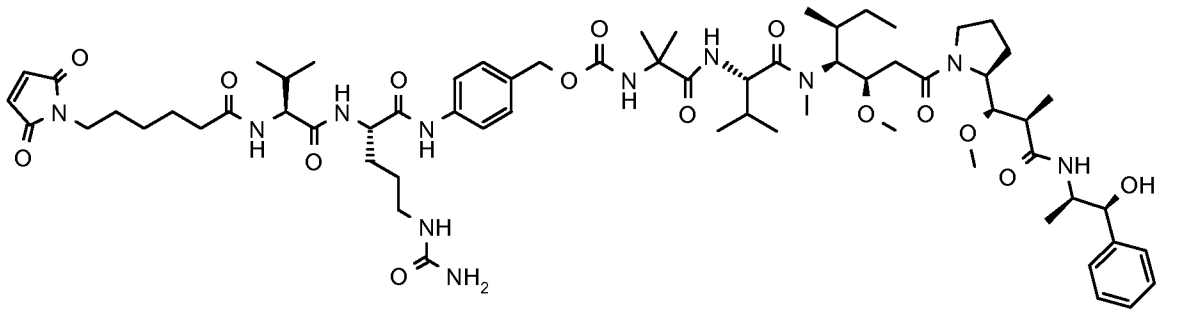
In another embodiment, the present invention provides antibody-drug conjugates wherein D is selected from the group consisting of: (a) 0101 (2-Methylalanyl-N-[(3R,4S,5S)-3-methoxy-1-{(2S)-2-[(1R,2R)-1-methoxy-2-methyl-3-oxo-3-{[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide), (b) 6780 (2-methylalanyl-N-[(3R,4S,5S)-1-{(2S)-2-[(1R,2R)-3-{[(1S,2R)-1-hydroxy-1-phenylpropan-2-yl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide), (c) 0131 (2-methyl-L-prolyl-N-[(3R,4S,5S)-1-{(2S)-2-[(1R,2R)-3-{[(1S)-1-carboxy-2-phenylethyl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide, trifluoroacetic acid salt), (d) 3377 (N,2-dimethylalanyl-N-{(1S,2R)-4-{(2S)-2-[(1R,2R)-3-{[(1S)-1-carboxy-2-phenylethyl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-2-methoxy-1-[(1S)-1-methylpropyl]-4-oxobutyl}-N-methyl-L-valinamide, trifluoroacetic acid salt), and (e) 8261 (2-Methylalanyl-N-[(3R,4S,5S)-1-{(2S)-2-[(1R,2R)-3-{[(1S)-1-carboxy-2-phenylethyl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide).

The present invention further provides for antibody-drug conjugates wherein L-D is selected from the group consisting of:

vc0101 having the formula:



and vc6780 having the formula:



In another embodiment, the present invention provides for antibody-drug conjugates, wherein Ab comprises (a) a heavy chain variable region having a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 13; and, (b) a light chain variable region having a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 25.

The present invention further provides for antibody-drug conjugates, wherein Ab comprises (a) a heavy chain CDR1 comprising SEQ ID NO: 15; (b) a heavy chain CDR2 comprising SEQ ID NO: 19; (c) a heavy chain CDR3 comprising SEQ ID NO: 23; (d) a light chain CDR1 comprising SEQ ID NO: 27; (e) a light chain CDR2 comprising SEQ ID NO: 29; and, (f) a light chain CDR3 comprising SEQ ID NO: 31.

In another embodiment, the present invention provides for antibody-drug conjugates, wherein Ab comprises (a) a heavy chain variable region comprising a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 37; and, (b) a light chain variable region comprising a CDR1, a CDR2, and a CDR3 of SEQ ID NO: 49.

The present invention further provides for antibody-drug conjugates, wherein Ab comprises (a) a heavy chain CDR1 comprising SEQ ID NO: 39; (b) a heavy chain CDR2 comprising SEQ ID NO: 43; (c) a heavy chain CDR3 comprising SEQ ID NO: 47; (d) a light chain CDR1 comprising SEQ ID NO: 51; (e) a light chain CDR2 comprising SEQ ID NO: 53; and, (f) a light chain CDR3 comprising SEQ ID NO: 55.

The present invention further provides for antibody-drug conjugates,wherein Ab comprises an engineered human IgG1 heavy chain constant domain (Cγ) polypeptide selected from the group consisting of: (a) one amino acid substitution at position L443 as set forth in SEQ ID NO: 61 and (b) two amino acid substitutions at positions L443 and K392 as set forth in SEQ ID NO: 65, according to the EU index of Kabat.

The present invention further provides for antibody-drug conjugates, wherein Ab comprises an engineered human kappa light chain constant domain (CK) polypeptide having one amino acid substitution at position κK183 as set forth in SEQ ID NO: 63, according to the EU index of Kabat.

The present invention further provides a pharmaceutical composition having any antibody, or antigen-binding fragment thereof, of the present invention or any antibody-drug conjugate of the present invention and a pharmaceutically acceptable carrier.

Further, the present invention provides for a method of treating a condition associated with Notch3 expression in a patient in need thereof, comprising administering to the patient any antibody-drug conjugate or a pharmaceutical composition of the present invention. The present invention also provides for a method of treating cancer, wherein the cancer is a solid tumor cancer. Further, the present invention provides for methods treating solid tumor cancers including, but not limited to, lung cancer, breast cancer, ovarian cancer, stomach cancer, esophageal cancer, cervical cancer, head and neck cancer, bladder cancer, liver cancer, skin cancer and sarcoma. The present invention also provides for methods of treating cancer, wherein the cancer is a blood cancer including, but limited to, T-cell malignancies, T-cell leukemia, T-cell lymphoma, T-cell acute lymphoblastic leukemia, multiple myeloma, B-cell malignancies, myeloid malignancies, acute myeloid leukemia and chronic myeloid leukemia.

The present invention provides any antibody-drug conjugate or pharmaceutical composition of the present invention for use in therapy. The present invention further provides for use in a therapy, wherein the cancer is a solid tumor cancer. The present invention also provides for use in therapy wherein the solid tumor cancers includes but is not limited lung cancer, breast cancer, ovarian cancer, stomach cancer, esophageal cancer, cervical cancer, head and neck cancer, bladder cancer, liver cancer, skin cancer and sarcoma. The present invention also provides for use in a therapy, wherein the cancer is a blood cancer including, but limited to, T-cell malignancies, T-cell leukemia, T-cell lymphoma, T-cell acute lymphoblastic leukemia, multiple myeloma, B-cell malignancies, myeloid malignancies, acute myeloid leukemia and chronic myeloid leukemia. The invention further provides for use of any antibody-drug conjugate of the

present invention in the manufacture of a medicament for therapy. The invention further provides the use of any antibody-drug conjugate of the present invention, wherein said use is for the treatment of a Notch3 expressing cancer.

The invention further provides a nucleic acid that encodes Notch3 antibodies, or antibody-binding fragments thereof, of the present invention, a vector comprising said nucleic acid, and a host cell comprising said vector. The invention also provides a process for producing Notch3 antibodies of the present invention wherein said process comprises cultivating the host cell comprising the above mentioned vector and recovering the antibody from the cell culture.

In another embodiment, the invention provides a process for producing antibody-drug conjugates of the present invention comprising: linking L to D; conjugating the L-D to an antibody recovered from the culture of the present invention; and purifying the antibody-drug conjugate.

The invention further provides for antibody-drug conjugates having antibody, or antigen-binding fragments thereof, of the present invention that specifically binding to Notch3.

In a further embodiment, the present invention provides a method for predicting whether a subject with cancer will respond to any antibody-drug conjugates of the present invention by determining whether a biological sample from the subject expresses Notch3.

The invention further provides a process of determining the level of Notch3 in a biological sample comprising the steps of: contacting a sample from a subject suspected to have cancer with any antibody, or antigen-binding fragment thereof, of the present invention; determining the cell surface levels of Notch3 on the sample; and comparing the cell surface levels of Notch3 with that of a reference subject or standard.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a schematic diagram of recombinant, S1-cleaved, heterodimeric Notch3 NRR protein immunogen with Avi and His tags.

FIG. 2 shows the amino acid and nucleotide sequences of purified human and mouse Notch3 NRR recombinant proteins.

FIG. 3 shows the amino acid and nucleotide sequences of r28 and r75 antibody variable regions (CDRs underlined).

FIGS. 4A through 4C show the amino acid and nucleotide sequences of [A] hu28 VH 1.0 and CDRs (Kabat and Chothia), [B] hu28 VL 1.0 and CDRs (Kabat and Chothia), and [C] hu28 HC 1.0 and LC 1.0.

FIGS. 5A through 5C show the amino acid and nucleotide sequences of [A] hu75 VH 1.9 and CDRs (Kabat and Chothia), [B] hu75 VL 1.3 and CDRs (Kabat and Chothia), and [C] hu75 HC 1.9 and LC 1.3.

FIG. 6 shows recombinant human Notch1 NRR and Notch3 NRR domain swap chimeric constructs for epitope mapping of the anti-Notch3 antibodies ch28 and ch75.

FIG. 7 shows Western blot analysis of Notch3 positive and negative cell lines using the D11B8 antibody.

FIGS. 8A through 8E show [A] cell membrane localization of anti-Notch3 antibody hu75-Alexa 488 and labeling of acidic vesicles with pHrodo™ red dextran in MDA-MB-468 breast cancer cells at hour 0, [B] Intracellular trafficking of anti-Notch3 antibody hu75-Alexa 488 and co-localization with pHrodo™ red dextran in MDA-MB-468 breast cancer cells at hour 5 (arrows), [C] cell membrane localization of anti-Notch3 antibody hu28-DyLight650 and labeling of acidic vesicles with pHrodo™ red dextran (intracellular puncta) in MDA-MB-468 breast cancer cells at hour 0, [D] intracellular trafficking of anti-Notch3 antibody hu28-DyLight650 and co-localization with pHrodo™ red dextran in MDA-MB-468 breast cancer cells at hour 8 (arrows) and [E] Pearson's correlation coefficient demonstrating the degree of overlap or co-localization of hu28-DyLight650 and pHrodo™ red dextran fluorescent labels in MDA-MB-468 cells over time.

FIG. 9 shows Western blot analysis of S2-cleavage assay using HCC2429 and MDA-MB-468 cells treated with anti-Notch3 hu28 and hu75. M.W. = molecular weight.

FIG 10 shows the treatment of OVCAR3 ovarian cancer cells with anti-Notch3 hu28-vc0101 disrupts microtubules (stained with anti-alpha-tubulin antibody) in mitotic cells that were identified by phospho-Histone H3 staining.

FIG. 11 shows Western blot analysis of Notch3-ECD from xenografts harvested from 2-3 mice (M).

FIG. 12 shows the efficacy of anti-Notch3 hu28-vc0101 and hu75-vc0101 at a dose of 3 mg/kg compared to Cisplatin at a dose of 5 mg/kg in the 37622A1 NSCLC patient derived xenograft model.

FIGS. 13A and 13B show [A] the efficacy of anti-Notch3 hu28-vc0101 in the MDA-MB-468 breast model and [B] the efficacy of anti-Notch3 hu75-vc0101 in the MDA-MB-468 breast model.

FIGS. 14A and 14B show [A] the efficacy of anti-Notch3 hu28-vc6780 in the MDA-MB-468 breast model and [B] the efficacy of anti-Notch3 hu75-vc6780 in the MDA-MB-468 breast model.

FIG. 15 shows the efficacy of anti-Notch3 hu28-vc0101 in the OVCAR3 ovarian model.

FIGS. 16A through 16E show [A] the efficacy of rat-human chimeric anti-Notch3 antibody-drug conjugates dosed at 5mg/kg in HCC2429 lung xenografts; [B and C] the efficacy of rat-human chimeric anti-Notch3 antibody-drug conjugates dosed at 5mg/kg in MDA-MB-468 breast xenografts; [D and E] the efficacy of rat-human chimeric anti-Notch3 antibody-drug conjugates dosed at 5mg/kg in N87 gastric xenografts.

FIGS. 17A and 17B show the amino acid and nucleotide sequences of [A] single cysteine mutants hu28 HC 1.0 L443C and hu28 LC 1.0 κK183C and [B] double cysteine mutants hu28 HC 1.0 L443C/K392C.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides anti-Notch3 antibodies, or antigen-binding fragment thereof, and antibody-drug conjugates (ADCs) for the treatment of cancer. In order that the present invention is more readily understood, certain terms and general techniques are first defined.

All amino acid abbreviations used in this disclosure are those accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. § 1.822 (d)(1).

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the

singular. Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art.

The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Sambrook J. & Russell D. *Molecular Cloning: A Laboratory Manual,* 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000); Ausubel et al., *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Wiley, John & Sons, Inc. (2002); Harlow and Lane *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1998); and Coligan et al., *Short Protocols in Protein Science*, Wiley, John & Sons, Inc. (2003).

"Notch3" or "Notch-3" refers to native, variants, isoforms and species homologs of human Notch3 protein. Native human Notch3 protein, for example, is made up of a leader peptide, a large epidermal growth factor (EGF)-like repeat region, three Lin12 repeats, a N terminal heterodimerization domain (HD-1), a C terminal heterodimerization domain (HD-2), a transmembrane (TM) sequence and an intracellular domain ($Notch3^{ICD}$). The NCBI/GenBank accession number of the full length human Notch3 is NM_000435.2.

"Notch3 negative regulatory region", or "Notch3 NRR" as used herein, unless otherwise indicated, refers to any native or synthetic polypeptide region of Notch3 consisting of the three Lin12 domains and the amino acid sequence or sequences located between the three Lin12 domains, plus the HD1 and HD2 domains of Notch3. In one embodiment, the "Notch3 NRR" includes the three Lin12 domains and two heterodimerization domains HD-1, and HD-2, wherein the HD-1 and HD-2 domains of Notch3 are covalently bonded and not yet cleaved by the furin-like protease (before S1 cleavage). In another embodiment, the "Notch3 NRR" includes the three Lin12 domains and the two heterodimerization domains HD-1, and HD-2, wherein the HD-1 and HD-2 domains are non-covalently bonded (after S1 cleavage). In one aspect of this embodiment, the S2 site within the HD-2 domain has not been cleaved by the ADAM-type

metalloproteases. In another particular aspect of this embodiment, the S2 site within the HD-2 domain is being cleaved or has already been cleaved by the ADAM-type metalloproteases. (Gordon, W.R., et.al, Nature Structural & Molecular Biology, 2007, volume 14, 295-300).

An “antibody” or “Ab” is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term “antibody” encompasses not only intact polyclonal or monoclonal antibodies, but also any antigen binding portion (e.g., "antigen-binding fragment") thereof of an intact antibody that retains the ability to specifically bind to a given antigen (e.g., target Notch3) or single chain thereof, fusion proteins comprising an antibody, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site, for example without limitation, Fab; Fab’; $F(ab')_2$; an Fd fragment; an Fv fragment; a single domain antibody (dAb) fragment; an isolated complementarity determining region (CDR); single chain (scFv) and single domain antibodies (*e.g.*, shark and camelid antibodies), maxibodies, minibodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (see, e.g., Hollinger and Hudson, 2005, Nature Biotechnology 23(9): 1126-1136). An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant region of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy chain (HC) constant regions that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

An "isolated antibody” refers to an antibody that is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds Notch3 is substantially free of antibodies that specifically bind antigens other than Notch3). An isolated antibody that specifically binds Notch3 may, however,

have cross-reactivity to other antigens, such as Notch3 molecules from other species. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

A "variable region" of an antibody refers to the variable region of the antibody light chain (VL) or the variable region of the antibody heavy chain (VH), either alone or in combination. As known in the art, the variable regions of the heavy and light chain each consist of four framework regions (FRs) connected by three complementarity determining regions (CDR1, CDR2, CDR3) also known as hypervariable regions, contribute to the formation of the antigen binding site of antibodies. If variants of a subject variable region are desired, particularly with substitution in amino acid residues outside of a CDR region (i.e., in the framework region), appropriate amino acid substitution, preferably, conservative amino acid substitution, can be identified by comparing the subject variable region to the variable regions of other antibodies which contain CDR1 and CDR2 sequences in the same canonincal class as the subject variable region (Chothia and Lesk, J Mol Biol 196(4): 901-917, 1987). When choosing FR to flank subject CDRs, e.g., when humanizing or optimizing an antibody, FRs from antibodies which contain CDR1 and CDR2 sequences in the same canonical class are preferred.

A "CDR" of a variable domain are amino acid residues within the variable region that are identified in accordance with the definitions of the Kabat, Chothia, the accumulation of both Kabat and Chothia, AbM, contact, and/or conformational definitions or any method of CDR determination well known in the art. Antibody CDRs may be identified as the hypervariable regions originally defined by Kabat et al. See, e.g., Kabat et al., 1992, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington D.C. The positions of the CDRs may also be identified as the structural loop structures originally described by Chothia and others. See, e.g., Chothia et al., 1989, Nature 342:877-883. Other approaches to CDR identification include the "AbM definition," which is a compromise between Kabat and Chothia and is derived using Oxford Molecular's AbM antibody modeling software (now Accelrys®), or the "contact definition" of CDRs based on observed antigen contacts, set forth in MacCallum et al., 1996, J. Mol. Biol., 262:732-745. In another approach,

referred to herein as the “conformational definition” of CDRs, the positions of the CDRs may be identified as the residues that make enthalpic contributions to antigen binding. See, e.g., Makabe et al., 2008, Journal of Biological Chemistry, 283:1156-1166. Still other CDR boundary definitions may not strictly follow one of the above approaches, but will nonetheless overlap with at least a portion of the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. As used herein, a CDR may refer to CDRs defined by any approach known in the art, including combinations of approaches. The methods used herein may utilize CDRs defined according to any of these approaches. For any given embodiment containing more than one CDR, the CDRs may be defined in accordance with any of Kabat, Chothia, extended, AbM, contact, and/or conformational definitions.

The terms "IgG Fc region", “Fc region”, “Fc domain” and “Fc”, as interchangeably used herein refer to the portion of an IgG molecule that correlates to a crystallizable fragment obtained by papain digestion of an IgG molecule. The Fc region consists of the C-terminal half of the two heavy chains of an IgG molecule that are linked by disulfide bonds. It has no antigen binding activity but contains the carbohydrate moiety and the binding sites for complement and Fc receptors, including the FcRn receptor (see below). The Fc fragment contains the entire second constant domain CH2 (residues 231-340 of human IgG1, according to the Kabat numbering system) and the third constant domain CH3 (residues 341-447).

By “engineered Fc polypeptide”, “engineered Fc region” and “engineered Fc” as the terms are interchangeably used herein, is meant an Fc polypeptide, or portion thereof, comprising at least one mutation, e.g., an amino acid substitution, introducing a site for conjugation. Preferably, the mutation introduces a cysteine in place of the naturally-occurring amino acid residue at that position, where the mutation creates a reactive site (e.g., a reactive sulfhydryl group) for conjugation of a moiety to the Fc.

The term "monoclonal antibody" or “mAb” refers to an antibody that is derived from a single copy or clone, including e.g., any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. Preferably, a monoclonal antibody of the invention exists in a homogeneous or substantially homogeneous population.

"Humanized" antibody refers to forms of non-human (*e.g.* rat) antibodies that are chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. Preferably, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity.

“Human antibody or fully human antibody” refers to those antibodies derived from transgenic mice carrying human antibody genes or from human cells.

The term “chimeric antibody” is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a rat antibody and the constant region sequences are derived from a human antibody.

A "therapeutic agent" is an agent that exerts a cytotoxic, cytostatic, and/or immunomodulatory effect on cancer cells or activated immune cells. Examples of therapeutic agents include cytotoxic agents, chemotherapeutic agents, cytostatic agents, and immunomodulatory agents.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer.

A "cytotoxic effect" refers to the depletion, elimination and/or the killing of a target cell(s). A "cytotoxic agent" refers to an agent that has a cytotoxic and/or cytostatic effect on a cell.

A "cytostatic effect" refers to the inhibition of cell proliferation. A "cytostatic agent" refers to an agent that has a cytostatic effect on a cell, thereby inhibiting the growth and/or expansion of a specific subset of cells.

“Antibody-drug conjugate” or ”ADC” refers to antibodies or antibody fragments thereof, including antibody derivatives that bind to Notch3 and are conjugated to cytotoxic, cytostatic, and/or therapeutic agents.

"Anti-Notch3 antibody-drug conjugate" or "anti-Notch3 ADC" refers to an anti-Notch3 antibody or antigen binding fragment thereof, as described herein linked to a drug (D) via a linker (L).

"Linker (L)" describes the direct or indirect linkage of the antibody to the drug. Attachment of a linker to an antibody can be accomplished in a variety of ways, such as through surface lysines, reductive-coupling to oxidized carbohydrates, and through cysteine residues liberated by reducing interchain disulfide linkages. A variety of antibody-drug conjugate linkage systems are known in the art, including hydrazone-, disulfide- and peptide-based linkages.

"Drug (D)" is any substance having biological or detectable activity, for example, therapeutic agents, detectable labels, binding agents, etc., and prodrugs, which are metabolized to an active agent *in vivo*. The terms drug, payload and compound are used interchangeably.

"L-D" is a linker-drug moiety resulting from a drug (D) linked to a linker (L).

The term "epitope" refers to that portion of a molecule capable of being recognized by and bound by an antibody at one or more of the antibody's antigen-binding regions. Epitopes often consist of a chemically active surface grouping of molecules such as amino acids or sugar side chains and have specific three-dimensional structural characteristics as well as specific charge characteristics. The term "antigenic epitope" as used herein, is defined as a portion of a polypeptide to which an antibody can specifically bind as determined by any method well known in the art, for example, by conventional immunoassays. A "nonlinear epitope" or "conformational epitope" comprises noncontiguous polypeptides (or amino acids) within the antigenic protein to which an antibody specific to the epitope binds. Once a desired epitope on an antigen is determined, it is possible to generate antibodies to that epitope, e.g., using the techniques described in the present specification. During the discovery process, the generation and characterization of antibodies may elucidate information about desirable epitopes. From this information, it is then possible to competitively screen antibodies for binding to the same epitope. An approach to achieve this is to conduct competition and cross-competition studies to find antibodies that compete or cross-compete with one another *e.g.*, the antibodies compete for binding to the antigen.

The term "binding affinity (K_D)" as used herein, is intended to refer to the dissociation rate of a particular antigen-antibody interaction. The K_D is the ratio of the rate of dissociation, also called the "off-rate (k_d)", to the association rate, or "on- rate (k_a)". Thus, K_D equals k_d/ k_a and is expressed as a molar concentration (M). It follows that the smaller the K_D, the stronger the affinity of binding. Therefore, a K_D of 1 μM indicates weak binding affinity compared to a K_D of 1 nM. K_D values for antibodies can be determined using methods well established in the art. One method for determining the K_D of an antibody is by using surface plasmon resonance, typically using a biosensor system such as a Biacore® system.

An antibody, an antibody conjugate, or a polypeptide that “preferentially binds” or “specifically binds” (used interchangeably herein) to a target (e.g., Notch3 protein) is a term well understood in the art, and methods to determine such specific or preferential binding are also well known in the art. A molecule is said to exhibit “specific binding” or “preferential binding” if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular cell or substance than it does with alternative cells or substances. An antibody “specifically binds” or “preferentially binds” to a target if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antibody that specifically or preferentially binds to a Notch3 epitope is an antibody that binds this epitope with greater affinity, avidity, more readily, and/or with greater duration than it binds to other Notch3 epitopes or non- Notch3 epitopes. It is also understood that by reading this definition, for example, an antibody (or moiety or epitope) that specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, “specific binding” or “preferential binding” does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding. “EC_{50}” is a measurement of binding capacity and is defined as the half maximal effective concentration of an antibody or antibody-drug conjugate that is needed to produce a response halfway between the baseline and maximum.

“Pharmaceutically acceptable salt” as used herein refers to pharmaceutically acceptable organic or inorganic salts of a molecule or macromolecule.

The term "potency" is a measurement of biological activity and may be designated as IC_{50}, or inhibitory concentration of an antibody or antibody drug conjugate to the antigen Notch3, needed to inhibit 50% of Notch3-dependent reporter gene activity or growth of a Notch3 positive cell line as described in Examples 9 and 12 respectively.

The phrase “effective amount” or “therapeutically effective amount” as used herein refers to an amount necessary (at dosages and for periods of time and for the means of administration) to achieve the desired therapeutic result. An effective amount is at least the minimal amount, but less than a toxic amount, of an active agent which is necessary to impart therapeutic benefit to a subject.

The terms “inhibit” or “neutralize” as used herein with respect to bioactivity of an antibody of the invention mean the ability of the antibody to substantially antagonize, prohibit, prevent, restrain, slow, disrupt, eliminate, stop, reduce or reverse e.g. progression or severity of that which is being inhibited including, but not limited to , a biological activity.

The term “compete” or “competes”, as used herein with regard to an antibody, means that a first antibody, or an antigen-binding fragment thereof, binds to an epitope in a manner sufficiently similar to the binding of a second antibody, or an antigen-binding fragment thereof, such that the result of binding of the first antibody with its cognate epitope is detectably decreased in the presence of the second antibody compared to the binding of the first antibody in the absence of the second antibody. The alternative, where the binding of the second antibody to its epitope is also detectably decreased in the presence of the first antibody, can, but need not be the case. That is, a first antibody can inhibit the binding of a second antibody to its epitope without that second antibody inhibiting the binding of the first antibody to its respective epitope. However, where each antibody detectably inhibits the binding of the other antibody with its cognate epitope or ligand, whether to the same, greater, or lesser extent, the antibodies are said to “cross-compete” with each other for binding of their respective epitope(s). Both competing and cross-competing antibodies are encompassed by the present invention. Regardless of the mechanism by which such competition or cross-competition occurs (e.g., steric hindrance, conformational change, or binding to a common epitope, or fragment thereof), the skilled artisan would appreciate, based upon

the teachings provided herein, that such competing and/or cross-competing antibodies are encompassed and can be useful for the methods disclosed herein.

The terms “polynucleotide” or "nucleic acid molecule", as used herein, are intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The polynucleotides that encode the antibodies of the present invention may include the following: only the coding sequence for the variant, the coding sequence for the variant and additional coding sequences such as a functional polypeptide, or a signal or secretory sequence or a pro-protein sequence; the coding sequence for the antibody and non-coding sequence, such as introns or non-coding sequence 5’ and/or 3’ of the coding sequence for the antibody. The term ‘polynucleotide encoding an antibody” encompasses a polynucleotide which includes additional coding sequence for the variant but also a polynucleotide which includes additional coding and/or non-coding sequence. It is known in the art that a polynucleotide sequence that is optimized for a specific host cell/expression system can readily be obtained from the amino acid sequence of the desired protein (see GENEART AG, Regensburg, Germany).

A “host cell” includes an individual cell or cell culture that can be or has been a recipient for vector(s) for incorporation of polynucleotide inserts. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected in vivo with a polynucleotide(s) of this invention.

The term "vector" means a construct, which is capable of delivering, and, preferably, expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

The term "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. The

expression control sequence is operably linked to the nucleic acid sequence to be transcribed.

The polynucleotides encoding the antibodies of the present invention will typically include an expression control polynucleotide sequence operably linked to the antibody coding sequences, including naturally-associated or heterologous promoter regions known in the art. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host cell line, the host cell is propagated under conditions suitable for expressing the nucleotide sequences, and, as desired, for the collection and purification of the antibodies. Preferred eukaryotic cell lines include CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, transformed B-cells, or human embryonic kidney cell lines. The most preferred host cell is a CHO cell line.

Antibodies

Antibodies of the invention can be produced using techniques well known in the art, *e.g.*, recombinant technologies, phage display technologies, synthetic technologies or combinations of such technologies or other technologies readily known in the art (see, for example, Jayasena, S.D., Clin. Chem., 45: 1628-50 (1999) and Fellouse, F.A., et al, J. Mol. Biol., 373(4):924-40 (2007)).

An embodiment of the invention is an antibody that specifically binds to the same Notch3 epitope as an antibody comprising a first amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 13 and a second amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 25.

Another embodiment of the invention is an antibody that specifically binds to the same Notch3 epitope as an antibody comprising a first amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 37 and a second amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 49.

In some embodiments, the antibody, or antigen-binding fragment thereof, specifically binds to Notch3, and the antibody, or antigen-binding fragment thereof, competes with the binding of an antibody comprising a first amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 13 and a second amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 25.

In some embodiments, the antibody, or antigen-binding fragment thereof, specifically binds to Notch3, and the antibody ,or antigen-binding fragment thereof, competes with the binding of an antibody comprising a first amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 37 and a second amino acid sequence that is at least 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 49.

Conjugation of a Drug to an Antibody

The drug has, or is modified to include, a group reactive with a conjugation point on the antibody. For example, a drug can be attached by alkylation (*e.g.*, at the epsilon-amino group lysines or the N-terminus of antibodies), reductive amination of oxidized carbohydrate, transesterification between hydroxyl and carboxyl groups, amidation at amino groups or carboxyl groups, and conjugation to thiols. In some embodiments, the number of drug, *p*, conjugated per antibody molecule ranges from an average of 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8; 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2. In some embodiments, *p* ranges from an average of 2 to 12, 2 to 11, 2 to 10, 2 to 9, 2 to 8, 2 to 7, 2 to 6, 2 to 5, 2 to 4 or 2 to 3. In other embodiments, *p* is an average of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12. In some embodiments, *p* ranges from an average of about 1 to about 12, about 1 to about 11, about 1 to about 10, about 1 to about 9, about 1 to about 8; about 1 to about 7, about 1 to about 6, about 1 to about 5, about 1 to about 4, about 1 to about 3, or about 1 to about 2. In some embodiments, *p* ranges from about 2 to about 12, about 2 to about 11, about 2 to about 10, about 2 to about 9, about 2 to about 8, about 2 to about 7, about 2 to about 6, about 2 to about 5, about 2 to about 4 or about 2 to about 3. For examples of chemistries that can be used for conjugation, see, *e.g.*,

Current Protocols in Protein Science (John Wiley & Sons, Inc.), Chapter 15 (Chemical Modifications of Proteins).

Linkers

A linker is a bifunctional compound which can be used to link a drug and an antibody to form an antibody drug conjugate (ADC). Such conjugates are useful, for example, in the formation of immunoconjugates directed against tumor associated antigens. Such conjugates allow the selective delivery of cytotoxic drugs to tumor cells. Suitable linkers include, for example, cleavable and non-cleavable linkers. A cleavable linker is typically susceptible to cleavage under intracellular conditions. Suitable cleavable linkers include, for example, a peptide linker cleavable by an intracellular protease, such as lysosomal protease or an endosomal protease. In exemplary embodiments, the linker can be a dipeptide linker, such as a valine-citrulline (val-cit), a phenylalanine-lysine (phe-lys) linker, or maleimidocaproyl–valine-citrulline-p-aminobenzyloxycarbonyl (vc) linker. Another linker is Sulfosuccinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (smcc). Sulfo-smcc conjugation occurs via a maleimide group which reacts with sulfhydryls (thiols, -SH), while its Sulfo-NHS ester is reactive toward primary amines (as found in Lysine and the protein or peptide N-terminus). Yet another linker is maleimidocaproyl (mc). Other suitable linkers include linkers hydrolyzable at a specific pH or a pH range, such as a hydrazone linker. Additional suitable cleavable linkers include disulfide linkers. The linker may be covalently bound to the antibody to such an extent that the antibody must be degraded intracellularly in order for the drug to be released *e.g.* the mc linker and the like.

Linkers of the present invention include maleimidocaproyl–valine-citrulline-p-aminobenzyloxycarbonyl (vc) and maleimidocaproyl (mc), maleimido-heptanoyl (me) and maleimido-Peg6C2 (MalPeg6C2).

Engineered Fc Polypeptide

It has been previously reported that certain residues presumably present on the surface of the CH2 or CH3 domain of the heavy chain of antibodies, or on the constant domain of the light chain, or otherwise accessible, are suitable for the substitution of the naturally-occurring wild type amino acid with, for example, cysteine, and are therefore

useful to engineer a site capable of conjugation to various agents, as described in International Publication No. WO/2013/093809, which is incorporated herein by reference.

Amino acid modifications can be made by any method known in the art and many such methods are well known and routine for the skilled artisan. For example, but not by way of limitation, amino acid substitutions, deletions and insertions may be accomplished using any well-known PCR-based technique. Amino acid substitutions may be made by site-directed mutagenesis (see, for example, Zoller and Smith, 1982, Nucl. Acids Res. 10:6487-6500; and Kunkel, 1985, Proc. Natl. Acad. Sci USA 82:488).

In some embodiments, the engineered Fc polypeptide of the disclosure may be used to prepare an antibody, or antigen binding fragment thereof, such that the antibody or fragment thereof thereby comprises the engineered Fc region which can be used to conjugate, at the engineered residue (i.e., the amino acid substituted compared to wild type unmodified Fc), a wide variety of moieties.

In some embodiments, the engineered kappa light chain constant polypeptide of the disclosure may be used to prepare an antibody, or antigen binding fragment thereof, such that the antibody or fragment thereof thereby comprises an engineered CL region comprising an amino acid mutation, or fragment thereof, which can be used to conjugate, at the engineered amino acid residue, a wide variety of moieties.

It should be noted that a single substitution in an Fc polypeptide, for example of a cysteine residue, normally results in the display of two corresponding residues in the resultant IgG antibody due to the homodimeric nature of IgG antibody molecules. Thus, the resultant engineered IgG antibodies of the invention may display at least 1, 2, 3, 4, or more reactive groups for the purpose of conjugation to a drug or compound. In an embodiment, one or more of the substitutions is with a cysteine residue, and the resulting engineered antibodies may display at least 1, 2, 3, 4, or more thiol groups for the purpose of conjugation to a drug or compound.

In some embodiments, the engineered Fc polypeptide of the present invention comprises one or more substitutions selected from the positions 443 and 392, of the heavy chain of an antibody, and wherein the numbering system of the constant region is that of the EU index as set forth in Kabat et al. (supra).

In some embodiments, the engineered Fc polypeptide comprises one amino acid substitution (L443C) as provided in SEQ ID NO: 61. In another embodiment, the engineered Fc polypeptide comprises two amino acid substitutions (L443C/K392C) as provided in SEQ ID NO: 65.

Engineered Cκ polypeptide

The anti-Notch3 antibodies of the present invention may encompass an engineered antibody light chain constant region (LC), or a fragment thereof, where 1, 2, or 3 amino acids of the antibody light chain, wherein the numbering system of the light chain constant region is that of the Kabat numbering system as set forth in Kabat et al. (1991, NIH Publication 91- 3242, National Technical Information Service, Springfield, VA, hereinafter "Kabat"), of a parent, native, or wild type antibody, substituted with another amino acid (including natural and non-natural/synthetic amino acids).

In other embodiments, due to the dimeric nature of many antibodies (e.g., IgGs comprise two light chains and two heavy chains each heavy chain comprising an Fc polypeptide), an antibody of the invention may comprise at least one engineered Fc polypeptide and may further comprise at least one engineered light chain constant polypeptide thereby providing at least two site-specific conjugation sites – one in the Fc polypeptide and another in the CL polypeptide.

In some embodiments, the engineered Cκ polypeptide of the present invention comprises at least one substitution at position 183 of the light chain of the antibody. In some embodiments, the engineered Cκ polypeptide comprises one amino acid substitution (κK183C) as provided in SEQ ID NO. 63.

Therapy for Cancer

Cancers, including, but not limited to, a tumor, metastasis, or other disease or disorder characterized by uncontrolled cell growth, can be treated or prevented by administration of an antibody, or antigen-binding fragment thereof, or antibody-drug conjugate (ADC) of the present invention.

Exemplary anti-Notch3 antibodies, or antigen-binding fragments thereof, and antibody-drug conjugates are useful for treating cancer in which Notch3 is expressed or

overexpressed, relative to a reference subject or standard (e.g., non-cancerous or normal tissue). Treatment or prevention of a Notch3 -expressing cancer, according to the methods described herein, can be achieved by administering to a subject in need of such treatment an effective amount of an anti-Notch3 antibody, or antigen-binding fragment thereof, and/or antibody-drug conjugate. In some embodiments, an anti-Notch3 full length antibody, or antigen-binding fragment thereof, that is conjugated to a cytotoxic agent will be administered. In some exemplary embodiments, an anit-Notch3 antibody-drug conjugate of the present invention will (i) bind to Notch3 expressing cancer cells, and (ii) exert a cytotoxic or cytostatic effect to, for example, inhibit the proliferation of the Notch3 expressing cancer cells, or kill Notch3 expressing cancer cells.

In other embodiments, the anti-Notch3 antibodies, or antigen-binding fragment thereof, and/or anti-Notch3 antibody-drug conjugates are co-administered with another therapeutic agent, or administered sequentially with another therapeutic agent. In some embodiments, the anti-Notch3 antibodies, or antigen-binding fragment thereof, and/or anti-Notch3 antibody-drug conjugates are co-administered with chemotherapeutics, including standard of care chemotherapeutics, or administered sequentially.

In some embodiments, the other therapeutic agent will be an agent that is standard of care for the specific disease to be treated or is part of a salvage regimen for the specific disease to be treated. Anti-cancer agents and chemotherapeutic regimens include, for example, anti-cancer antibodies, including, for example, anti-CD52 antibodies (e.g., Alemtuzumab), anti-CD20 antibodies (e.g., Rituximab), and anti-CD40 antibodies (e.g., SGN40); chemotherapeutic regimens including, for example, CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone); CVP (cyclophosphamide, vincristine, and prednisone); RCVP (Rituximab+CVP); RCHOP (Rituximab+CHOP); RICE (Rituximab+ifosamide, carboplatin, etoposide); RDHAP, (Rituximab+dexamethasone, cytarabine, cisplatin); RESHAP (Rituximab+etoposide, methylprednisolone, cytarabine, cisplatin); gemcitabine; combination treatment with vincristine, prednisone, and anthracycline, with or without asparaginase; combination treatment with daunorubicin, vincristine, prednisone, and asparaginase; combination treatment with teniposide and Ara-C (cytarabine); combination treatment with

methotrexate and leucovorin; combination treatment with bleomycin, doxorubicin, etoposide, mechlorethamine, prednisone, vinblastine, and vincristine; small molecule inhibitors; and proteosome inhibitors including, for example, bortezomib.

In some embodiments, methods for treating cancer including administering to a patient in need thereof an effective amount of an anti-Notch3 antibody, or antigen-binding fragment thereof, and/or anti-Notch3 antibody-drug conjugate in combination with radiation treatment, and optionally another therapeutic agent. In some embodiments, the anti-Notch3 antibody, or antigen-binding fragment thereof, and/or anti-Notch3 antibody-drug conjugate is administered concurrently or sequentially with an anti-cancer agent (e.g., a chemotherapeutic agent) and/or with radiation therapy. In some embodiments, the chemotherapeutic agent or radiation therapy is administered at least an hour, five hours, 12 hours, a day, a week, a month, several months (e.g., up to three months), prior or subsequent to administration of a compound of the present invention.

Generally, for administration of an anti-Notch3 antibody and/or an anti-Notch3 antibody-drug conjugate, an initial candidate dosage can be about 2 mg/kg. For the purpose of the present invention, a typical daily dosage might range from about any of 3 µg/kg to 30 µg/kg to 300 µg/kg to 3 mg/kg, to 30 mg/kg, to 100 mg/kg or more, depending on the factors mentioned above. For example, dosage of about 1 mg/kg, about 2.5 mg/kg, about 5 mg/kg, about 10 mg/kg, and about 25 mg/kg may be used. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of symptoms occurs or until sufficient therapeutic levels are achieved, for example, to inhibit or delay tumor growth/progression or metatstasis of cancer cells. An exemplary dosing regimen comprises administering an initial dose of about 2 mg/kg, followed by a weekly maintenance dose of about 1 mg/kg of the anti-Notch3 antibody or anti-Notch3 antibody-drug conjugate, or followed by a maintenance dose of about 1 mg/kg every other week. Other exemplary dosing regimens comprise administering increasing doses (e.g., initial dose of 1 mg/kg and gradual increase to one or more higher doses every week or longer time period). Other dosing regimens may also be useful, depending on the pattern of pharmacokinetic decay that the practitioner wishes to

achieve. For example, in some embodiments, dosing from one to four times a week is contemplated. In other embodiments dosing once a month or once every other month or every three months is contemplated as well as weekly, bi-weekly and every three weeks. The progress of this therapy may be monitored by conventional techniques and assays. The dosing regimen (including the anti-Notch3 antibody or the anti-Notch3 antibody-drug conjugate used) can vary over time.

For the purpose of the present invention, the appropriate dosage of an anti-Notch3 antibody or an anti-Notch3 antibody-drug conjugate will depend on the anti-Notch3 antibody or the anti-Notch3 antibody-drug conjugate (or compositions thereof) employed, the type and severity of symptoms to be treated, whether the agent is administered for therapeutic purposes, previous therapy, the patient's clinical history and response to the agent, the patient's clearance rate for the administered agent, and the discretion of the attending physician. The clinician may administer an anti-Notch3 antibody or an anti-Notch3 antibody-drug conjugate until a dosage is reached that achieves the desired result and beyond. Dose and/or frequency can vary over course of treatment, but may stay constant as well. Empirical considerations, such as the half-life, generally will contribute to the determination of the dosage. For example, antibodies that are compatible with the human immune system, such as humanized antibodies or fully human antibodies, may be used to prolong half-life of the antibody and to prevent the antibody being attacked by the host's immune system. Frequency of administration may be determined and adjusted over the course of therapy, and is generally, but not necessarily, based on treatment and/or suppression and/or amelioration and/or delay of symptoms, e.g., tumor growth inhibition or delay, etc. Alternatively, sustained continuous release formulations of anti-Notch3 antibodies or anti-Notch3 antibody-drug conjugates may be appropriate. Various formulations and devices for achieving sustained release are known in the art.

In one embodiment, dosages for anti-Notch3 antibody or anti-Notch3 antibody-drug conjugate may be determined empirically in individuals who have been given one or more administration(s) of the anti-Notch3 antibody or its anti-Notch3 antibody-drug conjugate. Individuals are given incremental dosages of an anti-Notch3 antibody or a Notch3 antagonist. To assess efficacy, an indicator of the disease can be followed.

Administration of an anti-Notch3 antibody or an anti-Notch3 antibody-drug conjugate in accordance with the method in the present invention can be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of an anti-Notch3 antibody or an anti-Notch3 antibody-drug conjugate may be essentially continuous over a preselected period of time or may be in a series of spaced doses.

In some embodiments, more than one anti-Notch3 antibody or anti-Notch3 antibody-drug conjugate may be present. At least one, at least two, at least three, at least four, at least five different or more anti-Notch3 antibody or anti-Notch3 antibody-drug conjugate can be present. Generally, those anti-Notch3 antibodies or anti-Notch3 antibody-drug conjugates may have complementary activities that do not adversely affect each other. For example, one or more of the following anti-Notch3 antibody may be used: a first anti-Notch3 antibody directed to one epitope on Notch3 and a second anti- Notch3 antibody directed to a different epitope on Notch3.

The anti-Notch3 antibodies, or antigen-binding fragment thereof, and/or anti-Notch3 antibody-drug conjugates of the present invention can be in the form of a pharmaceutical composition for administration that are formulated to be appropriate for the selected mode of administration, and pharmaceutically acceptable diluent or excipients, such as buffers, surfactants, preservatives, solubilizing agents, isotonicity agents, stabilizing agents, carriers, and the like. Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton Pa., 18th ed., 1995, provides a compendium of formulation techniques as are generally known to practitioners.

These pharmaceutical compositions may be administered by any means known in the art that achieve the generally intended purpose to treat cancer. The in one embodiment, the route of administration is parenteral, defined herein as referring to modes of administration that include but not limited to intravenous, intramuscular, intraperitoneal, subcutaneous, and intraarticular injection and infusion. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

Compositions within the scope of the invention include all compositions wherein an anti-Notch3 antibody, or antigen-binding fragment thereof, and/or anti-Notch3 antibody-drug conjugate is present in an amount that is effective to achieve the desired medical effect for treating cancer. While individual needs may vary from one patient to another, the determination of the optimal ranges of effective amounts of all of the components is within the ability of the clinician of ordinary skill.

Diagnostic

The antibodies, or antigen-binding fragment thereof, of the invention can also be used to detect Notch3 in a biological sample in vitro or in vivo. In one embodiment, the anti- Notch3 antibodies, or antigen-binding fragment thereof, of the invention are used to determine the level of Notch3 in a tissue or in cells derived from the tissue. In a one embodiment, the tissue is a diseased tissue. In some embodiments, the tissue is a tumor or a biopsy thereof. In a one embodiment, the levels of Notch3 in a tissue or biopsy from a patient can be determined in an immunoassay with the antibodies, or antigen-binding fragment thereof, of the invention. The tissue or biopsy thereof can be excised from the patient and can be frozen or fixed. The same method can be used to determine other properties of the Notch3 protein, such as its level of cell surface levels, or cellular localization.

The above-described method can be used to diagnose a cancer in a subject known to or suspected to have a cancer, wherein the level of Notch3 measured in the patient is compared with that of a reference subject or standard. The method can then be used to determine whether a tumor expresses Notch3, which may suggest that the tumor will respond well to treatment with the antibody-drug conjugates of the present invention. In one embodiment of the invention, the tumor is a solid tumor cancer, including but not limited to, lung cancer, breast cancer, ovarian cancer, stomach cancer, esophageal cancer, cervical cancer, head and neck cancer, bladder cancer, liver cancer, skin cancer and sarcoma or a blood cancer, including but not limited to, T-cell malignancies, T-cell leukemia, T-cell lymphoma, T-cell acute lymphoblastic leukemia, multiple myeloma, B-cell malignancies, myeloid malignancies, acute myeloid leukemia

and chronic myeloid leukemiain which Notch3 is expressed, and other cancers yet to be determined in which Notch3 is expressed predominantly.

An embodiment of the invention is a method of treating a Notch3 expressing cancer, the method comprising: determining the level of Notch3 in a biological sample comprising the steps of: contacting a sample obtained from a subject suspected to have cancer with an anit-Notch3 antibody, or antigen-binding fragment thereof; determining the cell surface levels of Notch3 in the sample; comparing the cell surface levels of Notch3 with that of a reference subject or standard; and administering an antibody–drug conjugate of the present invention to the subject. The method may optionally comprise a step of obtaining the sample from a subject suspected to have cancer;

Another embodiment of the invention is a method of treating a Notch3 expressing cancer the method comprising: determining the level of Notch3 in a biological sample comprising the steps of: subjecting a sample obtained from a subject suspected to have cancer to in-situ hybridization (ISH); determining the level of Notch3 mRNA in the sample; comparing the levels of Notch3 mRNA with that of a reference subject or standard; and administering an antibody-drug conjugate of the present invention to the subject. The method may optionally comprise a step of obtaining the sample from a subject suspected to have cancer.

The present invention further provides for monoclonal antibodies, humanized antibodies and epitope-binding fragments thereof that are further labeled for use in research or diagnostic applications. In some embodiments, the label is a radiolabel, a fluorophore, a chromophore, an imaging agent or a metal ion.

A method for diagnosis is also provided in which the labeled antibodies or epitope-binding fragments thereof are administered to a subject suspected of having a cancer, and the distribution of the label within the body of the subject is measured or monitored.

Kit

The present invention also includes kits, e. g. comprising a described cytotoxic conjugate and instructions for the use of the cytotoxic conjugate for killing of particular cell types. The instructions may include directions for using the cytotoxic conjugates in

vitro, in vivo or ex vivo. Typically, the kit will have a compartment containing the cytotoxic conjugate. The cytotoxic conjugate may be in a lyophilized form, liquid form, or other form amendable to being included in a kit. The kit may also contain additional elements needed to practice the method described on the instructions in the kit, such a sterilized solution for reconstituting a lyophilized powder, additional agents for combining with the cytotoxic conjugate prior to administering to a patient, and tools that aid in administering the conjugate to a patient.

All publications and patent documents cited above or in the following Examples are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

The following examples of specific aspects for carrying out the present invention are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Example 1

Generation of Recombinant Notch3 Protein Immunogens

cDNA constructs encoding the human Notch3 NRR region with a signal peptide at N-terminus, and Avi and His6 tag at C-terminus, were cloned into the expression vector pSMED2. These constructs were transiently transfected into Chinese hamster ovary (CHO) cells and the secreted proteins in conditioned media were analyzed on SDS-PAGE. After processing at the S1 cleavage site, the N-terminal ~26kDa (LNR-A, B, C and HD1) and C-terminal ~12 kDa (HD2 and Avi_His tag) halves of the Notch3 NRR domain remain associated through non-covalent interactions to form a heterodimeric complex, as shown in **FIG. 1.** S1 processing of the Notch3 NRR was determined to be about 50% or less in samples prepared from CHO cells. To enhance processing at the S1 cleavage site, the Notch3 NRR expression construct was transfected into CHO-PACE cells (Harrison et, al, Semin Hematol. 1998 Apr;35(2 Suppl 2):4-10) and stable cell lines with the highest

expression and complete processing of Notch3 NRR were selected. Culture of these cell lines was scaled up for the collection of conditioned media from which Notch3 NRR proteins were purified.

The amino acid and nucleotide sequences of purified human and mouse Notch3 NRR_Avi_His tag proteins (hereinafter "Notch3 NRR recombinant proteins") are provided in **FIG. 2.** Lower case type represents the Avi_His tag. SDS-PAGE analysis showed that > 90% of the purified protein was correctly cleaved into the predicted Notch3 NRR N-terminal and C-terminal peptide sizes (data not shown).

Example 2

Generation and Cloning of Rat Anti-Notch3 Antibodies

A. Immunization and Hybridoma Generation

Sprague-Dawley rats were immunized by subcutaneous injections with a mixture containing 20 µg each of human (SEQ ID NO. 1) and mouse (SEQ ID NO. 3) Notch3 recombinant proteins in Freund's complete adjuvant. Immunizations were repeated at 2-week intervals for 12 weeks. Collected sera samples at day 0, 35, 49, and 63 after the first injection were tested for circulating anti-Notch3 antibody titer activity by enzyme-linked immunosorbent assay (ELISA). When optimal titers were reached, a final dose of the protein mixture was injected intravenously (tail vein) into a rat having optimal antibody titer 4 days before it was to be sacrificed for splenocyte collection. Total splenocytes (2×10^8) from the rat were fused with mouse myeloma cell line P3X63.Ag8.653 (2.5×10^7) using PEG 1500. Fused cells were plated out in 96-well plates (0.2 mL/well) and subjected to HAT selection (RPMI 1640 containing 5×10^{-4} M Hypoxanthine, 1.6×10^{-5} M Thymidine, 4×10^{-4} M Aminopterin, and 20% Heat Inactivated FCS).

Fourteen days post fusion, hybridoma supernatants were harvested and tested for the presence of Notch3 binding activities. Two parental rat clones 28 and 75 (hereinafter r28 and r75, respectively) exhibited binding activity to human Notch3 NRR recombinant protein by ELISA and to the cell surface of U-2 OS cells stably over-expressing human full length Notch3 by a cell-based ELISA with an O.D. 450nM value of 1 or above at 0.1-1nM

antibody concentrations. Further, r28, but not r75, exhibited binding activity to mouse Notch3 NRR recombinant protein by ELISA and to the cell surface of U-2 OS cells stably over-expressing mouse full length Notch3 by a cell-based ELISA with an O.D. 450nM value of 1 or above at 0.1-1nM antibody concentrations.

B. Cloning and Sequencing of anti-Notch3 antibodies

r28 and r75 variable regions were subcloned for further analysis. RNAs from the subclones were extracted and the variable region DNA sequences from the expressed antibodies were obtained via RT-PCR cloning. One to five million of the subcloned hybridoma cells were homogenized for total RNA isolation with QIAGEN RNAeasy Mini kit. First strand cDNA was then produced using SuperScript III RT kit (Invitrogen). Double stranded cDNAs for variable regions of anti-Notch3 IgGs were subsequently generated and amplified by PCR using primers from the rat IgG heavy chain (IgG1, 2a, 2b) and light chain (kappa or lambda) constant regions, as described below. PCR cycling conditions: 1 cycle at 95°C for 1 min; 25 cycles at 95°C for 1 min, 63°C for 1 min and 72 °C for 1 min. The resulting RT-PCR products were cloned into TOPO-Blunt cloning vector (Invitrogen) and sequenced by conventional methods. The amino acid and nucleotide sequences of r28 and r75 variable regions are provided in **FIG. 3.** CDRs are underlined.

Example 3

Generation and Characterization of Chimeric Anti-Notch3 Antibodies

Variable region cDNAs from r28 and r75 were subcloned into mammalian expression vectors where rat variable heavy chain (VH) were fused in frame with human IgG1 (hIgG1) and rat variable light chain (VL) were fused with human kappa. Rat-human chimeric antibodies 28 and 75 (hereinafter ch28 and ch75, respectively) were generated from these constructs by transient transfection in HEK293 cells and further analyzed.

ch28 and ch75 were tested for binding activity in recombinant protein ELISA. Purified human or mouse Notch3 NRR recombinant proteins were coated on CoStar hi-bound 96-well ELISA plates in 100µl of PBS with Mg/Ca at a concentration of 1µg/ml

overnight. The plates were washed with PBS-Mg/Ca and blocked for 1 hour with 1% BSA in PBS-Mg/Ca. Blocking solution was decanted from the plate and 1:3 serial dilutions of antibodies in blocking solutions were applied to the plate. After incubation at room temperature for 1 hour, plates were washed again with PBS-Mg/Can before HRP (horse radish peroxidase) -conjugated secondary antibody diluted (1:20,000) in blocking buffer was applied. When the primary antibody tested was rat IgG, the secondary antibody was goat anti-rat IgG Fc (Bethyl Biotech); and when the primary antibody was human IgG, the secondary antibody was goat anti-human IgG Fc (Southern Biotech).

After 1 hour incubation with the secondary antibody, plates were washed again, as described above, and TMB substrate solution was added. The developing reaction was allowed for 10 minutes before the stopping solution, 0.18M H_2SO_4, was added. Absorbance at O. D. 450 nM was measured and data was plotted and analyzed with Microsoft Excel and Graphpad-Prism software. ch28 and ch75 exhibited strong binding activity to human Notch3 NRR recombinant protein, having an EC50 of 1nM or lower, and ch28, but not ch75, also exhibited strong binding activity to mouse Notch3 NRR recombinant with an EC50 of 1nM or below. ch28 and ch75 were selected for further cell based ELISA, as described below.

Example 4

Humanization

r28 and r75 were selected to be humanized for further development. Humanization was performed by CDR grafting onto human acceptor frameworks, DP-54 for heavy chain and DPK9 for light chain, followed by selected back mutations in human acceptor framework to recover full activity of parental rat antibodies. **Table 1** shows selected back mutations in the human acceptor framework for different variants.

Table 1: Selected back mutations for humanized anti-Notch3 antibody variants.

Variant	Back Mutations (Kabat)
VH	
hu28 VH1.0	none
hu28 VH1.1	A93T, V37I

VL	
hu28 VL1.0	none
hu28 VL1.1	Y36F
VH	
hu75 VH1.8	R71V
hu75 VH 1.9	V34M (CDR1), R71V
VL	
hu75 VL 1.1	S60D
hu75 VL 1.2	S60D, T85F
hu75 VL 1.3	S67Y

cDNAs containing grafted CDR donor sequences of r28 and r75 onto human acceptor frameworks, DP-54 and DPK9, with selected back mutations were synthesized by Genewiz. Synthesized cDNA products were subcloned and fused in frame with human IgG1 heavy chain constant region for the heavy chain and human kappa for the light chain in mammalian expression vectors pSMED2 and pSMEN3, respectively.

Humanized r28 variant VH1.0/VL1.0 (hereinafter hu28) fully retained the antigen binding epitope and affinity of ch28 and lacked potent signaling inhibition activity observed in ch28. The CDRs of the VH and VL regions of hu28 are the same as the CDRs of the VH and VL regions of r28, respectively. Humanized r75 variant VH1.9/VL1.3 (hereinafter hu75) fully retained the antigen binding epitope and affinity of ch75 and potent signaling inhibition activity observed in ch75. CDR2 and CDR3 of the VH region of hu75 are the same as CDR2 and CDR3 of the VH region of r75, respectively. CDR1 of the VH region of hu75 contains one back mutation V34M. The CDRs of the VL region of hu75 are the same as the CDRs of the VL region of r28. **FIGS 4A-4C** provide amino acid and nucleotide sequences of hu28 with CDRs identified (Kabat and Chothia), and **FIGS. 5A-5C** provide amino acid and nucleotide sequences of hu75 with CDRs identified (Kabat and Chothia).

Alignment of the VH and VL regions of the human acceptor framework for r28 and hu28, and r75 and hu75 are shown in **Table 2.** The Kabat CDRs are underlined. The differences in residues of the framework between rat and humanized sequences are indicated by lower case text. The homology between the human acceptor framework for r28 and hu28 variable regions are 73% for VH and 76% for VL. The homology between the human acceptor framework for r75 and hu75 variable regions are 46% for VH and 64% for VL. Kabat CDRs are underlined.

Table 2: Alignment of human acceptor frameworks for anti-Notch3 antibodies.

r28 VH:

```
DP54_JH4  EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYWMS WVRQAPGKGLEWVA NIKQDGSEKYYVDSVKG RFTISRDNAKNSLY
r28VH     EVQLVESGGGLVQPGRSLTLSCVASGFTFR DYGMT WIRQAPGKGLTWVA YISSGSNYIYYAEAVKG RFTISRDNAKNTLY
hu28VH1.0 EVQLVESGGGLVQPGGSLRLSCAASGFTFR DYGMT WVRQAPGKGLEWVA YISSGSNYIYYAEAVKG RFTISRDNAKNSLY

DP54_JH4  LQMNSLRAEDTAVYYCAR ----YFDY WGQGTLVTVSS  (SEQ ID NO: 67)
r28VH     LQMTSLRSEDTALYFCTR RGPFVLDA WGQGASVTVSS  (SEQ ID NO: 5)
hu28VH1.0 LQMNSLRAEDTAVYYCAR RGPFVLDA WGQGTLVTVSS  (SEQ ID NO: 13)
```

r28 VL:

```
DPK9_Jk4  DIQMTQSPSSLSASVGDRVTITC RASQSISSYLN WYQQKPGKAPKLLIY AASSLQS GVPSRFSGSGSGTDFTLTISSLQP
r28VL     DIQMTQSPSFLSASVGDRVTINC KASQSINRYLH WFQQKLGEAPKLLIY NANGLQT GIPSRFSGSGSGTDFTLTISSLQS
hu28VL1.0 DIQMTQSPSSLSASVGDRVTITC KASQSINRYLH WYQQKPGKAPKLLIY NANGLQT GVPSRFSGSGSGTDFTLTISSLQP

DPK9_Jk4  EDFATYYC QQSYSTPLT FGGGTKVEIK  (SEQ ID NO: 68)
r28VL     EDVATYFC LQHNTWPDT FGAGTKLELK  (SEQ ID NO: 7)
hu28VL1.0 EDFATYYC LQHNTWPDT FGGGTKVEIK  (SEQ ID NO: 25)
```

r75 VH:

```
DP54_JH4  EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYWmS WVRQAPGKGLEWVANIKQDGSEKYYVDSVKG RFTISrDNAKNSLY
r75VH     QVKLLQSGAALVKPGASVKMSCKASGYAFT DYWvT WVKQSHGKSLEWIGEISPNSGGTNFNEKFKG KATLTvDKSTSTAY
hu75VH1.9 EVQLVESGGGLVQPGGSLRLSCAASGYAFT DYWmT WVRQAPGKGLEWVAEISPNSGGTNFNEKFKG RFTISvDNAKNSLY

DP54_JH4  LQMNSLRAEDTAVYYCAR ------YFDY WGQGTLVTVSS  (SEQ ID NO: 67)
r75VH     MELSRLTSEDSAIYYCTR GEIRYNWFAY WGQGTLVTVSS  (SEQ ID NO: 9)
hu75VH1.9 LQMNSLRAEDTAVYYCAR GEIRYNWFAY WGQGTLVTVSS  (SEQ ID NO: 37)
```

r75 VL:

```
DPK9_Jk4  DIQMTQSPSSLSASVGDRVTITC RASQSISSYLN WYQQKPGKAPKLLIY AASSLQS GVPSRFSGSGsGTDFTLTISSLQP
r75VL     NIVMTQSPKSMSISVGDRVTMNC KASQNVGNNIA WYQQKPGQSPKLLIY YASNRYT GVPDRFTGGGyGTDFTLTINSMQA
hu75VL1.3 DIQMTQSPSSLSASVGDRVTITC KASQNVGNNIA WYQQKPGKAPKLLIY YASNRYT GVPSRFSGSGyGTDFTLTISSLQP

DPK9_Jk4  EDFATYYC QQSYSTPLT FGGGTKVEIK  (SEQ ID NO: 68)
r75VL     EDAAFYYC QRLYNSPFT FGSGTKLEIK  (SEQ ID NO: 11)
hu75VL1.3 EDFATYYC QRLYNSPFT FGGGTKVEIK  (SEQ ID NO: 49)
```

Example 5

Characterization of hu28 and hu75

A. Cell-based ELISA

Humanized hu28 and hu75, and rat-human chimeric, ch28 and ch75, anti-Notch3 antibodies were screened for cell surface Notch3 binding in a cell-based ELISA. U-2 OS cells stably over-expressing human or mouse full length Notch3 protein on cell surface (hereinafter U2OS/hNotch3 and U2OS/mNotch3, respectively) were plated at 50,000 cells/well in 96 well plates (white opaque, BD/VWR) the day before ELISA assay. On the

day of the ELISA, culture media was removed from wells and serially diluted (1:3 in blocking buffer) antibody solutions were applied to the plate. Plates were incubated at room temperature for 2 hours before being washed with PBS-Mg/Ca. HRP-conjugated secondary antibody was then applied and incubated with cells for 1 hour as described above for recombinant protein ELISA. Plates were washed with PBS-Mg/Ca before being developed with Pico-Chemiluminescent developing kit (Thermal Scientific), and chemiluminescence measurements were performed per manufacturer's instructions. Data plotting and analyses were performed with Microsoft Excel and Graphpad-Prism software. EC_{50} (nM) values were calculated from cell surface Notch3 binding ELISAs and provided in **Table 3**.

The data demonstrates that hu28 is similar to ch28 in binding to full-length human Notch3 expressed on the cell surface U2OS/hNotch3 cells. Further, the data demonstrates that hu28 fully retained the cross-reactivity of ch28 to mouse Notch3 expressed on the cell surface of U2OS/mNotch3 cells. The data also demonstrates that hu75 is similar to ch75 in binding to full-length human Notch3 expressed on the cell surface of U2OS/hNotch3 cells. Further, the data demonstrates that hu75 fully retained specificity of ch75 to human Notch3; no cross-reactivity was observed to mouse Notch3 expressed on the cell surface of U2OS/mNotch3 cells. N/B represents non-binding.

Table 3. EC_{50} (nM) values of cell surface Notch3 binding ELISAs for anti-Notch3 antibodies.

	EC_{50} (nM)			
Antibody	**ch28**	**hu28 VH1.0/VL1.0**	**ch75**	**hu75 VH1.9/VL1.3**
Full-length Human Notch3	0.098	0.093	0.20	0.22
Full-length Mouse Notch3	0.13	0.13	N/B	N/B

B. Competition ELISA

Competition ELISAs were performed for hu28 and ch28, and hu75 and ch75 on cell surface expressed full-length human Notch3. 96 well cell culture plates were seeded with U2OS/hNotch3 cells (cell culture plate, Co-star). Serially diluted (1:3 in blocking buffer) antibody solutions, in the presence of 0.8 nM of ch28 (biotinylated) or

ch75 (biotinylated) antibodies were applied to the plate. After incubation for 2 hours, the plates were washed, as described above, and HRP-conjugated streptavidin (Southern Biotech) diluted 1:5000 in blocking buffer was applied. After incubation with streptavidin for 30 minutes, the plates were washed again before being developed with TMB solution for 10 minutes. The developing reaction was stopped by adding 0.18M H_2SO_4 and absorbance at 450 nM was measured. Data plotting and analyses were performed with Microsoft Excel and Graphpad-Prism software.

Table 4 shows EC_{50} (nM) values from competition ELISAs for binding to full-length human Notch3 expressed on the cell surface of U2OS/hNotch3 cells. The data shows that hu28 has a similar EC_{50} value to the unlabelled r28, demonstrating that hu28 competes as well as unlabelled ch28 with biotinylated ch28 for binding to full-length human Notch3 expressed on the cell surface of U2OS/hNotch3 cells. The data indicates that hu28 binds to the same, or a highly similar, epitope on full-length human Notch3 expressed on the cell surface of U2OS/hNotch3 cells as ch28.

The data further shows that hu75 has a similar EC_{50} value to unlabelled ch75, demonstrating that hu75 competes as well as unlabelled ch75 with biotinylated ch75 for binding to full-length human Notch3 expressed on the cell surface of U2OS/hNotch3 cells. The data indicates that hu75 binds to the same, or highly similar, epitope on full-length human Notch3 expressed on the cell surface of U2OS/hNotch3 cells as ch75.

Table 4. EC_{50} (nM) values for competition ELISAs of anti-Notch3 antibodies.

	EC_{50} (nM)				
Antibody	**ch28**	**hu28 VH1.0/VL1.0**	**ch75**	**hu75 VH1.9/VL1.3**	**Anti-E. Tenella Control**
Full-length Human Notch3	3.0	3.1	1.3	1.7	Non-Competing

C. Specificity of binding to other human Notch homologues

Other members of the Notch receptor family play important roles in biological processes. For example, a Notch1 or Notch2 deficiency leads to embryonic death in mouse models. In contrast, a Notch4 deficiency results in no detectable phenotype in mouse models. The closest homologues of the Notch3 NRR region are Notch1 and

Notch2 (~50% homology), and Notch4 is a more distant homologue (~30% homology). Cross-reactivity of anti-Notch3 antibodies to other members of the Notch family, especially Notch1 and 2, may lead to undesired effects in patients. Therefore, the potential cross-reactivity of r28 and hu28, along with r75 and hu75 to other Notch family members were assessed.

Expression constructs encoding human Notch1 and Notch2 NRR regions, fused with human IgG1 Fc fragments were stably introduced into CHO-PACE cells. Conditioned media from these cells expressing NRR-Fc fusions were collected. Human Notch2 NRR-Fc and human Notch1 NRR-Fc were purified by protein A affinity followed by size exclusion chromatography (SEC). Purified preparations were dialysed into TBS with 1mM $CaCl_2$ and analyzed on analytical SEC to be >99% in purity.

As shown in **Table 5**, r28 and hu28, along with r75 and hu75 all lacked detectable binding to human Notch2 NRR-Fc fusion proteins. Further, hu28 and hu75 also lacked detectable binding to full-length human Notch1 NRR-Fc. This demonstrates that hu28 and hu75 do not cross-react with Notch1 or Notch2. N/B represents non-binding.

Table 5. Binding of anti-Notch3 antibodies to Notch2 NRR-Fc and Notch1 NRR-Fc.

	Antibody	Binding
Notch2 NRR-Fc	ch28	N/B
	hu28 VH1.0/V1.0	N/B
	ch75	N/B
	hu75 VH1.9/V1.3	N/B
Notch1 NRR-Fc	**ch28**	N/B
	hu28 VH1.0/V1.0	N/B
	ch75	N/B
	hu75 VH1.9/V1.3	N/B

D. Binding Affinity to Human Notch3 NRR

The kinetic constants of the anti-Notch3 NRR interactions were determined by surface plasmon resonance (Biacore® T100, Biacore Inc., Piscataway, NJ). Flow cells of a CM5 chip were immobilized with approximately 10,000 response units (RU) of anti-human IgG-Fc (Biacore®) in 10mM Glycine, pH 5.0 at 10µl/min for 600 seconds. 10µg/ml of anti-Notch3 antibodies ch28 and hu28, and ch75 and hu75 were diluted in TBS with 1mM $CaCl_2$ were captured at 10µl/min. Association of four concentrations of

human Notch3 NRR recombinant protein (from 3.7-100nM) and a zero concentration (running buffer) at 100µl/min were recorded for 3 minutes in TBS with 1mM $CaCl_2$. Dissociation of the complexes was measured for 10 minutes. The surface of the chip was regenerated by injecting 3M $MgCl_2$ with 3mM EGTA for 60 seconds at 10µl/min. Curves obtained after subtraction of the reference and buffer signals were fitted to a 1:1 Langmuir binding model with Biacore® T100 Evaluation Software (Biacore®).

K_a, K_d and K_D are shown in **Table 6**. Kinetic analysis shows similar ka (on) and kd (off) rates for ch28 and hu28. Further, a higher k_a (on) and lower k_d (off) rates for hu75 than ch75 were observed, resulting in a lower K_D value for hu75.

Table 6. Kinetic analysis of anti-Notch3 antibodies.

Antibody	k_a (1/Ms)	k_d (1/s)	K_D (nM)
ch28	4.09E+05	2.49E-04	0.61
hu28 VH1.0/VL1.0	3.86E+05	3.47E-04	0.90
ch75	5.70E+04	9.92E-04	17.40
hu75 VH1.9/VL1.3	3.48E+04	3.40E-04	9.76

D. Thermal Stability

There is a positive correlation between the thermal stability of a protein or protein domain with the overall stability of the protein or protein domain. A higher melting point of a protein or protein domain often provides improved manufacturability and longer shelf life. Differential scanning calorimetry (DSC) was used to assess the thermal stability of hu28 and hu75 versus ch28 and ch75, respectively. Protein samples were diluted in PBS to 0.3 mg/ml in a volume of 250 µl. The corresponding formulation buffer blank was used for the reference sample. Both samples were thoroughly degassed using a MicroCal ThermoVac Sample Degassing and Thermostat (Microcal, Inc., Northampton, MA) set to 8°C. Samples were dispensed into the appropriate cells of a MicroCal VP-DSC Capillary Cell MicroCalorimter (MicroCal, Inc., Northampton, MA). Samples were equilibrated for 4 minutes at 15°C and then scanned up to 100°C at a rate of 100°C per hour. A filtering period of 20 seconds was selected. Raw data was baseline corrected and the protein concentration was normalized. Origin Software (OriginLab Corporation, Northampton, MA) was used to fit the data to an MN2-State Model with an appropriate number of transitions.

As shown in **Table 7** below, both hu28 and hu75 had higher thermostability, as displayed by higher melting point, in their Fab region (all above 77°C) compared to ch28 and ch75, respectively.

Table 7. Thermal Stability (DSC) analysis of anti-Notch3 antibodies.

	Tm (°C) ± Standard Deviation		
Antibody	**CH2**	**Fab**	**CH3**
ch28	70.20 ± 0.05	73.22 ± 0.73	84.24± 0.07
hu28 VH1.0/VL1.0	72.63±0.03		83.45±0.17
ch75	73.32±0.01		84.59±0.03
hu75 VH1.9/VL1.3	70.46±0.02	74.63±0.17	84.17±0.05

Example 6

Domain Swap Chimeric Constructs

As described in Example 3, both hu28 and hu75 lacked cross-reactivity with the Notch1 protein. Domain swap chimeric constructs for the Notch1 and Notch3 NRR were prepared for epitope mapping of the anti-Notch3 ch28 and ch75 antibodies. Expression constructs encoding human Notch1-Notch3 (hereinafter Notch 1-3) NRR region domain swap chimera with C-terminal Fc fusion (human IgG1 Fc fragment) were individually transfected into CHO-PACE and stable pools expressing each chimera were established. Conditioned media from each stable pool were applied to protein A affinity chromatography, followed by size exclusion chromatography (SEC) for the purification of the chimeric fusion protein. Purified preparations were dialysed into TBS with 1mM $CaCl_2$ and analyzed on analytical SEC. **FIG. 6** shows the recombinant NRR chimeric proteins consist of various Notch1 (shown in grey) and Notch3 (shown in black) domains fused to human Fc (not shown).

Relative binding capacities of ch28 and ch75 to Notch 1-3 NRR domain swap chimeras were tested in ELISAs as described in Examples 3 and 5. The Notch 1-3 NRR domain swap chimera were coated on ELISA plates at 1ug/ml, followed by blocking with 1% BSA in PBS with 0.9mM Mg^{2+} and Ca^{2+}. ch28 and ch75 were then applied to the blocked plates at 5ug/ml diluted in the blocking buffer. After 1 hour of incubation, plates were washed with PBS with 0.9mM Mg^{2+} and Ca^{2+}, before secondary

goat anti-human Fc antibody conjugated with HRP were applied and incubated on the plates. After washing again, the plates were developed with TMB and the developing reactions were stopped by adding 0.18M H_2SO_4. O.D. 450nM were read on plate reader and relative binding capacities indicated by O.D. values.

As shown in **FIG. 6**, the epitope binding profile of ch28 and ch75 to domains of the Notch3 NRR are distinct. More specifically, the binding of ch28 to the Notch3 NRR was more dependent on the LNR-C and HD-1 domains, while ch75 was more dependent on the LNR-A and both the HD-1 and HD-2 domains. The observed binding profile for ch28 and ch75 was consistent with the signaling inhibition activities of the antibodies. In particular, ch28 only interacted with the N-terminus of the Notch3 NRR heterodimer (HD-1) which would not maintain the Notch3 NRR region in the auto-inhibitory conformation that is required for inhibiting the activation of Notch3 signaling. In contrast, ch75 interacted with both the HD-1 and HD-2 domains of the Notch3 NRR heterodimer which would maintain the Notch3 NRR region in the auto-inhibitory conformation, thereby inhibiting the activation of Notch3 signaling.

Example 7

Identification of Notch3 Expressing Cancer Cell Lines

Expression of Notch3 was determined in a panel of cancer cell lines by western blot analysis to identify Notch3 positive cells for further analysis and testing of anti-Notch3 antibodies and anti-Notch3 antibody-drug conjugates. The panel included HCC2429 lung cancer cell line, OVCAR3 ovarian cancer cell line, MDA-MB-468 breast cancer cell line, N87 gastric cancer cell line, along with cell lines engineered to over-express human Notch3 including U-2 OS and MDA-MB-468 cells, hereinafter referred to as U2OS/hNotch3 and MDAMB468/hNotch3, respectively. Notch3 was detected with a rabbit monoclonal anti-Notch3 antibody D11B8 (Cell Signaling Technologies) or a mouse monoclonal 1G5 (Abnova) using standard western blot procedures.

The D11B8 anti-Notch3 antibody binds to an epitope surrounding Glu2312 within the C-terminal tail of the human Notch3 protein and recognizes both uncleaved, full-

length Notch3 (~270 kDa) and cleaved, C-terminal domain containing Notch3 protein fragments at (~80-90 kDa). Notch3 bands at the ~80-90 kDa molecular weights represent the TMIC (Transmembrane and intracellular domain) and/or NEXT (Notch extracellular truncation) proteolytic fragments, as shown in **FIG. 7**. The 1G5 antibodies bind an epitope within amino acids 47-156 of the Notch3 extracellular domain (ECD) and recognize both the uncleaved, full-length (~270kDa) and the cleaved, N-terminal fragment (~210 kDa) (data not shown).

Both the Notch3-ECD (data not shown) and the cleaved C-terminal domain containing Notch3 protein fragments were detected in HCC2429, OVCAR3, MDA-MB-468, N87, MDAMB468/hNotch3 and U2OS/hNotch3, as shown in **FIG. 7**. Further, Notch3 was not detected in the SW900 lung cancer cell line and thus represents a Notch3 negative control cell line.

Example 8

Internalization and Trafficking of Anti-Notch3 Antibodies

Anti-Notch3 antibody-drug conjugates consist of a peptide cleavable maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl (vc) linker or a non-cleavable, thioether-based maleimidocaproyl (mc) linker conjugated to a series of cytotoxic agents. Release of payloads from the antibody requires trafficking and localization of the antibody-drug conjugate to lysosomes that possess proteolytic enzymes, such as cathepsin B, to cleave the vc-type linker or late lysosomes for complete catabolism of the antibody in order to release the mc-linked payloads. The internalization and intracellular trafficking of anti-Notch3 antibodies to lysosomal vesicles were monitored by indirect and direct immunofluoresence microscopy-based assays. To directly visual intracellular trafficking of anti-Notch3 antibodies, they were conjugated to fluorescent dyes and incubated with live cells in the presence of pHrodo™ red dextran (Life Technologies) to stain acidic vesicles such as lysosomes. Live cell imaging was performed to identify co-localization of the fluorescence-labeled antibody with lysosomes and other acidic vesicles. Further, indirect immunofluorescence

microscopy-based assays were performed on cells to confirm co-localization of anti-Notch3 antibodies and LAMP1, a lysosomal-associated protein.

A. Internalization and lysosomal trafficking

HCC2429 or MDA-MB-468 cells were cultured in a Lab Tec II 4 chambered coverglass with cover #1.5 borosilicate sterile slides (Thermo Fisher Scientific Inc.). On day 1, pHrodo™ red dextran (Life Technologies) was added to the medium at 10 µg/ml concentration and incubated for 16 hours in order to stain acidic vesicles such as lysosomes. On day 2, cells were washed twice with HBSS++ (Gibco Life Technologies). Anti-Notch3 antibody hu75 was conjugated to Alexa Fluor 488 (hereinafter "hu75-Alexa488") (Life Technologies Protein Labeling kit) and hu28 was directly conjugated to the DyLight650 maleimide reagent (hereinafter "hu28-DyLight650") (Thermo Scientific) according to manufacturer's instructions . Hu75-Alexa488 or hu28-DyLight650 labeled antibodies were added to the cells at a concentration 5 µg/ml in 2% bovine serum albumin (BSA)/HBSS++ for 25 minutes on wet ice. Cells were washed twice with ice cold HBSS++ on ice, placed in 2% BSA/HBSS++ and imaged on a spinning disk CSU-X1M 5000 microscope (Yokogawa) equipped with a eXcelon Evolve 512 camera (Photometrics) and a XL S Series chamber (Zeiss) for temperature, humidity and 5% CO_2 control, from 5 minutes to 12-18 hours. Images were captured every 5 minutes and combined using the Zen CZI file format (Zeiss). A Pearson's correlation coefficient was calculated using the Volocity v6.3 software (PerkinElmer) to determine the degree of colocalization between hu28-DyLight650 and pHrodo™ red dextran.

Once cells were prepared for live cell imaging, the earliest time point that could be assessed was 10 minutes due to set up and optimization of instrument setting for image acquisition. Approximately 10 minutes after cells were place in the humidified, 37°C, 5% CO_2 chamber, hu75-Alexa488 could be observed at the cell surface as well as in several punctuate-like structures inside the cells, as shown in **FIG. 8A**. This result indicates that anti-Notch3 antibody hu75 rapidly underwent internalization upon binding the Notch3 receptor at the cell membrane. At the early time points (10 minutes to about 65 minutes), intracellular anti-Notch3 antibody hu75-Alexa488 and acidic vesicles that were stained with pHrodo™ red dextran appeared as discrete puncta that did not co-

localize. From time about 70 minutes and later, anti-Notch3 antibody hu75 and pHrodo™ red dextran-labeled vesicles co-localized to the same discrete punctate-like structures, as shown by arrows in **FIG. 8B**. This data suggests that anti-Notch3 antibody hu75 internalized and trafficked to acidic vesicles such as lysosomes in Notch3 expressing HCC2429 (data not shown) and MDA-MB-468 cells, as shown in **FIG 8B**.

About 10 minutes after cells were place in the humidified, 37°C, 5% CO_2 chamber, hu28-DyLight650 could be observed at the cell surface, as shown in **FIG. 8C**. Intracellular anti-Notch3 antibody hu28-DyLight650 and pHrodo™ red dextran-stained acidic vesicles appeared as discrete puncta and gradually co-localized over time, as shown by arrows in **FIG. 8D**. A Pearson's correlation coefficient (PCC) was calculated to determine the degree of overlap or co-localization between hu28-DyLight650 and pHrodo™ red dextran fluorescent labels in MDA-MB-468 cells over time. The higher the PCC number, the greater degree of co-localization between the hu28-DyLight650 and pHrodo™ red dextran fluorescence labels. **FIG 8E** demonstrates a steady increase in co-localization up to about 360 minutes and a maximal colocalization between hu28-DyLight650 and pHrodo™ red dextran occurs at about 420 minutes and then plateaus. The data suggests that anti-Notch3 antibody hu28-DyLight650 internalized and trafficked to acidic vesicles such as lysosomes in Notch3 expressing MDA-MB-468 cells.

B. Co-localization with lysosomal-associated membrane protein 1 (LAMP1)

HCC2429 or MDA-MB-468 cells were cultured in a Lab Tec II 4 chambered coverglass with cover #1.5 borosilicate sterile slides (Thermo Fisher Scientific Inc.). For binding and internalization assays, cells were washed twice with HBSS++ and unconjugated anti-Notch3 antibodies, hu75 and hu28, were added to the cells at a concentration 10 µg/ml in 2% bovine serum albumin (BSA)/HBSS++ for 25 minutes on ice. To visual membrane binding at time 0 minutes, control cells were washed with ice cold HBSS++ and then fixed in 4% paraformaldehyde in PBS for 10 minutes. For antibody internalization, cells were washed twice with ice cold HBSS++ and then pre-warmed complete growth media was added to the cells and placed inside a humidified 37°C, 5% CO_2 incubator. Cells were removed from the incubator, washed and fixed as

before at multiple time points from 5 minutes to 18 hours. Cells were then washed 3 times with PBS, and permeabilized for 10 minutes with 0.3%Triton X-100 in PBS. Cells were washed 3 times with PBS for 5 minutes each time and then blocked for 1hour in 3% BSA/PBS. Anti-LAMP-1 mouse monoclonal antibody (H4A3, Abcam) was added at 1:100 in 2% BSA/PBS for overnight incubation at 4°C. Cells were washed twice with PBS for 5 minutes each and then secondary goat anti-human Alexa Fluor 488 and goat anti-mouse Alexa Fluor 555 (Life technologies) were added for 45 minutes in the dark. Cells were washed three times with PBS and were imaged on Zeiss LSM510 confocal microscope or a CSU-X1M 5000 (Yokogawa) spinning disk confocal microscope.

In control cells incubated on ice with anti-Notch3 antibodies and then immediately fixed, both hu28 and hu75 were localized at the cell surface of Notch3 expressing cells and no staining was observed inside the cells. In control cells, lysosomes were stained with an anti-LAMP1 antibody and appeared as discrete punctuate-like structures inside the cells that did not co-localize with anti-Notch3 antibodies hu28 and hu75. After incubation at 37°C for 90 minutes, anti-Notch3 antibodies hu28 and hu75 were observed in punctuate-like structures inside the cells that co-localized with anti-LAMP1 antibodies. After incubation at 37°C, cell membrane staining of anti-Notch3 antibodies hu28 and hu75 was reduced and eventually became undetectable. This data suggested that anti-Notch3 antibodies hu28 and hu75 bound the cell surface after incubation with cells on ice and then underwent temperature-dependent internalization at 37°C. Once internalized, anti-Notch3 antibodies hu28 and hu75 co-localized with the LAMP1 protein indicating they trafficked specifically to the lysosome (data not shown).

Example 9

Effects of Anti-Notch3 Antibodies on Notch3 Signaling in Cell-Based Assays.

Notch3 signaling is initiated by ligand-induced proteolysis. The mature Notch3 heterodimer after furin-like protease cleavage at site S1 is held in an auto-inhibited state by the juxtamembrane negative regulatory region (NRR). Binding of ligands, such as

DLL4 or Jagged1, to the Notch3-ECD induces two successive additional cleavages at sites S2 and S3 that are catalyzed by ADAM-type metalloproteinase and gamma-secretase, respectively. The latter cleavage releases the intracellular domain of Notch3 (NICD3), permitting it to translocate to the nucleus and activate the transcription of target genes containing consensus DNA binding site motifs for the CSL protein.

The ability of anti-Notch3 chimeric antibodies, ch28-huIgG1 and ch75-huIgG1, and humanized antibodies, hu28 and hu75, to inhibit Notch3 signaling were tested in a Notch3-dependent reporter gene co-culture assay. Anti-Notch3 antibodies were pre-incubated with Notch3 reporter cells and then co-cultured with DLL4-HEK293 cells to activate Notch3 signaling or with parental HEK293 cells as a control.

A. Cell line construction for Notch3 reporter gene co-culture assay

To generate the Notch3 reporter cell line, a series of three sequential, stable transfections were performed in the U-2 OS human osteosarcoma cell line (ATCC, Manassas, VA). The first transfection used a vector for expression of full-length human Notch3 based on the pCMV6-Entry-Myc-Flag backbone (Origene), and the correct DNA sequence of the Notch3 insert was confirmed. Following transfection with the TransIT-LT1 transfection reagent (Mirus, Madison, WI), U-2 OS cells were selected in G418 and clonal lines were isolated. Second, stable Notch3-expressing U-2 OS clones were re-transfected with the pGL4.27 [luc2P/minP/Hygro] vector (Promega, Madison, WI) containing eight tandem copies of the CSL enhancer sequence (CGTGGGAAAAT), selected in Hygromycin B plus G418 and clonal lines were isolated. The 8xCSL Firefly-luciferase reporter construct is responsive to activated Notch signaling (for example, see, Jeffries et al., Mol. Cell. Biol. 22(11):3927-3941, 2002). Thirdly, the human Notch3 8xCSL Firefly-Luciferase U-2 OS cells were transduced with Cignal Lenti Renilla Control (luc) (Qiagen, CA) lentiviral particles, selected in Puromycin, Hygromycin B and G418, and clonal lines were isolated. The Cignal Lenti Renilla control (luc) vector encoded the Renilla-luciferase gene that is constitutively expressed from a CMV promoter and served as an internal control. The triple stable transfected U-2 OS line (hereinafter termed “Notch3 reporter cells”) was maintained in McCoy’s 5A medium (Gibco, Grand

Island, NY) containing 10% FBS, 1X Penicillin/Streptomycin/L-Glutamine (Gibco), 0.25 mg/ml G418 sulfate, 0.3 mg/ml Hygromycin B and 0.001 mg/ml Puromycin.

To generate the ligand-expressing cells, HEK293 cells (ATCC) were transfected with a vector for expression of human DLL4. The vector was based on the pCMV6-AC-HA-His backbone (Origene, Rockville, MD), and the correct DNA sequence of the DLL4 insert was confirmed. Following transfection, HEK293 cells were selected in 0.5 mg/ml G418, and clonal lines were isolated, expanded and analyzed for DLL4 expression. Clones with high DLL4 expression and high induction of Notch3 reporter activity in the U-2 OS cells were used to assess the inhibitory effect of anti-Notch3 antibodies.

The luminescent readings from Firefly-luciferase were divided by the internal control Renilla-luciferase reading to normalize the signals (termed hereinafter "F/R ratio"). To calculate the fold-induction of Notch3 signaling, the F/R ratios generated from the DLL4-HEK293 co-culture reporter assays were divided by the F/R ratios from the parental HEK293 co-cultures and termed relative luciferase unit (RLU) or activity.

B. Reporter gene co-culture assays

Human Notch3 reporter cells were trypinized and harvested from culture plate in assay medium which consisted 50% complete McCoy's 5A media (McCoy's 5A with 10% FBS and penicillin, streptomycin, Invitrogen) and 50% of complete MEM media (MEM with 10% FBS and penicillin, streptomycin, Invitrogen) and counted. Appropriate dilutions of cells were made with the same medium to allow for 10,000 cells/well in a total volume of 45µl/well on a 96 well culture plate (white opaque, BD/VWR), in the presence of serially diluted (1:3 in complete McCoy's 5A media) antibody solutions or hybridoma culture supernatants. The mixture of cells and antibody dilutions were incubated on the plates in a sterile hood at room temperature for 1hr before 30,000/45µl of human DLL4-HEK293 cells were added to each well. After addition of hDLL4-HEK293 cells, the plates were further incubated for 20hrs in the incubator and Dual-Glo Luciferase assay system (Promega) was used to measure the firefly luciferase and internal control Renilla luciferase activity per manufacturer's instructions. Data was plotted and analyzed using Microsoft Excel and Graphpad-Prism software.

A titration of ch75-huIgG1 and hu75 in the human Notch3 reporter co-culture assay demonstrated potent inhibition of Notch3 signaling in a dose-dependent manner. **Table 8** shows the inhibitory activities of ch75-huIgG1 and hu75 antibodies against Notch3 dependent signaling in human Notch3 reporter cells. ch75-huIgG1 and hu75 showed similar neutralization activities in human Notch3 dependent signaling reporter assays. Therefore, hu75 fully retained the inhibitory activity of ch75-huIgG1. Both ch28-huIgG1 and hu28 weakly inhibited Notch3 signaling at a level similar to control ch2H6-huIgG1 and huNeg8.8 antibodies. This indicates that the inhibitory activity of ch28-huIgG1 and hu28 were non-specific. Further, the data demonstrates that anti-Notch3 antibodies ch28-huIgG1 and hu28 do not inhibit Notch3 signaling and therefore are functionally distinct from ch75-huIgG1 and hu75.

Table 8. Dose-dependent inhibitory activity of humanized and chimeric antibodies.

	% inhibition (SD)					
nM	**hu28 VH1.0/VL1.0**	**hu75 VH1.9/VL1.3**	**huNeg-8.8**	**ch28-hIgG1**	**ch75-hIgG1**	**ch2H6-hIgG1**
0	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
0.27	99.9 (6.2)	104.4 (4.9)	84.5 (8.6)	102.8 (6.1)	88.4 (8.7)	95.7 (6.9)
0.82	88.6 (11.8)	103.0 (17.8)	88.2 (9.0)	83.2 (11.7)	70.6 (4.1)	93.1 (11.0)
2.47	77.7 (9.5)	65.6 (12.3)	84.6 (7.5)	75.0 (5.2)	48.4 (4.2)	92.4 (9.0)
7.41	71.9 (4.7)	40.5 (4.1)	79.3 (10.4)	74.0 (2.9)	26.8 (1.9)	84.8 (9.4)
22.2	68.6 (7.9)	22.7 (3.2)	83.5 (8.8)	76.5 (6.2)	20.3 (2.1)	94.8 (8.6)
66.7	66.8 (2.0)	14.2 (1.1)	81.4 (4.3)	72.3 (8.9)	13.2 (1.0)	83.1 (2.8)
200	71.1 (10.3)	13.7 (0.9)	75.7 (5.1)	70.3 (4.1)	13.9 (1.4)	78.2 (5.1)

The IC_{50} (nM) values of ch75-huIgG1 and hu75 variants were calculated from the inhibition of Notch3-dependent signaling of the Notch3 reporter gene co-culture assays. IC_{50} (nM) data from two or three independent experiments were averaged, as provided in **Table 9**. Both hu75 and ch75-huIgG1 have low IC_{50} values indicating they are potent inhibitors of Notch3 signaling.

Table 9. IC_{50} values (nM) for inhibitory activity of hu75 and ch75-hIgG1.

Antibody	IC_{50} (nM)	SD
hu75 VH1.9/VL1.3	4.43	2.36
ch75-hIgG1	2.10	0.66

C. Effect of anti-Notch3 antibodies on Site 2 cleavage by Metalloprotease

To confirm that binding of anti-Notch3 antibody hu75, but not hu28, to the NRR domain of Notch3 is accompanied by a decrease in S2-cleavage, western blot analysis was performed. The rabbit monoclonal anti-Notch3 antibody D11B8 (Cell Signaling Technologies) recognizes the Notch3 C-terminal fragments that are products of S1 and S2 proteolytic cleavage events. As demonstrated by NRR domain swap experiments, the anti-Notch3 antibody hu75 bound simultaneously to LNR-A, HD-1 and HD-2 domains located on two non-covalently linked regions of the NRR that are separated by furin-cleavage at site 1 (S1). The anti-Notch3 antibody hu28 bound to LNR-C and HD1 domains that are located on a linear, covalently linked region of the NRR domain that is N-terminal to the S1-site. An inhibitory antibody is expected to decrease the detection of S2-cleaved Notch3 by stabilizing the NRR domain in an auto-inhibitory conformation thus preventing S2-cleavage, while a non-inhibitory antibody is not expected to have an effect on S2-cleavage.

Site 2 (S2)-cleavage of the Notch3 receptor was assessed by western blot analysis of the protein using the D11B8 (Cell Signaling Technologies) antibody which recognizes an epitope surrounding Glu2312 in the C-terminal domain. HCC2429 and MDA-MB-468 breast cancer cells were used to examine the effects of inhibitory anti-Notch3 antibody hu75 and non-inhibitory anti-Notch3 antibody hu28 on S2-cleavage. For the assay, 1-2.5X10^6 cells were plated in complete growth medium. Hu75, hu28 and huNeg8.8 control antibody were added at a concentration of 5 µg/ml. Cells were incubated at 37°C in a 5% CO_2 incubator for 24 hours and then directly lysed in buffer. Extracts were resolved by denaturing SDS-PAGE on a 7.5% polyacrylamide gel (Bio-Rad criterion gel) and transferred to nitrocellulose paper using an iBlot Gel transfer system (Invitrogen). Notch3 was detected with D11B8 antibody and, as a loading control, anti-GAPDH (Sigma) using standard western blot procedures.

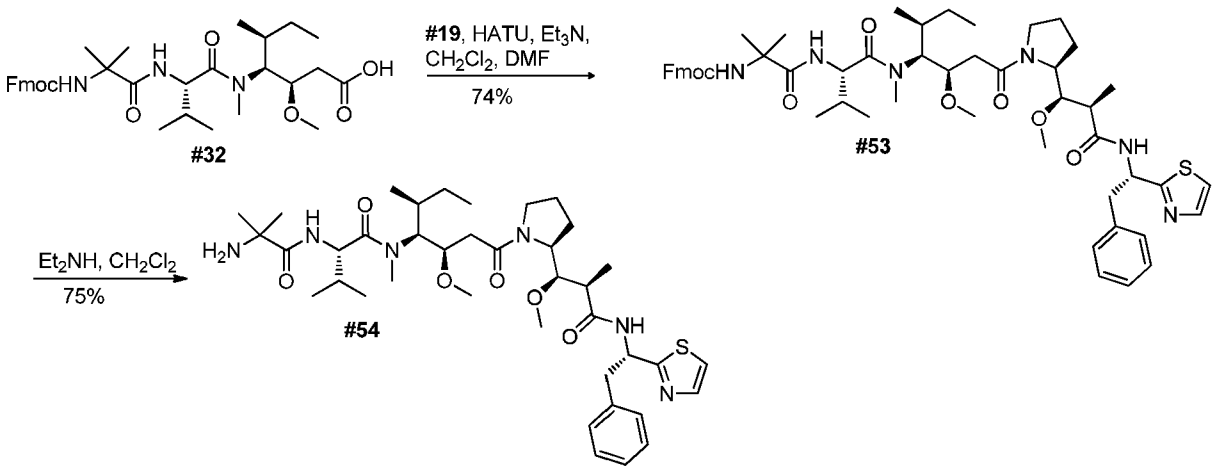
FIG. 9 shows a Western blot analysis of the S2-cleavage assay. In untreated and control huNeg8.8-treated HCC2429 and MDA-MB-468 cells for 24 hours, both S1- and S2-cleaved C-terminal Notch3 protein fragments could be detected by western blot analysis with D11B8 antibody, as expected. Further, in cells treated with inhibitory anti-Notch3 antibody hu75, the S1-cleaved Notch3 C-terminal fragment was detected, but the S2-cleaved fragment was not detected indicating that metalloprotease cleavage was blocked by hu75. Furthermore, in cells treated with non-inhibitory anit-Notch3 antibody hu28, both S1- and S2-cleaved Notch3 C-terminal fragments were detected indicating that hu28 does not inhibit proteolysis of the receptor. Therefore, hu28 binds with high affinity to the Notch3-NRR as demonstrated in Example 5 and had a high binding activity to full-length Notch3 expressed on the cell surface, but does not inhibit Notch3 signaling indicating it is functionally distinct from inhibitory anti-Notch3-NRR antibodies, including hu75.

Example 10
Synthesis of Compounds

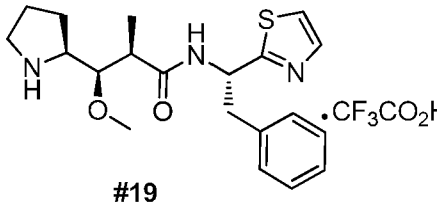
Compounds 0101, 6780, 0131, 3377 and 8261 were prepared according to the methods described in International Publication No. WO/2013/072813, which is incorporated herein by reference.

A. Experimental for compound 0101 (#54 in the schematic)

Preparation of 2-Methylalanyl-N-[(3R,4S,5S)-3-methoxy-1-{(2S)-2-[(1R,2R)-1-methoxy-2-methyl-3-oxo-3-{[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide (#54)



Step 1. Synthesis of *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide (#53). According to general procedure D (below), from #32 (2.05 g, 2.83 mmol, 1 eq.) in dichloromethane (20 mL, 0.1 M) and *N*,*N*-dimethylformamide (3 mL), the amine #19 (2.5 g, 3.4 mmol, 1.2 eq.), HATU (1.29 g, 3.38 mmol, 1.2 eq.)



#19

and triethylamine (1.57 mL, 11.3 mmol, 4 eq.) was synthesized the crude desired material, which was purified by silica gel chromatography (Gradient: 0% to 55% acetone in heptane), producing #53 (2.42 g, 74%) as a solid. LC-MS: *m/z* 965.7 [M+H$^+$], 987.6 [M+Na$^+$], retention time = 1.04 minutes; HPLC (Protocol A): *m/z* 965.4 [M+H$^+$], retention time = 11.344 minutes (purity > 97%); ^{1}H NMR (400 MHz, DMSO-d_6), presumed to be a mixture of rotamers, characteristic signals: δ 7.86-7.91 (m, 2H), [7.77 (d, *J*=3.3 Hz) and 7.79 (d, *J*=3.2 Hz), total 1H], 7.67-7.74 (m, 2H), [7.63 (d, *J*=3.2 Hz) and 7.65 (d, *J*=3.2 Hz), total 1H], 7.38-7.44 (m, 2H), 7.30-7.36 (m, 2H), 7.11-7.30 (m, 5H), [5.39 (ddd, *J*=11.4, 8.4, 4.1 Hz) and 5.52 (ddd, *J*=11.7, 8.8, 4.2 Hz), total 1H], [4.49 (dd, *J*=8.6, 7.6 Hz) and 4.59 (dd, *J*=8.6, 6.8 Hz), total 1H], 3.13, 3.17, 3.18 and 3.24 (4 s, total 6H), 2.90

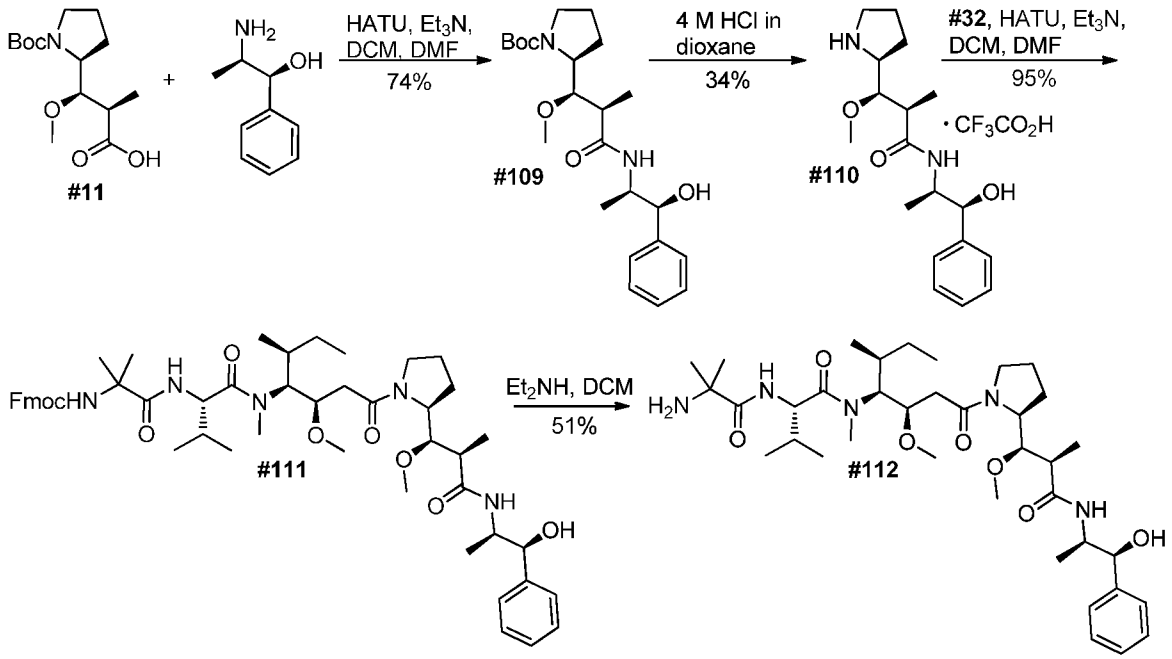
and 3.00 (2 br s, total 3H), 1.31 and 1.36 (2 br s, total 6H), [1.05 (d, *J*=6.7 Hz) and 1.09 (d, *J*=6.7 Hz), total 3H].

Step 2. Synthesis of 2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide (#54)

According to general procedure A (below), from #53 (701 mg, 0.726 mmol) in dichloromethane (10 mL, 0.07 M) was synthesized the crude desired material, which was purified by silica gel chromatography (Gradient: 0% to 10% methanol in dichloromethane). The residue was diluted with diethyl ether and heptane and was concentrated *in vacuo* to afford #54 (406 mg, 75%) as a white solid. LC-MS: *m/z* 743.6 [M+H$^+$], retention time = 0.70 minutes; HPLC (Protocol A): *m/z* 743.4 [M+H$^+$], retention time = 6.903 minutes, (purity > 97%); ^{1}H NMR (400 MHz, DMSO-d_6), presumed to be a mixture of rotamers, characteristic signals: δ [8.64 (br d, *J*=8.5 Hz) and 8.86 (br d, *J*=8.7 Hz), total 1H], [8.04 (br d, *J*=9.3 Hz) and 8.08 (br d, *J*=9.3 Hz), total 1H], [7.77 (d, *J*=3.3 Hz) and 7.80 (d, *J*=3.2 Hz), total 1H], [7.63 (d, *J*=3.3 Hz) and 7.66 (d, *J*=3.2 Hz), total 1H], 7.13-7.31 (m, 5H), [5.39 (ddd, *J*=11, 8.5, 4 Hz) and 5.53 (ddd, *J*=12, 9, 4 Hz), total 1H], [4.49 (dd, *J*=9, 8 Hz) and 4.60 (dd, *J*=9, 7 Hz), total 1H], 3.16, 3.20, 3.21 and 3.25 (4 s, total 6H), 2.93 and 3.02 (2 br s, total 3H), 1.21 (s, 3H), 1.13 and 1.13 (2 s, total 3H), [1.05 (d, *J*=6.7 Hz) and 1.10 (d, *J*=6.7 Hz), total 3H], 0.73-0.80 (m, 3H).

B. Experimental for compound 6780 (#112 in the schematic)

Preparation of 2-methylalanyl-N-[(3R,4S,5S)-1-{(2S)-2-[(1R,2R)-3-{[(1S,2R)-1-hydroxy-1-phenylpropan-2-yl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide (#112)

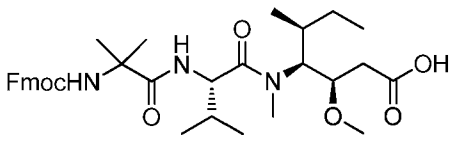


Step 1. Synthesis of *tert*-butyl (2*S*)-2-[(1*R*,2*R*)-3-{[(1*S*,2*R*)-1-hydroxy-1-phenylpropan-2-yl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidine-1-carboxylate (#109). To a solution of #11 (2.00 g, 6.96 mmol, 1 eq.) in dichloromethane (21 mL, 0.3 M) and *N,N*-dimethylformamide (3 mL) was added HATU (3270 mg, 8.35 mmol, 1.2 eq.). After two minutes, the amine (1*R*,2*S*)-(+)-norephedrine (1.07 mg, 6.96 mmol, 1 eq.) and triethylamine (1.94 mL, 13.9 mmol, 2 eq.) were added. After two hours, the reaction mixture was diluted with ethyl acetate (100 mL), washed with a 1 M aqueous solution of hydrochloric acid and with brine, dried over sodium sulfate, filtered, concentrated *in vacuo*, and purified by silica gel chromatography (Gradient: 0% to 60% ethyl acetate in heptane) to provide #109 (2.18 g, 74%) as a white solid. LC-MS: *m/z* 321.3 [(M - Boc)+H^+], retention time = 3.14 minutes; ^{1}H NMR (400 MHz, DMSO-d_6), presumed to be a mixture of rotamers, characteristic signals: δ 7.64 (d, *J*=8.6 Hz, 1H), 7.24-7.33 (m, 4H), 7.15-7.21 (m, 1H), 5.35 (br d, *J*=5 Hz, 1H), 4.45 (br dd, *J*=5, 5 Hz, 1H), 3.91-4.00 (m, 1H), 3.30-3.39 (m, 1H), 3.26 (s, 3H), 2.94-3.07 (m, 1H), 2.04-2.14 (m, 1H), 1.46-1.78 (m, 4H), 1.40 (s, 9H), 0.97-1.04 (m, 6H).

Step 2. Synthesis of (2*R*,3*R*)-*N*-[(1*S*,2*R*)-1-hydroxy-1-phenylpropan-2-yl]-3-methoxy-2-methyl-3-[(2*S*)-pyrrolidin-2-yl]propanamide, trifluoroacetic acid salt (#110). According to general procedure C (below), at 0 °C from #109 (414 mg, 0.984 mmol, 1

eq.), dioxane (5 mL, 0.2 M) and a 4 M solution of hydrogen chloride in dioxane (15 mL, 60 mmol, 60 eq.) was synthesized the crude desired compound, which was purified by reverse phase chromatography (Method C) to give #110 (120 mg, 34%) as a viscous liquid. LC-MS: *m/z* 321.1 [M+H$^+$], retention time = 0.55 minutes; ^{1}H NMR (400 MHz, DMSO-d_6), characteristic signals: δ 7.90 (d, *J*=8.6 Hz, 1H), 7.28-7.36 (m, 4H), 7.20-7.27 (m, 1H), 4.46 (d, *J*=6.2 Hz, 1H), 3.48 (dd, *J*=8.6, 2.3 Hz, 1H), 3.38 (s, 3H), 2.92-3.16 (m, 3H), 2.24-2.35 (m, 1H), 1.49-1.88 (m, 4H), 1.09 (d, *J*=6.6 Hz, 3H), 1.01 (d, *J*=6.6 Hz, 3H).

Step 3. Synthesis of *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-1-{(2*S*)-2-[(1*R*,2*R*)-3-{[(1*S*,2*R*)-1-hydroxy-1-phenylpropan-2-yl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide (#111). According to general procedure D (below), from #32 (140 mg, 0.230 mmol, 1 eq.),



#32

#110 (110 mg, 0.253 mmol, 1.1 eq.), dichloromethane (3 mL, 0.08 M), *N,N*-dimethylformamide (0.5 mL), HATU (96.2 mg, 0.253 mmol, 1.1 eq) and triethylamine (96 µL, 0.69 mmol, 3 eq.) was synthesized the crude desired product, which was purified by silica gel chromatography (Gradient: 0% to 40% acetone in heptane) to give #111 (220 mg, 95%). LC-MS: *m/z* 912.4 [M+H$^+$], 935.4 [M+Na$^+$], retention time = 2.15 minutes; HPLC (Protocol B): *m/z* 912.5 [M+H$^+$], 934.5 [M+Na$^+$], retention time = 10.138 minutes (purity >94%); ^{1}H NMR (400 MHz, DMSO-d_6), presumed to be a mixture of rotamers, characteristic signals: δ 7.89 (d, *J*=7.8 Hz, 2H), 7.66-7.75 (m, 2H), 7.41 (dd, *J*=7.4, 7.4 Hz, 2H), 7.12-7.20 (m, 1H), [5.33 (d, *J*=4.7 Hz) and 5.38 (d, *J*=4.7 Hz), total 1H], 3.15, 3.18, 3.22 and 3.23 (4 s, total 6H), 1.30, 1.33, 1.36 and 1.39 (4 s, total 6H), 0.95-1.06 (m, 6H).

Step 4. Synthesis of 2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-1-{(2*S*)-2-[(1*R*,2*R*)-3-{[(1*S*,2*R*)-1-hydroxy-1-phenylpropan-2-yl]amino}-1-methoxy-2-methyl-3-

oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide (#112). According to general procedure A (below), from #111 (210 mg, 0.230 mmol) in dichloromethane (5 mL, 0.05 M) and diethylamine (5 mL) was synthesized the crude desired material, which was purified by silica gel chromatography (Gradient: 0% to 10% methanol in dichloromethane) to give a mixture of an oil and solid. Diethyl ether and heptane were added and the mixture was concentrated *in vacuo*, producing #112 (81 mg, 51%) as a white solid. LC-MS: *m/z* 690.4 [$M+H^+$], retention time = 1.10 minutes; HPLC (Protocol A): *m/z* 690.5 [$M+H^+$], 712.4 [$M+Na^+$], retention time = 7.229 minutes (purity > 90%); ^{1}H NMR (400 MHz, DMSO-d_6), presumed to be a mixture of rotamers, characteristic signals: δ [7.62 (br d, *J*=8 Hz), 7.88 (br d, *J*=8 Hz), 8.07 (br d, *J*=9 Hz) and 8.11 (br d, *J*=9 Hz), total 2H], 7.15-7.34 (m, 5H), [5.34 (d, *J*=4 Hz) and 5.41 (d, *J*=5 Hz), total 1H], 3.18, 3.21, 3.23 and 3.25 (4 s, total 6H), 2.93 and 3.08 (2 br s, total 3H), 1.15, 1.18, 1.21 and 1.25 (4 s, total 6H).

C, General Procedures

General Procedure A: Fmoc removal using diethylamine or piperidine. To a solution of the Fmoc-containing compound in dichloromethane or *N*,*N*-dimethylformamide (also referred to as DMF), was added an equal volume of diethylamine or piperidine. Reaction progress was monitored by LC-MS (or HPLC or TLC). Solvents were removed *in vacuo*, and in some cases the residue was azeotroped one to four times with heptane. Residue was usually diluted with dichloromethane and a small amount of methanol before being reduced down onto silica and purified by chromatography on silica gel, eluting with methanol in dichloromethane (or other appropriate mixture of solvents) to afford the desired material (or crude material was used as is).

General Procedure C: Boc removal or *tert*-butyl ester (also refers to *t*-Bu ester) cleavage using hydrochloric acid in dioxane. To either a solution of Boc-containing compound or *tert*-butyl ester-containing compound in dioxane (or in some cases no solution, or other relevant solvent) was added a 4 M solution of hydrochloric acid in dioxane. Reaction progress was monitored by LC-MS (or HPLC or TLC). The reaction

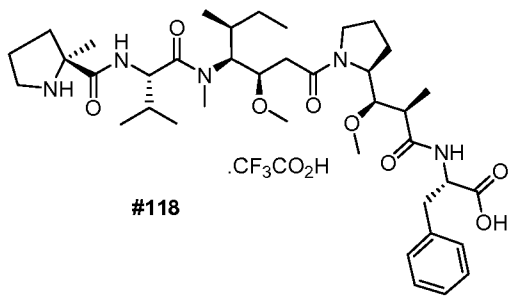
was concentrated *in vacuo* and in some cases azeotroped one to four time with heptanes.

General Procedure D: coupling with *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU). To a stirring solution of the amine (1.0 eq.) and acid (1.0-2.0 eq.) in dichloromethane, *N,N*-dimethylformamide (also referred to as DMF), or a mixture of both, HATU (1.0-2.0 eq.) was added followed by triethylamine (2.0-4.0 eq.) or diisopropylethylamine (2.0-4.0 eq., also referred to as Hunig's base). Reaction progress was monitored by LC-MS (or HPLC or TLC); the reaction was usually completed within three hours. Solvents were removed *in vacuo*. The residue was purified by silica gel or reverse phase chromatography or in some cases azeotroped three times with heptanes, diluted with a small amount of ethyl acetate before being reduced down onto silica or C18 bonded silica and purified by silica gel or reverse phase chromatography.

D. Other Compounds

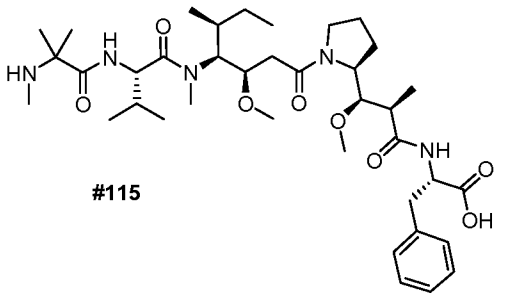
Further compounds used in the present invention are described in International Publication No. WO/2013/072813 and shown below.

As used herein, compound 0131 or 2-methyl-L-prolyl-N-[(3R,4S,5S)-1-{(2S)-2-[(1R,2R)-3-{[(1S)-1-carboxy-2-phenylethyl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide, trifluoroacetic acid salt (#118) has the formula:



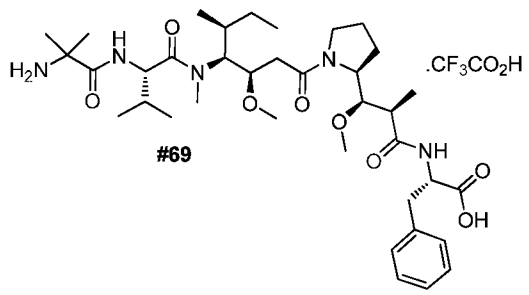
#118

As used herein, compound 3377 or N,2-dimethylalanyl-N-{(1S,2R)-4-{(2S)-2-[(1R,2R)-3-{[(1S)-1-carboxy-2-phenylethyl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-2-methoxy-1-[(1S)-1-methylpropyl]-4-oxobutyl}-N-methyl-L-valinamide, trifluoroacetic acid salt (#115) has the formula:



#115

As used herein, compound 8261 or 2-Methylalanyl-N-[(3R,4S,5S)-1-{(2S)-2-[(1R,2R)-3-{[(1S)-1-carboxy-2-phenylethyl]amino}-1-methoxy-2-methyl-3-oxopropyl]pyrrolidin-1-yl}-3-methoxy-5-methyl-1-oxoheptan-4-yl]-N-methyl-L-valinamide (#69) has the formula:



#69

Example 11

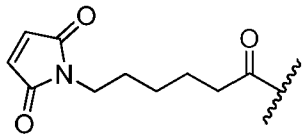
Preparation of Anti-Notch3 Antibody-Drug Conjugates (ADCs)

The ADCs of the present invention were prepared using a section of a linker having a reactive site for binding to a chemical compound and introducing another section of the linker unit having a reactive site for an antibody. In one aspect, the linker

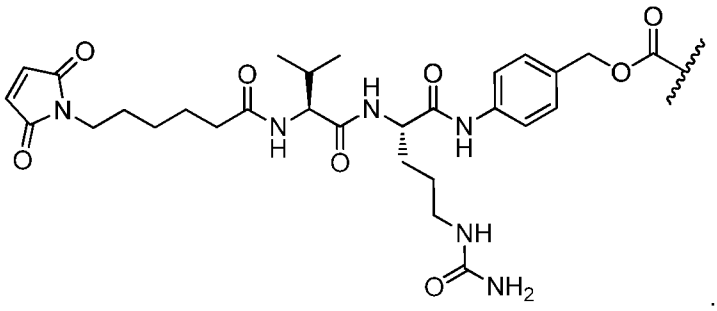
unit has a reactive site which has an electrophilic group that is reactive with a nucleophilic group present on an antibody unit, such as an antibody. Useful nucleophilic groups on an antibody include but are not limited to, sulfhydryl, hydroxyl and amino groups. The heteroatom of the nucleophilic group of an antibody is reactive to an electrophilic group on a linker unit and forms a covalent bond to a linker unit. Useful electrophilic groups on the linker include, but are not limited to, maleimide and haloacetamide groups.

The linker unit has a reactive site which has a nucleophilic group that is reactive with an electrophilic group present on an antibody unit. The electrophilic group on an antibody provides a convenient site for attachment to a linker unit. Useful electrophilic groups on an antibody include, but are not limited to, aldehyde and ketone carbonyl groups. The heteroatom of a nucleophilic group of a linker unit can react with an electrophilic group on an antibody and form a covalent bond to the antibody. Useful nucleophilic groups on a linker unit include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide.

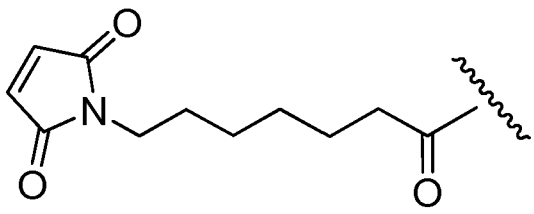
As used herein, "mc-" also known as "MalC-" refers to:



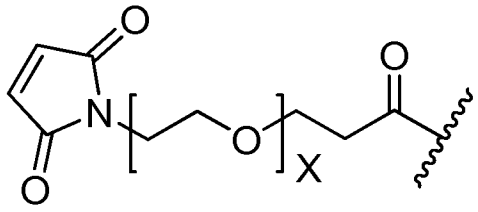
As used herein, "vc-", also known as "mcValCitPABC-" or "MalCValCitPABC-" refers to:



As used herein, "me-" refers to



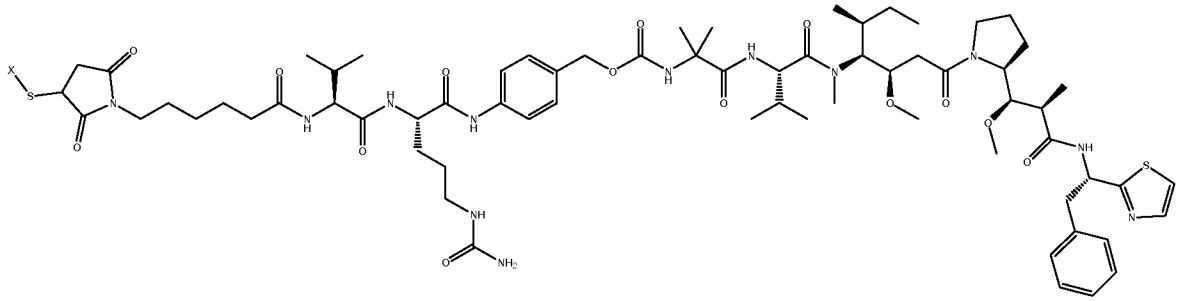
As used here, "MalPeg6C2-" refers to "MalPegXC2-" shown below, wherein X=6:



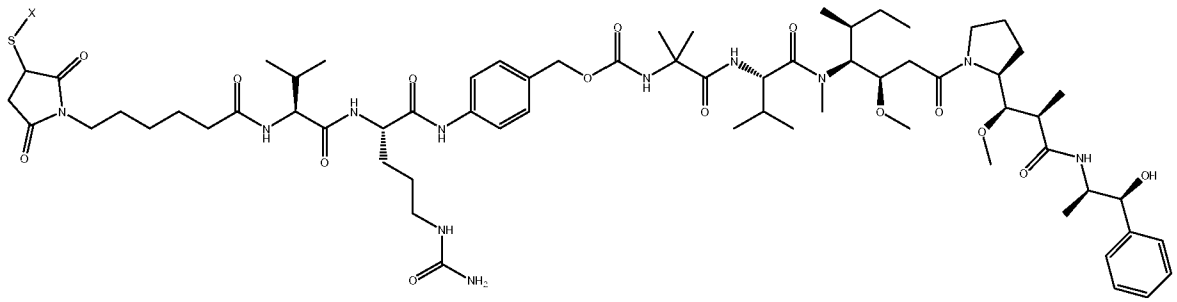
The ADCs of the present invention were prepared via partial reduction of the antibody with tris(2-carboxyethyl)phosphine (TCEP) followed by a reaction of reduced cysteine residues with the desired maleimide terminated linker-payload. Specifically, the antibodies were partially reduced via addition of about 2.3 – 3.0-fold molar excess of tris(2-carboxyethyl)phosphine (TCEP) in 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer), pH 7.0 and 1 mM diethylenetriaminepentaacetic acid (DTPA) for 2 hours at 37 °C. The desired linker-payload was then added to the reaction mixture at a linker-payload/antibody molar ratio of about 7–8 and reacted for an additional 1 hour at 25°C in the presence of 15% v/v of dimethylacetamide (DMA). After the 1 hour incubation period, 3-fold excess of N-ethylmaleimide was added to cap the unreacted thiols and was allowed to react for 15 minutes, followed by addition of 6-fold excess L-Cys to quench any unreacted linker-payload. The reaction mixture was dialyzed overnight at 4°C in phosphate buffered saline (PBS), pH 7.4, and purified via SEC (AKTA explorer, Superdex 200). The hydrolysis of the succinimide ring was further

achieved via incubating the purified ADC in a 100mM borate, pH 9.2 buffer for 24-72 hours at 37°C. The ring opening was monitored via liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI MS) and purified via size exclusion chromatography (SEC). The ADC was further characterized via SEC for purity, hydrophobic interaction chromatography (HIC), and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI MS) to calculate drug-antibody ratio (loading). The protein concentration was determined via UV spectrophotometer.

vc0101 (shows conjugation to antibody X through cysteine residue)



vc6780 (shows conjugation to antibody X through cysteine residue)



Humanized anti-Notch antibodies, hu28 and hu75, and rat-human chimeric anti-Notch antibodies, ch28 and ch75, were conjugated to various linker-payload combinations as provided in **Table 10.** The ADCs and elements thereof were prepared according to the methods of the present invention and according to International Publication No. WO/2013/072813.

Table 10. Anti-Notch3 ADC nomenclature.

ADC Nomenclature	Corresponding ADC Linker-Payload #
hu28-vc0101	Notch3-28-v1010-hG1-(C)_mcValCitPABC-#54
hu28-vc6780	Notch3-28-v1010-hG1-(C)_mcValCitPABC-#112
hu75-vc0101	Notch3-75-v1913-hG1-(C)_mcValCitPABC-#54
hu75-vc6780	Notch3-75-v1913-hG1-(C)_mcValCitPABC-#112
ch28-vc0101	Notch3-28-cG1-(C)_mcValCitPABC-#54
ch28-vc6780	Notch3-28-cG1-(C)_mcValCitPABC-#112
ch28-mc0101	Notch3-28-cG1-(C)_mc-#54
ch28-mc0131	Notch3-28-cG1-(C)_mc-0#118
ch28-mc3377	Notch3-28-cG1-(C)_mc-#115
ch28-mc8261	Notch3-28-cG1-(C)_mc-#69
ch28-MalPeg6C2-0131	Notch3-28-cG1-(C)_MalPeg6C2-0#118
ch28-MalPeg6C2-8261	Notch3-28-cG1-(C)_MalPeg6C2-#69
ch28-me0131	Notch3-28-cG1-(C)_me-0#118
ch28-m(H2O)c-0131	Notch3-28-cG1-(C)_m(H2O)c-0#118
ch75-vc0101	Notch3-75-cG1-(C)_mcValCitPABC-#54
ch75-vc6780	Notch3-75-cG1-(C)_mcValCitPABC-#112
ch75-mc0131	Notch3-75-cG1-(C)_mc-0#118
ch75-mc3377	Notch3-75-cG1-(C)_mc-#115
ch75-MalPegC2-0131	Notch3-75-cG1-(C)_MalPeg6C2-0#118
ch75-MalPeg6C2-8261	Notch3-75-cG1-(C)_MalPeg6C2-#69
ch75-me0131	Notch3-75-cG1-(C)_me-0#118
ch75-m(H2O)c-0131	Notch3-75-cG1-(C)_m(H2O)c-0#118
huNeg8.8-vc0101	huNeg8.8-(C)_mcValCitPABC-#54
huNeg8.8-vc6780	huNeg8.8-(C)_mcValCitPABC-#112
huNeg8.8-mc0131	huNeg8.8-(C)_mc-0#118
huNeg8.8-mc3377	huNeg8.8-(C)_mc-#115
huNeg8.8-me0131	huNeg8.8-(C)_me-0#118
huNeg8.8-MalPeg6C2-8261	huNeg8.8-(C)_MalPeg6C2-#69
ch2H6-mc8261	ch2H6-(C)_mc-#69

5

Example 12

Binding Activity and Specificity of Anti-Notch3 Antibodies and ADCs

A. Cell-based ELISA

Unconjugated anti-Notch3 antibodies, anti-Notch3 ADCs and a negative control antibody (huNeg8.8) were screened for cell surface binding activity to Notch3 expressing cell lines in a cell-based ELISA. Over-expressing Notch3 cell line, U2OS/hNotch3, and endogenous Notch3 expressing cell lines, HCC2429 and MDA-MB-468, were plated at 50,000, 200,000 and 100,000 cells/well, respectively, in 96 well plates (white opaque, BD/VWR) the day before ELISA assay. On the day of the ELISA, culture media was removed from wells and serially diluted (1:3 in DPBS with calcium chloride and magnesium chloride (Ca/Mg) and 1% BSA) antibody and ADC solutions were applied to the plate. Plates were incubated at room temperature for 2 hours before washed with DPBS with Ca/Mg and 1% BSA. HRP-conjugated secondary antibody was then applied and incubated with cells for 1 hour. Plates were washed with DPBS with Ca/Mg and 1% BSA before being developed with Pico-Chemiluminescent developing kit (Thermal Scientific), and chemiluminescence measurements were performed per manufacturer's instructions. Data plotting and analyses were performed with Microsoft Excel and Graphpad-Prism software.

Table 11 shows EC_{50} (nM) values and standard deviations (SD) calculated for two to four independent experiments from cell surface Notch3 binding ELISAs for unconjugated anti-Notch3 antibodies, hu28 and hu75, and anti-Notch3 ADCs, hu28-vc0101, hu28-vc6780, hu75-vc0101 and hu75-vc6780. The data demonstrates that hu28-ADCs and hu75-ADCs are similar to unconjugated antibodies hu28 and hu75, respectively, in binding to full-length human Notch3 expressed on the cell surface of U2OS/hNotch3, HCC2429 and MDA-MB-468 cells. Further, the data demonstrates that conjugation of various linker-payloads to the hu28 and hu75 antibodies did not affect or alter the binding characteristics. Furthermore, the data demonstrates that hu28 and both hu28-vc0101 and hu28-vc6780 have a higher binding capacity for cell surface Notch3, as demonstrated by their lower EC_{50} values, than hu75 and hu75-vc0101 and hu75-vc6780. The negative control, unconjugated huNeg8.8 antibody, did not bind any of the

cell lines tested. For the control antibody that lacked binding (LB), EC_{50} values were not generated as indicated. (SD = Standard Deviation)

Table 11. EC_{50} (nM) values for unconjugated anti-Notch3 antibodies and anti-Notch3 ADCs. (SD = Standard Deviation)

	EC_{50} (nM) and (SD)					
	HCC2429		**MDA-MB-468**		**U2OS/ hNotch3**	
	EC50	**SD**	**EC50**	**SD**	**EC50**	**SD**
hu28 VH1.0/VL1.0	0.132	0.009	0.192	0.054	0.172	0.077
hu28-vc0101	0.176	0.025	0.273	0.063	0.266	0.029
hu28-vc6780	0.154	0.034	0.242	0.046	0.236	0.008
hu75 VH1.9/VL1.3	0.335	0.053	1.284	0.176	0.585	0.160
hu75-vc0101	0.427	0.115	2.022	0.167	0.798	0.057
hu75-vc6780	0.343	0.063	1.804	0.062	0.723	0.041
huNeg8.8	LB	-	LB	-	LB	-

B. Flow cytometry

Unconjugated anti-Notch3 antibodies, hu28 and hu75, were examined for cell surface binding activity to Notch3 expressing cell lines by flow cytometry. Fluorescence activated cell sorting (FACS) analysis was conducted according to standard procedures. Cells were rinsed in HBSS with calcium chloride and magnesium chloride (herein termed HBSS++), harvested using trypsin without EDTA and neutralized with medium containing FBS. Cells were incubated on ice for 30 minutes with 4 µg/mL anti-Notch3 antibodies, hu28 and hu75, in HBSS++ containing 3% HICS (heat-inactivated calf serum). Cells were washed three times with cold HBSS++, 3% HICS buffer. Cells were incubated in allophycocyanin-conjugated AffiniPure F(ab')2 fragment goat anti-human IgG, Fc fragment secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 10 µg/mL for 30 minutes on ice in the dark. Cells were washed once with cold HBSS++/3% HICS buffer. Cells were resuspended in HBSS++/3% HICS, 25mM HEPES, 1 mM $MgCl_2$ and 25 µg/ml DNaseI. 7-AAD (7-Amino-Actinomycin D) was added for exclusion of nonviable cells. Live cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Mean fluorescence

intensity (MFI) from channel FL-4 was calculated with FlowJo flow cytometry analysis software (Ashland, OR).

Table 12 shows the binding activity of unconjugated anti-Notch3 antibodies hu28 and hu75 by FACS analysis to a panel of Notch3 expressing cell lines. The U-2 OS cell line was used as a Notch3 negative/low control cell line. Both hu28 and hu75 had low levels of binding to U-2 OS that were minimally higher than the negative control huNeg8.8. Further, the data demonstrates that hu28 and hu75 bound specifically to the Notch3 over-expressing cell, U2OS/hNotch3 and MDAMB468/hNotch3, and Notch3 endogenously expressing cells MDA-MB-468, HCC2429 and OVCAR3. Furthermore, the data demonstrates that the binding activity of hu28 was greater than hu75 to all Notch3 expressing cell lines similar to the observation of binding activity by cell-based ELISA.

Table 12. MFI values by FACS analysis for anti-Notch3 antibodies.

	MFI		
Cell line	**hu28 VH1.0/VL1.0 (anti-Notch3)**	**hu75 VH1.9/VL1.3 (anti-Notch3)**	**huNeg8.8 (negative control)**
U2OS/hNotch3(positive control)	256	171	6
MDA-MB-468	557	274	6
MDA-MB-468/hNotch3	1192	530	6
HCC2429	564	322	3
OVCAR3	116	80	5
U-2 OS (negative/low control)	17	18	7

Example 13

In vitro Cytotoxicity Assay

The effects of anti-Notch3 ADCs were assessed on 1) cell lines endogenously expressing Notch3 protein: HCC2429 (lung cancer), OVCAR3 (ovarian cancer) and MDA-MB-468 (breast cancer), 2) cell lines engineered to over-express full length human Notch3 protein: MDA-MB-468/hNotch3 and U2OS/hNotch3, and 3) a negative control cell line (SW900) using an MTS cellular viability indicator (Promega, Madison, WI).

These cell lines were cultured with increasing concentrations of anti-Notch3 ADCs comprising rat-human chimeric anti-Notch3 antibodies, ch28 and ch75, and humanized anti-Notch3 antibodies, hu28 and hu75, conjugated to various linker-payload combinations of the present invention. As a specificity control for the anti-Notch3-ADCs, non-targeted control-ADCs (huNeg8.8-ADCs or ch2H6-ADCs) were also tested on the same cell lines. After four days, viability of each culture was assessed. IC_{50} values were calculated by logistic non-linear regression, model #203 with XL fit v4.2 (IDBS, Guildford, Surry, UK) and presented as ng Ab/mL. The drug antibody ratio (DAR) is also provided.

Table 13 shows IC_{50} (ng Ab/mL) values of the rat-human chimeric anti-Notch3 ADC treatments. For experiments with 2-4 individual repeats, average IC_{50} values were calculated along with standard error of the mean (S.E.M.). The data demonstrates that the rat-human chimeric anti-Notch3 ADCs with various linker-payloads were active and induced cell death in the Notch3 expressing and over-expressing cancer cell lines HCC2429, OVCAR3, MDA-MB-468, MDA-MB-468/hNotch3, U2OS/hNotch3. The non-targeted control-ADCs either lacked potency (LP) and therefore IC_{50} values were not generated as indicated, or were minimally active at the highest doses tested. Anti-Notch3 ADCs having IC_{50} values equal to or higher than IC_{50} values for control-ADCs were considered to lack potency in vitro and indicted as LP.

Table 13. IC_{50} (ng Ab/mL) values of rat-human chimeric anti-Notch3 ADCs (nd= not determined).

ADC	DAR	IC_{50} (ng Ab/mL) ± S.E.M.				
		HCC2429	OVCAR3	MDA-MB-468	MDA-MB-468/ hNotch3	U2OS/ hNotch3
ch28-mc8261	3.7	LP	nd	12147±4806.4	nd	nd
ch2H6-mc8261	4.1	LP	nd	LP	nd	nd
ch28-MalPeg6C2-8261	4.3	LP	nd	83±35.5	nd	nd
ch75-MalPeg6C2-8261	3.8	LP	nd	4255±2375	nd	nd
huNeg8.8-MalPeg6C2-8261	4.1	LP	nd	LP	nd	nd
ch28-mc0131	3.4	251±77.5	6±1.0	35±18.5	nd	3±0.5
ch75-mc0131	3.3	671±406.5	289	8202±2773.0	nd	19
huNeg8.8-mc0131	3.9	nd	LP	LP	nd	LP
ch28-me0131	3.9	30	8±2.0	24±14.0	nd	3±1.15

ch75-me0131	3.5	nd	nd	259	nd	nd
huNeg8.8-me0131	3.7	nd	LP	LP	nd	LP
ch28-mc3377	3.7	LP	14±5.5	27±11.3	nd	3±0.5
ch75-mc3377	3.7	nd	nd	560	nd	nd
huNeg8.8-mc3377	3.6	nd	LP	LP	nd	LP
ch28-MalPeg6C2-0131	4.1	LP	10±2.0	10±1.0	nd	3±0.85
ch28-vc0101	3.8	3230±1116.5	635	5443±2630.9	4±0.5	95±18.2
ch75-vc0101	2.7	2112±826.0	LP	4064±1793.9	24±4.0	LP
huNeg8.8-vc0101	3.7	15341	LP	4523±2789.5	8833	LP
ch28-vc6780	4.1	324±78.9	90±48.5	4407±2128.2	3±0.5	LP
ch75-vc6780	2.8	1004±177.0	922	6873±4230.0	21±3.5	LP
huNeg8.8-vc6780	4.1	LP	LP	LP	LP	LP

Table 14 shows IC_{50} (ng Ab/mL) values of the humanized anti-Notch3 ADC treatments. HCC2429 and MDA-MB-468/hNotch3 cell lines had two individual repeats. The data demonstrates that the humanized anti-Notch3 ADCs with various linker-payloads were active and induced cell death in the Notch3 expressing and over-expressing cancer cell lines HCC2429, OVCAR3, MDA-MB-468, MDA-MB-468/hNotch3, U2OS/hNotch3, but not in the negative control cell line SW900 lacking Notch3 expression. The non-targeted control-ADCs either lacked potency (LP) and therefore IC_{50} values were not generated as indicated, or were minimally active at the highest doses tested. Anti-Notch3 ADCs having IC_{50} values equal to or higher than IC_{50} values for control-ADCs were considered to lack potency in vitro and indicted as LP. Unconjugated humanized anti-Notch3 antibodies hu28 and hu75 did not affect the viability of HCC2429 or MDAMB468/hNotch3, indicating cytotoxicity can be specifically attributed to payloads (data not shown).

Table 14. IC_{50} (ng Ab/mL) values of humanized anti-Notch3 ADCs.

ADC	DAR	IC_{50} (ng Ab/mL)							
		HCC2429		OVCAR3	MDA-MB-468	MDA-MB-468/ hNotch3		U2OS/ hNotch3	SW900
hu28-vc0101	3.9	473	2940	306	6545	3.2	3	1330	LP
hu75-vc0101	3.8	611	3295	515	7001	37	36	523	LP
huNeg8.8-vc0101	3.7	18417	23978	3770	LP	5122	LP	LP	23379
hu28-vc6780	3.9	148	2050	17	LP	1.3	3	LP	LP

hu75-vc6780	4.2	214	630	254	LP	26	25	LP	LP
huNeg8.8-vc6780	4.2	LP	LP	9238	LP	LP	LP	LP	LP

Notch3 knockdown with siRNAs was used to confirm that the in vitro cytotoxicity of anti-Notch3 ADCs was dependent on the expression of the Notch3 protein. siRNA transfections were generated using ON-TARGET plus SMART pool human Notch3 (L-011093-00), ON-TARGET plus Control Non-Targeting pool (D-001810-10) (Thermo Scientific Dharmacon) and Lipofectamine RNAiMAX Reagent (Invitrogen). Two to $2.5x10^6$ cells of HCC2429, OVCAR3 and MDA-MB-468/hNotch3 cells were plated in 10cm dishes in growth medium without antibiotics the day before transfection. The next day fresh medium without antibiotics was added. The siRNAs and the Lipofectamine RNAiMAX were diluted in OPTI-MEM media and used as per the manufacturer's specifications. Each of the cell lines was transfected with control and Notch3 siRNAs. The cells were incubated with the transfection mixture for 24 hours in a humidified, 37°C, 5% CO_2 incubator. After 24 hours, cells were trypsinized and plated for assessment using MTS cellular viability indicator (Promega, Madison, WI).

Depending on the cell line, cells were then seeded at a density of 2,500 to 5,000 cells per well 24 hours before treatment. Cells were treated with 3-fold serially diluted humanized anti-Notch3 ADCs in triplicates at 10 concentrations (range 0-30 µg Ab/ml). Relative cell viability was determined as percentage of untreated control 96 hours after treatment IC_{50} values were calculated by logistic non-linear regression, model #203 with XL fit v4.2 (IDBS, Guildford, Surry, UK) and presented as ng Ab/mL. For experiments with 2 individual repeats, average IC_{50} values were calculated along with standard error of the mean (S.E.M.). Western blot analysis on extracts prepared from control and Notch3 siRNA-treated cells was performed to confirm that Notch3 knockdown occurred for up to 144 hours (data not shown). Cell lines that expressed Notch3 (herein termed control siRNA) or had reduced Notch3 expression after siRNA knockdown (herein termed Notch3 siRNA) were cultured with increasing concentrations of humanized anti Notch3 ADCs. As a specificity control for the anti-Notch3 ADCs, non-targeted control-ADCs (huNeg8.8-ADCs) were also tested on the same cell lines. After four days, viability of each culture was assessed. IC_{50} values were calculated by logistic non-

linear regression, model #203 with XL fit v4.2 (IDBS, Guildford, Surry, UK) and presented as ng Ab/mL. The drug antibody ratio (DAR) is also provided.

Table 15 shows IC_{50} (ng Ab/mL) values of humanized anti-Notch3 ADC treatments of a panel of control siRNA or Notch3 siRNA treated cancer cell lines. The data demonstrates that humanized anti-Notch3 ADCs with various linker-payloads were active and induced cell death in the Notch3-expressing cancer cell lines (control siRNA). In cells that had reduced Notch3 expression after siRNA knockdown (Notch3 siRNA), IC_{50} values were greater than control siRNA indicating that a reduction in Notch3 expression was accompanied by a reduction in cytotoxicity of anti-Notch3 ADCs. Control-ADCs lacked potency (LP) and therefore IC_{50} values were not generated as indicated, or were minimally active at the highest doses tested. The data further demonstrates that humanized anti-Notch3 ADCs specifically induced cell death on Notch3 expressing and over-expressing cancer cell lines. The observed cytotoxicity was dependent on Notch3 expression because Notch3 siRNA knockdown on the same cells reduced cell death by anti-Notch3 ADCs. Therefore, humanized anti-Notch3 ADCs were dependent on Notch3 expression for their in vitro cytotoxicity.

Table 15. IC_{50} (ng Ab/ml) values of humanized anti-Notch3 ADCs.

ADC	DAR	IC_{50} (ng Ab/mL) ± S.E.M.					
		HCC2429		OVCAR3		MDA-MB 468 /hNotch3	
		Control siRNA	Notch3 siRNA	Control siRNA	Notch3 siRNA	Control siRNA	Notch3 siRNA
hu28-vc0101	3.9	930±529	7138±827	684±183	1994±262	3.0	3340
hu75-vc0101	3.8	956±259	8972±891	1056±158	2408±27.5	29.0	3187
huNeg8.8-vc0101	3.7	17832±2319	21374±1614	3751±922	2902±654	4226	5580
hu28-vc6780	3.9	259±179	14120±1175	189±6.0	6100±200	2.0	LP
hu75-vc6780	4.2	236±30.5	LP	690±137	7300±147	17.0	7981
huNeg8.8-vc6780	4.2	LP	LP	8163±1031	7674±705	LP	LP

Example 14

In vitro Assessment of Anti-Notch3 ADC Mechanism of Action

A. Anti-Notch3 ADC disruption of microtubules

Following internalization and intracellular release of the payload from the Notch3-ADC, the presumed mechanism of action of the released payload is disruption of microtubules that are required for cell division, thereby leading to cell cycle arrest, induction of apoptosis and cell death. To confirm this mechanism of action, OVCAR3 ovarian cancer cells were treated with hu28-vc0101, huNeg8.8-vc0101 (control ADC) or left untreated, and then subjected to immunofluorescence analysis by staining with an anti-alpha-tubulin antibody to mark microtubules and an anti-phospho-Histone H3 antibody to identify cells undergoing or arrested in mitosis.

OVCAR3 cells were seeded in a Lab Tec II 4 chambered coverglass with cover #1.5 borosilicate sterile slides (Thermo Fisher Scientific Inc.). Next day, cells were treated with 1.0 µg/ml of hu28-vc0101, control huNeg8.8-vc0101 or left untreated. Forty-eight hours later, cells were washed twice with HBSS++ and fixed in 4% paraformaldyhide for 10 minutes. Cells were washed three times with PBS and permeabilized with 0.5 % Triton X-100 for 2 minutes. After washing cells three times with PBS, cells were blocked with 3% BSA/PBS for 30 minutes at room temperature. Cells were then incubated with 1:100 of anti-phospho-Histone H3 (Ser10) (Cell Signaling) and 2 µg/ml of anti-alpha-Tubulin, (Millipore) in 2%BSA/PBS for 2 hours at room temperature. After 2 hours, cells were washed twice with PBS and then incubated with a 1:500 dilution of goat anti-mouse Alexa Fluor 488 and goat anti-rabbit 555 for 45 minutes at room temperature. Cells were washed twice with PBS and samples were imaged on an LSM710 confocal microscope (Zeiss).

As shown in **FIG. 10**, untreated OVCAR3 cells in the mitotic phase of the cell cycle stain positive for phospho-histone H3 and contain a normal bipolar spindle apparatus as demonstrated by anti-alpha-tubulin staining. Treatment with hu28-vc0101, but not control huNeg8.8-vc0101, disrupted the structure of the mitotic spindle apparatus as demonstrated by its abnormal morphology in phospho-Histone H3 stained

cells. The data demonstrates that hu28-vc0101 inhibited cell proliferation by disrupting microtubules that are required during mitosis to complete cell division.

B. Anti-Notch3 ADC induction of apoptosis

Caspase-3 and caspase-7 (caspase-3/7) proteases are key members of the programmed cell death machinery responsible for mediating late apoptotic events in mammalian cells. The activity of caspase-3/7 was measured in Notch3 expressing cell lines that were treated with Notch3-ADCs.

OVCAR3, HCC2429 and MDA-MB-468/hNotch3 cells were seeded onto opaque white tissue culture plates and incubated overnight. OVCAR3 and HCC2429 were treated at 1.1 µg/ml and MDA-MB-468/hNotch3 cells were treated 1µg/ml of hu28-vc0101 and huNeg8.8-vc010 (control ADC). After 48 hours incubation, cells were treated with the Caspase-Glo 3/7 reagent (Promega #G8090) for 2 hours at room temperature. Luminescence was measured with a luminometer and following subtraction of background, values were reported as relative luminescence units.

Table 16 shows that hu28-vc0101 induced the activity of caspase-3/7 in Notch3 expressing cell lines MDA-MB-468/hNotch3, HCC2429 and OVCAR3 cells from 2-3 fold over control Neg8.8-vc0101 treated cells. Thus, hu28-vc0101 inhibits cell growth by inducing apoptosis.

Table 16. Relative luminescence units for anti-Notch3 ADCs.

	hu28-vc0101	**huNeg8.8-vc0101**
MDA-MB-468/hNotch3	78,622 ± 1160	47,362 ± 678
HCC2429	128,518 ± 3483	55,255 ± 2965
OVCAR3	209, 715 ± 15572	118,150 ± 4280

Example 15

Notch3 Immunohistochemistry

The effects of anti-Notch3 ADCs were assessed in pre-clinical models that had detectable levels of Notch3 expression at the cell membrane of xenografted human

tumor cells. To identify pre-clinical models expressing Notch3, immunohistochemistry (hereinafter "IHC") using an anti-Notch3 antibody was performed on a panel of xenograft models including: 37622A1 NSCLC (patient derived), HCC2429 lung cancer, MDA-MB-468 breast cancer and N87 gastric cancer xenograft models.

A tissue fragment from each xenograft was formalin-fixed and paraffin embedded (FFPE) using standard histological procedures. Five micron FFPE sections were cut, dewaxed and hydrated to distilled water. Antigens were retrieved in EDTA buffer pH 8.0 in a pressure cooker. Endogenous peroxidase was blocked with 3.0 % H_2O_2 for 10 minutes. Sections were incubated with DAKO Protein block for 20 minutes. A 1:2000 dilution of rabbit anti-Notch3 (D11B8; Cell Signaling Technologies) was applied to the sections for 1 hour at room temperature. Signalstain Boost anti-rabbit IgG-HRP polymer (Cell Signaling Technologies) was applied to the sections for 30 minutes at room temperature. DAB was used to develop color for 5 minutes. Sections were briefly counterstained in Mayer's hematoxylin, dehydrated, cleared and coverslipped. **Table 17** shows the staining intensity and staining distribution grade that were scored on a scale of 0-4, with 0 being negative and 4 being the highest intensity.

Table 17. Staining intensity and staining distribution grade.

Staining Intensity Grade	Staining Distribution Grade
0 = negative	0 = negative
1 = minimal	1 = 0-25%
2 = mild	2 = 26-50%
3 = moderate	3 = 51-75%
4 = marked	4 = 76-100%

As shown in **Table 18**, Notch3 protein was detected on the cell membrane (Mem) and in the cytoplasm (Cyto) and/or nucleus (Nuc) of cells from the 37622A1 NSCLC, HCC2429, MDA-MB-468 and N87 xenografts. The data demonstrates that both HCC2429 and N87 xenografts had a homogenous distribution of Notch3 protein at the cell membrane of 76-100% of cells. Further, the data demonstrates that 37622A1 NSCLC and MDA-MB-468 xenografts had a heterogenous distribution of Notch3 protein at the cell membrane in 51-75% of cells. Furthermore, the detection of the Notch3 C-

terminal intracellular domain in the nucleus of some epithelial tumor cells with the D11B8 antibody suggests that Notch3 signaling was active in these cells.

Table 18. Subcellular localization, intensity and distribution scores of Notch3 immunohistochemistry on a panel of xenograft models.

Xenograft	Tissue Type	Cell type	Subcellular Localization	Intensity Score (Scale 0-4)	Distribution Grade (Scale 0-4)
37622A1 NSCLC	Lung	Human epithelial	Cyto / Mem / Nuc	Cyto/Mem/Nuc=1-3	Cyto/Mem/Nuc =3
HCC2429	Lung	Human epithelial	Cyto / Mem / Nuc	Cyto=2-3 Mem=2-4 Nuc=1	Cyto/Mem=4 Nuc=2
MDA-MB-468	Breast	Human epithelial	Cyto / Mem / Nuc	Cyto = 3-4 Mem/Nuc = 2	Mem = 3 Cyto/Nuc = 4
N87	Gastric	Human epithelial	Cyto / Mem / Nuc	Cyto/Mem = 2-4 Nuc= 1-2	Cyto/Mem/Nuc = 4
OVCAR3	Ovarian	Human epithelial	Cyto / Mem / Nuc	Cyto/Nuc =1 Mem = 1-3	Cyto/Nuc = 2 Mem = 4

As shown in **FIG. 11**, Western blot analysis confirmed the Notch3 expression levels in the panel of xenografts that were used for in vivo efficacy studies. An ~210 kDa Notch3 protein fragment of the extracellular domain (hereinafter Notch3-ECD) was detected in HCC2429, MDA-MB-468, N87 and 37622A1 xenograft extracts using a mouse monoclonal anti-Notch3 antibody 1G5 (Abnova). Therefore, the data from the IHC using the anti-Notch3 antibody D11B8 demonstrated Notch3 at the cell membrane of human epithelial tumor cells and the Western blot analysis using the anti-Notch3 antibody 1G5 demonstrated expression of the Notch3-ECD which contains the NRR domain, the target of anti-Notch3 ADCs.

Example 16

In vivo Tumor Xenograft Models

Humanized anti-Notch3 antibodies, hu28 and hu75, and rat-human chimeric anti-Notch3 antibodies, ch28 and ch75, were conjugated to various linker-payload combinations and tested in 37622A1 non-small cell lung cancer (NSCLC), HCC2429 lung cancer, MDA-MB-468 breast cancer and N87 gastric cancer xenograft models. For each model described below the first dose was given on Day 0. The tumors were measured at least once a week and their volume was calculated with the formula: tumor volume (mm^3) = 0.5 x (tumor $width^2$)(tumor length). The mean tumor volumes (±

S.E.M.) for each treatment group were calculated having a maximum of 10 animals and a minimum of 6 animals to be included.

A. 37622A1 Patient Derived NSCLC Xenografts

The effects of anti-Notch3 ADCs were examined in immunodeficient mice on the in vivo growth of human tumor xenografts that were established from fragments of freshly resected 37622A1 NSCLC tumors obtained in accordance with appropriate consent procedures (Asterand). The 37622A1 NSCLC patient-derived xenografts were subcutaneously passaged in vivo as fragments from animal to animal in nude (Nu/Nu) female mice. When the tumors reached a volume of 150 to 300 mm^3, they were staged to ensure uniformity of the tumor size among various treatment groups. The 37622A1 NSCLC patient-derived xenografts model was dosed intravenously four times every four days (Q4dx4) with PBS vehicle, humanized anti-Notch3 ADCs, control huNeg-8.8 ADCs and cisplatin at the doses provided in **Table 19**. **FIG. 12** shows a graph of the data from **Table 19** of the ADCs with vc0101 linker-payloads at 3 mg/kg dose compared to Cisplatin (5 mg/kg) and PBS vehicle.

Cisplatin is a platinum-based anti-cancer agent used in the treatment of cancer and considered a standard-of-care therapy. Cisplatin cross-links DNA thereby inducing apoptosis and cell growth inhibition. The data demonstrates that anti-Notch3 ADCs hu28-vc0101, hu28-vc6780, hu75-vc0101 and hu75-vc6780 inhibited growth of 37622A1 NSCLC patient-derived xenograft tumors. The 3 mg/kg dose of hu28-vc0101 was the most potent ADC tested in this study, and by day 84, four out of nine animals still on study remained tumor-free. Further, the data shows that anti-Notch3 ADCs inhibited tumor growth more potently than control huNeg8.8-ADCs. Furthermore, the data shows that anti-Notch3 ADCs inhibited tumor growth more potently than cisplatin indicating a greater potency than a platinum-based standard-of-care chemotherapeutic drug (**FIG. 12**).

Table 19. Efficacy of anti-Notch3 ADCs in 37622A1 NSCLC xenografts.

	37622A1 NSCLC patient-derived xenografts, tumor volume (mm^3 ± SEM)							
	PBS	hu28-vc0101	hu28-vc6780	hu75-vc0101	hu75-vc6780	huNeg-8.8-vc0101	huNeg-8.8-vc6780	Cisplatin
Dose mg/kg	0	3	10	3	10	3	10	5
DAY -1	187 ±10	186 ±13	182 ±16	185 ±17	183 ±17	184 ±18	182 ±17	185 ±11
DAY 4	227 ±19	202 ±16	176 ±13	200 ±16	205 ±23	225 ±17	226 ±26	226 ±15
DAY 7	279 ±24	202 ±15	176 ±19	227 ±16	195 ±22	274 ±18	265 ±28	280 ±29
DAY 11	371 ±42	130 ±11	122 ±10	175 ±20	147 ±23	309 ±26	246 ±30	301 ±34
DAY 14	419 ±49	119 ±11	95 ±7	156 ±19	118 ±18	303 ±26	277 ±41	345 ±47
DAY 18	516 ±63	71 ±6	65 ±6	112 ±16	93 ±14	298 ±28	219 ±31	309 ±37
DAY 21	562 ±65	55 ±6	56 ±6	122 ±27	98 ±20	320 ±41	218 ±42	373 ±50
DAY 25	610 ±78	49 ±6	51 ±6	137 ±33	93 ±24	315 ±52	264 ±52	401 ±58
DAY 28	624 ±94	41 ±7	51 ±8	161 ±53	99 ±26	358 ±61	246 ±51	446 ±64
DAY 32	817 ±99	42 ±13	72 ±15	175 ±52	165 ±45	398 ±64	332 ±77	482 ±62
DAY 35	900 ±104	42 ±11	92 ±21	271 ±79	229 ±59	487 ±79	384 ±94	587 ±80
DAY 39	960 ±117	62 ±26	120 ±31	319 ±103	294 ±78	569 ±102	431 ±114	591 ±83
DAY 42	931 ±108	75 ±34	151 ±37	357 ±113	318 ±71	590 ±101	495 ±128	612 ±92
DAY 46	1037 102	92 ±44	172 ±47	431 ±137	412 ±106	743 ±133	610 ±165	723 ±119
DAY 49	1119 ±120	120 ±63	248 ±62	519 ±135	521 ±132	810 ±121	718 ±202	853 ±139
DAY 53	1345 ±158	144 ±67	339 ±93	678 ±195	629 ±162	989 ±146	848 ±251	970 ±193
DAY 56	1485 ±185	126 ±51	376 ±100	818 ±251	808 ±196	1149 ±191	776 ±184	1215 ±231
DAY 60	1691 ±220	180 ±85	503 ±138	710 ±162	917 ±209	1287 ±194	964 ±232	1428 ±273
DAY 63	1736 ±193	223 ±111	604 ±160	824 ±191	917 ±147	1503 ±227	1097 ±254	-
DAY 67	-	296 ±152	888 ±272	938 ±202	1116 ±173	1600 ±251	1167 ±260	-
DAY 70	-	312 ±162	773 ±235	953 ±209	1181 ±203	-	1352 ±305	-
DAY 74	-	331 ±160	881 ±264	-	-	-	-	-
DAY 77	-	422 ±210	1029 ±325	-	-	-	-	-
DAY 81	-	510 ±248	-	-	-	-	-	-
DAY 84	-	622 ±322	-	-	-	-	-	-

B. HCC2429 Lung Xenografts

Similar in vivo experiments were performed with the HCC2429 lung cancer cell line as described above. To generate xenografts, nude (Nu/Nu) female mice were implanted subcutaneously with $3.5x10^6$ HCC2429 cells in 50% Matrigel (BD Biosciences). When the tumors reached a volume of 200 to 400 mm^3, the tumors were staged to ensure uniformity of the tumor mass among various treatment groups. The HCC2429 lung model was dosed intravenously Q4dx4 with PBS vehicle, humanized anti-Notch3 ADCs and control huNeg-8.8 ADCs at the doses provided in **Tables 20 and 21.** The data demonstrates that anti-Notch3 ADCs hu28-vc0101, hu28-vc6780, hu75-vc0101 and hu75-vc6780 inhibited growth of HCC2429 lung xenografts in a dose-dependent manner. Further, the data shows that anti-Notch3 ADCs inhibited tumor growth more potently than control huNeg8.8-ADCs at the 1 and 3 mg/kg doses for anti-Notch3 ADCs with vc0101 linker-payloads and at the 3 and 10 mg/kg doses for anti-Notch3 ADCs with vc6780 linker-payloads. Furthermore, the data demonstrates that a 3mg/kg dose of hu28-vc0101 was more potent than a 10 mg/kg dose of hu28-vc6780.

Table 20. Efficacy of anti-Notch3-vc0101 ADCs in HCC2429 lung xenografts.

	HCC2429 Lung xenografts, tumor volume (mm^3 +/- SEM)									
	PBS	hu28-vc0101			hu75-vc0101			huNeg-8.8-vc0101		
Dose mg/kg	0	3	1	0.3	3	1	0.3	3	1	0.3
DAY -1	245 ±24	245 ±23	246 ±26	246 ±30	245 ±28	246 ±23	247 ±29	244 ±30	245 ±33	246 ±27
DAY 1	529 ±52	548 ±52	532 ±36	528 ±50	498 ±39	548 ±37	524 ±66	482 ±59	519 ±72	514 ±50
DAY 3	742 ±73	606 ±78	757 ±68	733 ±78	498 ±44	753 ±93	713 ±74	695 ±91	756 ±97	724 ±73
DAY 6	1205 ±120	723 ±101	1095 ±119	1112 ±132	469 ±70	1096 ±146	1078 ±74	1075 ±132	1144 ±100	1207 ±100
DAY 8	1720 ±181	696 ±100	1324 ±173	1617 ±172	407 ±71	1428 ±200	1499 ±115	1404 ±183	1598 ±133	1683 ±165
DAY 10	2312 ±197	620 ±90	1606 ±250	2027 ±233	370 ±81	1611 ±189	1830 ±120	1735 ±253	1974 ±185	2163 ±260
DAY 13	3235 ±120	543 ±92	1717 ±223	2642 ±297	273 ±69	1803 ±208	2408 ±226	2162 ±376	2676 ±346	2589 ±287
DAY 15	-	512 ±111	1865 ±263	-	298 ±88	1871 ±232	-	-	-	-
DAY 17	-	442 ±114	2228 ±333	-	250 ±77	1948 ±228	-	-	-	-
DAY 20	-	428 ±144	-	-	177 ±44	-	-	-	-	-

DAY 23	-	405 ±149	-	-	160 ±35	-	-	-	-	-
DAY 27	-	422 ±164	-	-	174 ±51	-	-	-	-	-
DAY 30	-	394 ±182	-	-	196 ±72	-	-	-	-	-
DAY 34	-	505 ±236	-	-	295 ±121	-	-	-	-	-
DAY 37	-	606 ±283	-	-	433 ±179	-	-	-	-	-
DAY 41	-	750 ±361	-	-	606 ±259	-	-	-	-	-
DAY 45	-	872 ±415	-	-	836 ±359	-	-	-	-	-
DAY 49	-	558 ±303	-	-	732 ±350	-	-	-	-	-
DAY 52	-	571 ±310	-	-	-	-	-	-	-	-
DAY 56	-	704 ±399	-	-	-	-	-	-	-	-

Table 21. Efficacy of anti-Notch3-vc6780 ADCs in HCC2429 lung xenografts.

	HCC2429 Lung xenografts, tumor volume (mm^3 ± SEM)									
	PBS	**hu28-vc6780**			**hu75-vc6780**			**huNeg-8.8-vc6780**		
Dose mg/kg	**0**	**10**	**3**	**1**	**10**	**3**	**1**	**10**	**3**	**1**
DAY -1	245 ±28	244 ±22	245 ±24	245 ±27	244 ±19	246 ±30	245 ±16	244 ±22	244 ±26	245 ±20
DAY 1	398 ±50	369 ±31	379 ±45	400 ±66	407 ±43	403 ±51	418 ±34	429 ±56	427 ±49	402 ±53
DAY 3	701 ±102	318 ±31	493 ±65	579 ±113	339 ±36	526 ±74	629 ±65	619 ±62	689 ±83	655 ±97
DAY 5	949 ±140	228 ±28	609 ±82	826 ±191	251 ±33	615 ±98	916 ±97	808 ±101	965 ±114	837 ±117
DAY 7	1345 ±200	172 ±22	638 ±86	1023 ±259	225 ±24	615 ±115	1164 ±131	1072 ±154	1380 ±136	1099 ±172
DAY 10	2045 ±356	143 ±22	784 ±115	1439 ±398	198 ±24	717 ±129	1705 ±184	1452 ±210	2082 ±192	1722 ±363
DAY 12	-	134 ±20	883 ±132	1442 ±487	166 ±22	807 ±130	2029 ±270	1673 ±290	2701 ±228	1586 ±337
DAY 14	-	115 ±16	895 ±175	-	150 ±22	831 ±145	2294 ±287	1809 ±314	-	-
DAY 17	-	127 ±18	1105 ±253	-	158 ±32	1017 ±178	-	-	-	-
DAY 20	-	149 ±27	1219 ±311	-	164 ±48	1297 ±231	-	-	-	-
DAY 24	-	206 ±60	1618 ±468	-	261 ±89	1813 ±343	-	-	-	-
DAY 27	-	290 ±100	-	-	316 ±135	1970 ±462	-	-	-	-
DAY 31	-	378 ±150	-	-	438 ±201	-	-	-	-	-
DAY 34	-	551 ±244	-	-	423 ±177	-	-	-	-	-
DAY 38	-	718 ±332	-	-	504 ±203	-	-	-	-	-
DAY 42	-	1011 ±504	-	-	655 ±266	-	-	-	-	-

DAY 46	-	-	-	-	793 ±320	-	-	-	-	-
DAY 49	-	-	-	-	901 ±351	-	-	-	-	-
DAY 53	-	-	-	-	1228 ±472	-	-	-	-	-

The HCC2429 lung model was also dosed intravenously Q4dx4 with PBS vehicle, rat-human chimeric anti-Notch3 ADCs and control huNeg-8.8 ADCs, at a dose of 5mg/kg as provided in **FIG. 16A.** The data demonstrates that anti-Notch3 ADCs with non-cleavable (mc) and cleavable (vc) linkers and various payload combinations inhibited growth of HCC2429 lung xenografts. Further, the data shows that rat-human chimeric anti-Notch3 ADCs inhibited tumor growth more potently than control huNeg8.8-ADCs. Furthermore, the data demonstrates that rat-human chimeric anti-Notch3 ADCs with vc0101 linker-payloads were more potent than the other anti-Notch3 ADCs tested.

Additional in vivo experiments were performed with the HCC2429 lung cancer cell line using an unconjugated rat-human chimeric anti-Notch3 antibody, ch75-hIgG1, to determine whether ch75-hIgG1's Notch3 signaling inhibition contributed to the observed potency of the anti-Notch3 hu75-ADCs. To generate xenografts, nude (Nu/Nu) female mice were implanted subcutaneously with $3.5x10^6$ HCC2429 cells in 50% Matrigel (BD Biosciences). When the tumors reached a volume of 75 to 200 mm^3, the tumors were staged to ensure uniformity of the tumor mass among various treatment groups.

The HCC2429 lung model was dosed intravenously Q4dx4 with PBS vehicle, rat-human chimeric anti-Notch3 antibody, ch75-hIgG1, and humanized anti-Notch1 antibody, hu438 VH1.1/VL1.8, at the does provided in **Table 22.** From 8 animals, mean tumor masses (± SEM) for each treatment group were calculated and compared to the control PBS vehicle group. P-values based on analysis of variance (ANOVA) were calculated to determine statistical significance of observed growth inhibition of anti-Notch treatments versus control PBS using Excel built-in statistical functions. Percent (%) growth inhibition values were calculated from measurements on the final day of the study for drug-treated compared with vehicle-treated mice with the formula $100*\{1-[(Treated_{Day\ 14} - Treated_{Day\ 0})/(Control_{Day\ 14} - Control_{Day\ 0})]\}$.

The data demonstrates that anti-Notch1 humanized antibody hu438 VH1.1/VL1.8 inhibited tumor growth by 57% and unconjugated rat-human chimeric anti-Notch3 antibody ch75-hIgG1 did not inhibit tumor growth compared to PBS vehicle treated tumors. Further, the data demonstrates that the observed tumor growth inhibition reported in **Tables 20, 21 and FIG. 16A** in the HCC2429 xenografts with Notch3 ADCs generated with the ch75 antibodies were not due to signaling inhibition.

Table 22. Effects of inhibitory anti-Notch3 and anti-Notch1 antibodies in HCC2429 lung xenografts.

	HCC2429 Lung xenografts, tumor volume (mm^3 ± SEM)				
	PBS control	**ch75-hIgG1 (anti-Notch3)**	***p*-value (ch75 ch75-hIgG1 vs. PBS)**	**hu438 VH1.1/VL1.8 (anti-Notch1)**	***p*-value (hu438 VH1.1/VL1.8 vs. PBS)**
DAY 0	110±7	110±5	0.54	112±11	0.504757
DAY 3	218±24	211±24	0.39	173±26	0.036706
DAY 5	397±41	356±41	0.22	254±36	0.001177
DAY 7	665±61	574±59	0.19	311±60	0.000001
DAY 10	1267±139	1054±104	0.19	518±92	0.000001
DAY 12	1615±187	1441±139	0.34	701±132	0.000006
DAY 14	1985±234	1821±175	0.42	909±172	0.000048

C. MDA-MB-468 Breast Xenografts

Similar in vivo experiments were performed with the MDA-MB-468 breast cancer cell line as described above. MDA-MB-468 cells are classified as a triple-negative breast cancer (TNBC) basal-like subtype since they lack expression of the estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2) (Lehmann, BD, et al, *J Clin Invest.* 2011;121(7):2750–2767). To generate xenografts, female SCID Hairless Outbred (SHO) mice were orthotopically implanted with $10x10^6$ MDA-MB-468 cells containing 50% Matrigel (BD Biosciences) in the mammary fat pad. When the tumors reached a volume of 250 to 450 mm^3, the tumors were staged to ensure uniformity of the tumor mass among various treatment groups. The MDA-MB-468 breast model was dosed intravenously Q4dx4 with PBS vehicle, humanized anti-Notch3 ADCs and control huNeg-8.8 ADCs at the doses provided in **Tables 23 and 24. FIG. 13A** and **13B** show graphs of the data from **Table 23** of anti-Notch3 ADCs with vc0101 linker-payloads compared to control huNeg-8.8 ADCs and PBS vehicle. **FIG.**

14A and **14B** show graphs of the data from **Table 24** of the anti-Notch3 ADCs with vc6780 linker-payloads compared to control huNeg-8.8 ADC and PBS vehicle.

The data demonstrates that anti-Notch3 ADCs hu28-vc0101, hu28-vc6780, hu75-vc0101 and hu75-vc6780 inhibited growth of MDA-MB-468 breast xenografts in a dose-dependent manner. Further, the data shows that anti-Notch3 ADCs inhibited tumor growth more potently than control huNeg8.8-ADCs at the 1 and 3 mg/kg doses for ADCs with vc0101 linker-payloads and 1, 3 and 10 mg/kg doses for ADC with vc6780 linker-payloads. Furthermore, the data demonstrates that a 1 mg/kg dose of anti-Notch3 ADCs with vc0101 linker-payloads were more potent than a 3 mg/kg dose of anti-Notch3 ADCs with vc6780 linker-payloads.

Table 23. Efficacy of anti-Notch3-vc0101 ADCs in MDA-MB-468 breast xenografts.

	MDA-MB-468 Breast xenografts, tumor volume (mm^3 ± SEM)									
	PBS	hu28-vc0101			hu75-vc0101			huNeg-8.8-vc0101		
Dose mg/kg	0	3	1	0.3	3	1	0.3	3	1	0.3
DAY 0	343 ±12	347 ±15	348 ±22	336 ±19	347 ±20	347 ±22	348 ±21	334 ±23	346 ±16	344 ±19
DAY 4	441 ±24	359 ±24	439 ±21	403 ±28	444 ±28	410 ±32	439 ±31	424 ±29	447 ±32	442 ±19
DAY 7	469 ±32	326 ±27	415 ±25	395 ±38	338 ±20	383 ±33	435 ±27	411 ±26	449 ±20	438 ±23
DAY 11	495 ±28	227 ±27	372 ±34	412 ±42	277 ±22	373 ±33	504 ±38	439 ±36	538 ±23	496 ±37
DAY 14	581 ±35	147 ±20	314 ±27	488 ±45	181 ±19	350 ±40	507 ±30	445 ±29	592 ±47	560 ±36
DAY 18	639 ±43	77 ±10	261 ±33	497 ±55	90 ±12	296 ±33	587 ±44	479 ±42	619 ±42	578 ±36
DAY 21	638 ±46	16 ±8	219 ±41	509 ±60	60 ±9	260 ±49	590 ±55	481 ±34	676 ±46	627 ±30
DAY 26	707 ±41	0 ±0	253 ±61	590 ±66	16 ±10	267 ±59	652 ±64	548 ±41	793 ±54	671 ±56
DAY 29	749 ±59	0 ±0	238 ±64	-	8 ±8	261 ±62	675 ±63	-	819 ±73	669 ±37
DAY 32	812 ±80	0 ±0	266 ±67	-	7 ±7	264 ±67	738 ±70	-	913 ±72	758 ±44
DAY 35	891 ±79	0 ±0	271 ±73	-	0 ±0	326 ±86	821 ±69	-	1023 ±96	848 ±58
DAY 39	892 ±84	0 ±0	310 ±88	-	0 ±0	324 ±81	864 ±74	-	-	884 ±64
DAY 42	1037 ±104	0 ±0	349 ±95	-	0 ±0	381 ±94	997 ±84	-	-	1002 ±55
DAY 47	1173 ±134	0 ±0	394 ±123	-	0 ±0	442 ±69	-	-	-	1145 ±78
DAY 50	-	0 ±0	377 ±118	-	0 ±0	484 ±89	-	-	-	1120 ±67
DAY 53	-	0 ±0	414 ±127	-	0 ±0	452 ±78	-	-	-	1229 ±100

DAY 56	-	0 ±0	470 ±128	-	0 ±0	535 ±93	-	-	-	1314 ±120
DAY 60	-	0 ±0	532 ±140	-	0 ±0	603 ±98	-	-	-	-
DAY 63	-	0 ±0	509 ±117	-	0 ±0	-	-	-	-	-
DAY 67	-	0 ±0	611 ±148	-	0 ±0	-	-	-	-	-
DAY 70	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 76	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 83	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 90	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 97	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 103	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 110	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 118	-	0 ±0	-	-	0 ±0	-	-	-	-	-
DAY 125	-	0 ±0	-	-	0 ±0	-	-	-	-	-

Table 24. Efficacy of anti-Notch3-vc6780 ADCs in MDA-MB-468 breast xenografts.

		MDA-MB-468 Breast xenografts, tumor volume (mm^3 ± SEM)								
	PBS	**hu28-vc6780**			**hu75-vc6780**			**huNeg-8.8-vc6780**		
Dose mg/kg	**0**	**10**	**3**	**1**	**10**	**3**	**1**	**10**	**3**	**1**
DAY 0	342 ±9	335 ±9	342 ±18	342 ±16	343 ±10	344 ±11	340 ±14	339 ±18	341 ±12	346 ±16
DAY 4	466 ±20	395 ±19	394 ±33	462 ±22	418 ±15	406 ±22	423 ±27	432 ±45	457 ±23	466 ±29
DAY 7	481 ±17	350 ±19	399 ±24	452 ±30	370 ±18	378 ±21	434 ±29	449 ±45	529 ±24	528 ±25
DAY 11	611 ±44	248 ±26	380 ±25	512 ±35	302 ±21	403 ±21	471 ±39	504 ±38	599 ±23	621 ±43
DAY 14	610 ±19	154 ±23	401 ±30	507 ±38	228 ±19	370 ±28	470 ±44	503 ±64	622 ±31	639 ±48
DAY 19	707 ±34	65 ±17	438 ±39	538 ±47	112 ±23	339 ±19	536 ±49	437 ±54	697 ±36	713 ±48
DAY 22	-	25 ±16	414 ±41	551 ±48	52 ±21	360 ±17	552 ±44	415 ±54	719 ±40	-
DAY 25	-	26 ±19	491 ±37	575 ±55	63 ±25	381 ±23	597 ±48	421 ±76	773 ±39	-
DAY 28	-	15 ±15	497 ±68	654 ±74	64 ±26	443 ±33	660 ±53	451 ±84	808 ±58	-
DAY 32	-	0 ±0	524 ±69	653 ±82	71 ±31	437 ±28	634 ±74	456 ±94	-	-
DAY 35	-	0 ±0	-	734 ±89	85 ±38	495 ±33	742 ±80	541 ±108	-	-
DAY 40	-	0 ±0	-	761 ±99	125 ±44	535 ±41	794 ±87	563 ±109	-	-
DAY 43	-	0 ±0	-	816 ±122	134 ±42	619 ±47	832 ±82	581 ±120	-	-

DAY 46	-	0 ±0	-	859 ±126	143 ±42	636 ±38	868 ±99	617 ±116	-	-
DAY 49	-	0 ±0	-	948 ±178	159 ±44	723 ±71	996 ±109	733 ±129	-	-
DAY 53	-	0 ±0	-	1008 ±192	201 ±63	795 ±67	-	758 ±163	-	-
DAY 56	-	0 ±0	-	-	211 ±63	819 ±77	-	-	-	-
DAY 60	-	0 ±0	-	-	240 ±63	976 ±115	-	-	-	-
DAY 63	-	0 ±0	-	-	201 ±57	-	-	-	-	-
DAY 67	-	0 ±0	-	-	-	-	-	-	-	-
DAY 70	-	0 ±0	-	-	-	-	-	-	-	-
DAY 76	-	0 ±0	-	-	-	-	-	-	-	-
DAY 83	-	0 ±0	-	-	-	-	-	-	-	-
DAY 90	-	0 ±0	-	-	-	-	-	-	-	-
DAY 96	-	0 ±0	-	-	-	-	-	-	-	-
DAY 103	-	0 ±0	-	-	-	-	-	-	-	-
DAY 111	-	0 ±0	-	-	-	-	-	-	-	-
DAY 118	-	0 ±0	-	-	-	-	-	-	-	-
DAY 124	-	0 ±0	-	-	-	-	-	-	-	-

The MDA-MB-468 breast cancer model was also dosed intravenously Q4dx4 with PBS vehicle, rat-human chimeric anti-Notch3 ADCs and control huNeg-8.8 ADCs, at a dose of 5mg/kg as provided in **FIGS. 16B and 16C.** The data demonstrates that rat-human chimeric anti-Notch3 ADCs with non-cleavable (mc) and cleavable (vc) linkers and various payload combinations inhibited growth of MDA-MB-468 breast xenografts. Further, the data shows that rat-human chimeric anti-Notch3 ADCs inhibited tumor growth more potently than control huNeg8.8-ADCs. Furthermore, the data demonstrates that rat-human chimeric anti-Notch3 ADCs with vc0101 linker-payloads were more potent than the other rat-human chimeric anti-Notch3 ADCs tested.

The MDA-MB-468 breast cancer model was used to examine the in vivo mechanism of action of hu28-vc0101. The pharmacodynamics of hu28-vc0101 was visualized at the cellular level by staining with the mitotic marker phospho-Histone H3 in ADC treated xenografts. Histone H3 is phosphorylated on Ser-10 residues (hereinafter "pHH3") within chromatin during the mitotic phase of the cell cycle.

Mice bearing MDA-MB-468 breast xenografts were given a single 3 mg/kg dose intravenously with anti-Notch3 ADC hu28-vc0101, huNeg-8.8-vc0101 (control ADC) or PBS. Three xenografts were harvested after 6, 24 and 96 hours and processed for standard immunohistochemistry. Five micrometer thick formalin fixed, paraffin embedded tissue sections were deparaffinized in xylene substitute, rehydrated with graded alcohols to distilled water. To expose antigenic sites, tissue sections were heated in 10mM citrate buffer pH 6.0 (Labvision) in a pressure cooker (Retriever; Electron Microscopy Sciences) and cooled to room temperature. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide for 15 min. Non specific protein interactions were blocked by a 10 minute incubation with UV Block (Labvision). Tissue sections were incubated with anti-pHH3 antibody for one hour, detected with Signalstain Boost Reagent (8114, Cell Signaling Technologies) for 30 minutes and color was developed with DAB+ (DAKO) for 5 minutes. All sections were counterstained with Hematoxylin QS (Vector Laboratories), washed in tap water, dehydrated in graded alcohols, cleared in xylene substitute, and mounted with Permount Mounting Medium (FisherChemicals, Fair Lawn, NJ).

Immunohistochemically stained slides were evaluated by image analysis. Slides were imaged at 20X using a Hamamatsu NanoZoomer automated slide scanner. Once digitized, the virtual slides were analyzed using Definiens Tissue Studio software. Each xenograft section was regionally segmented and classified based on cellularity and morphology. Individual nuclei were identified within the viable regions of the xenograft and the positivity was determined using the average brown chromogen intensity of each nuclei. Data is presented as percent (%) pHH3 positivity which is calculated using the following equation: (Number of pHH3 positive nuclei (viable region)/Total number of nuclei (viable region))*100. Determination of statistical significance was determined for each treatment group and time point compared to PBS control at 24 hour time point with Graph Pad Prism using a 2 tailed T test.

ADCs generated with microtubule inhibitors like hu28-vc0101 are expected to arrest proliferating cells in the mitotic phase of the cell cycle when histone H3 is phosphorylated on Ser-10 residues within chromatin. An accumulation of pHH3 staining in ADC treated xenografts indicates that cancer cells were arrested in mitosis. **Table 25**

contains percentages of pHH3 stained cells in anti-Notch3 hu28-vc0101 and huNeg-8.8-vc0101 (control ADC) treated MDA-MB-468 xenografts and untreated MDA-MB-468 xenografts. The data demonstrates that hu28-vc0101, but not control huNeg8.8-vc0101 resulted in a statistically significant 2 fold increase in the percentage of pHH3 stained nuclei in MDA-MD-468 cancer cells at 24 and 96 hours post-treatment compared to the PBS control. The data indicates that anti-Notch3 hu28-vc0101 inhibits in vivo tumor growth, at least in part, by arresting cells in the mitotic phase of the cell cycle, thus preventing proliferation.

Table 25. Percentage of pHH3 stained nuclei in Notch3 ADC treated MDA-MB-468 breast cancer xenografts (n.d.=not determined)

	6 hr		24 hr		96 hr	
	% pHH3	**p-value**	**% pHH3**	**p-value**	**% pHH3**	**p-value**
PBS	n.d	n.d.	2.62	n.d.	n.d.	n.d.
hu28-vc0101	2.54	0.84	5.37	0.0046	5.19	0.0003
huNeg8.8-vc0101	2.74	0.78	3.67	0.1025	3.01	0.0730

D. N87 Gastric Xenografts

Similar in vivo experiments were performed with the N87 gastric cancer cell line as described above. To generate xenografts, nude (Nu/Nu) female mice were implanted subcutaneously with 8 $x10^6$ N87 cells in 50% Matrigel (BD Biosciences). When the tumors reached a volume of 250 to 450 mm^3, the tumors were staged to ensure uniformity of the tumor mass among various treatment groups. The N87 gastric model was dosed intravenously Q4dx4 with PBS vehicle, humanized anti-Notch3 ADCs, control huNeg-8.8 ADCs and cisplatin at the doses provided in **Tables 26 and 27.** The data demonstrates that anti-Notch3 ADCs hu28-vc0101, hu28-vc6780, hu75-vc0101 and hu75-vc6780 inhibited growth of N87 gastric xenografts in a dose-dependent manner. Further, the data shows that anti-Notch3 ADCs inhibited tumor growth more potently than control huNeg8.8-ADCs at the 1, 3, 5 mg/kg doses for ADCs with vc0101 linker-payloads and 3 and 10 mg/kg doses for ADCs with vc6780 linker-payloads. By day 133, the 5 mg/kg dose group of hu28-vc0101 contained 6 out of 9 animals that were tumor-free, hu75-vc0101 had 4 out of 9 animals and control huNeg8-8-vc0101 had 1 out

of 8 animals that were tumor-free. Furthermore, the data demonstrates that ADCs with vc0101 linker-payloads were in general more potent than cisplatin standard-of-care therapy and ADCs with vc6780 linker-payloads.

Table 26. Efficacy of anti-Notch3-vc0101 ADCs in N87 gastric xenografts.

	N87 Gastric xenografts, tumor volume (mm^3 ± SEM)										
	PBS	hu28-vc0101			hu75-vc0101			huNeg-8.8-vc0101			Cisplatin
Dose mg/kg	0	1	3	5	1	3	5	1	3	5	5
DAY 0	327 ±11	321 ±21	326 ±13	321 ±8	321 ±9	324 ±19	320 ±11	327 ±18	324 ±11	321 ±16	328 ±20
DAY 4	526 ±19	369 ±18	339 ±11	344 ±14	392 ±15	362 ±35	315 ±15	437 ±34	478 ±19	423 ±32	414 ±27
DAY 7	706 ±27	429 ±43	302 ±10	272 ±7	417 ±25	303 ±21	246 ±12	584 ±54	625 ±34	512 ±34	520 ±26
DAY 11	854 ±36	304 ±30	182 ±14	152 ±13	331 ±21	174 ±14	156 ±10	702 ±60	716 ±53	501 ±38	501 ±29
DAY 14	887 ±45	282 ±25	191 ±5	155 ±13	305 ±17	172 ±10	151 ±7	822 ±65	823 ±42	549 ±37	637 ±31
DAY 18	1045 ±68	263 ±24	161 ±7	138 ±11	267 ±17	151 ±10	128 ±6	823 ±73	789 ±33	491 ±51	-
DAY 21	1072 ±76	227 ±23	123 ±15	110 ±9	218 ±23	130 ±5	115 ±7	857 ±78	785 ±35	413 ±50	-
DAY 26	1303 ±140	205 ±32	108 ±16	69 ±16	185 ±24	92 ±14	82 ±10	895 ±126	825 ±62	343 ±63	-
DAY 29	1276 ±139	180 ±30	99 ±14	50 ±13	211 ±37	104 ±16	75 ±12	957 ±126	879 ±72	411 ±89	-
DAY 33	1480 ±183	211 ±43	106 ±17	43 ±14	251 ±53	91 ±18	73 ±12	988 ±180	966 ±98	411 ±89	-
DAY 36	-	215 ±42	122 ±22	52 ±16	272 ±59	86 ±18	85 ±9	884 ±143	1023 ±106	481 ±86	-
DAY 39	-	261 ±54	128 ±23	45 ±14	304 ±72	59 ±16	72 ±13	937 ±167	1142 ±121	535 ±128	-
DAY 42	-	283 ±52	149 ±25	34 ±15	314 ±73	81 ±22	74 ±13	1008 ±179	1240 ±143	596 ±119	-
DAY 47	-	262 ±64	105 ±19	25 ±14	334 ±95	80 ±25	36 ±8	1061 ±210	1380 ±153	621 ±137	-
DAY 53	-	302 ±75	104 ±30	29 ±16	393 ±115	86 ±24	69 ±13	-	-	757 ±189	-
DAY 62	-	415 ±111	116 ±47	33 ±18	463 ±155	106 ±35	50 ±15	-	-	690 ±122	-
DAY 70	-	521 ±135	139 ±54	58 ±30	658 ±241	148 ±54	76 ±22	-	-	852 ±150	-
DAY 78	-	622 ±169	172 ±69	67 ±40	531 ±152	161 ±60	90 ±25	-	-	937 ±168	-
DAY 84	-	709 ±200	149 ±73	85 ±49	602 ±185	200 ±76	96 ±37	-	-	1178 ±222	-
DAY 91	-	848 ±236	178 ±94	104 ±63	732 ±246	225 ±92	109 ±46	-	-	-	-
DAY 99	-	-	214 ±108	118 ±72	-	170 ±75	130 ±53	-	-	-	-
DAY 103	-	-	123 ±43	135 ±90	-	184 ±82	142 62	-	-	-	-
DAY 112	-	-	126 ±50	166 ±105	-	222 ±108	174 ±75	-	-	-	-

DAY 120	-	-	128 ±44	226 ±140	-	240 ±116	221 ±103	-	-	-	-
DAY 133	-	-	175 ±74	258 ±157	-	-	295 ±139	-	-	-	-

Table 27. Efficacy of anti-Notch3-vc6780 ADCs in N87 gastric xenografts.

	N87 Gastric xenografts, tumor volume (mm^3 ± SEM)								
	PBS	**hu28-vc6780**			**hu75-vc6780**			**huNeg8.8-vc6780**	
Dose mg/kg	**0**	**10**	**3**	**1**	**10**	**3**	**1**	**10**	**3**
DAY 0	345 ±14	350 ±14	349 ±10	348 ±13	349 ±8	351 ±20	357 ±14	356 ±20	344 ±14
DAY 4	600 ±16	434 ±24	552 ±24	560 ±26	468 ±18	545 ±37	546 ±35	581 ±60	537 ±36
DAY 8	675 ±20	379 ±12	545 ±37	592 ±44	351 ±24	511 ±31	563 ±55	605 ±67	670 ±45
DAY 11	763 ±54	315 ±18	511 ±25	617 ±48	316 ±25	544 ±43	582 ±56	636 ±79	706 ±38
DAY 14	886 ±72	292 ±24	564 ±29	782 ±60	269 ±27	558 ±36	667 ±68	717 ±118	917 ±36
DAY 18	997 ±93	199 ±18	479 ±29	797 ±88	224 ±26	494 ±41	638 ±80	665 ±112	958 ±57
DAY 21	1041 ±107	194 ±20	499 ±34	839 ±93	192 ±19	534 ±41	709 ±103	637 ±119	1002 ±59
DAY 25	1151 ±144	181 ±21	588 ±40	878 ±105	227 ±32	628 ±58	750 ±122	647 ±134	1075 ±82
DAY 28	1200 ±155	204 ±16	672 ±48	904 ±123	244 ±35	645 ±57	763 ±145	674 ±146	1148 ±77
DAY 33	1481 ±206	196 ±27	786 ±65	1043 ±152	267 ±52	730 ±66	991 ±239	733 ±195	1290 ±128
DAY 36	-	189 ±37	827 ±69	1108 ±185	300 ±64	850 ±74	-	817 ±222	1265 ±111
DAY 39	-	228 ±44	847 ±77	1204 ±209	323 ±69	881 ±88	-	880 ±247	1429 ±121
DAY 42	-	257 ±60	959 ±81	-	350 ±78	1020 ±99	-	797 ±244	-
DAY 46	-	253 ±59	1018 ±94	-	380 ±78	1097 ±129	-	874 ±267	-
DAY 50	-	253 ±67	1111 ±95	-	415 ±77	1162 ±134	-	-	-
DAY 56	-	298 ±85	1279 ±108	-	504 ±111	1331 ±187	-	-	-
DAY 63	-	345 ±93	1368 ±133	-	581 ±121	-	-	-	-
DAY 70	-	376 ±117	1483 ±154	-	726 ±163	-	-	-	-
DAY 77	-	388 ±123	-	-	797 ±184	-	-	-	-
DAY 84		463 ±149			995 ±239				
DAY 91		523 ±171							
DAY 98		701 ±251							

The N87 gastric model was also dosed intravenously Q4dx4 with PBS vehicle, rat-human chimeric anti-Notch3 ADCs and control huNeg-8.8 ADCs, at a dose of 5mg/kg as provided in **FIG. 16D.** The data demonstrates that rat-human chimeric anti-Notch3 ADCs with non-cleavable (mc) and cleavable (vc) linkers and various payload combinations inhibited growth of N87 gastric xenografts. Further, the data shows that rat-human chimeric anti-Notch3 ADCs inhibited tumor growth more potently than control huNeg8.8-ADCs. Furthermore, the data demonstrates that rat-human chimeric anti-Notch3 ADCs with vc0101 linker-payloads were more potent than the other anti-Notch3 ADCs tested.

The N87 gastric model was also dosed intravenously Q4dx4 with PBS vehicle and rat-human chimeric anti-Notch3 ADCs ch28-mc0131, ch75-mc0131, ch28-m(H2O)c-0131 and ch75-m(H2O)c-0131 at a dose of 5mg/kg as provided in **FIG 16E.** The data demonstrates that rat-human chimeric anti-Notch3 ADCs having mc0131 and m(H2O)c-0131 linker-payloads inhibited growth of N87 gastric xenografts. Further, the data demonstrates that rat-human chimeric anti-Notch3 ADCs having m(H2O)c-0131 linker-payloads were more potent than rat-human chimeric anti-Notch3 ADCs having mc0131 linker-payloads.

E. OVCAR3 Ovarian Xenografts

Similar in vivo experiments were performed with the OVCAR3 ovarian cell line as described above. To generate xenografts, SCID Hairless Outbred (SHO) female mice were implanted subcutaneously with 5 $x10^6$ OVCAR3 cells in 50% Matrigel (BD Biosciences). When the tumors reached a volume of 120 to 290 mm^3, the tumors were staged to ensure uniformity of the tumor mass among various treatment groups. The OVCAR3 model was dosed intravenously Q4dx4 with PBS vehicle, humanized anti-Notch3 ADC hu28-vc0101, and control ADC huNeg-8.8-vc0101; and dosed intraperitoneally Q7dx4 with paclitaxel (30 mg/kg twice a day) and Q7dx8 with carboplatin at the doses provided in **Table 28**. **FIG. 15** and **Table 28** demonstrate that hu28-vc0101 inhibited growth of OVCAR3 ovarian xenografts in a dose-dependent manner. Further, the data shows that hu28-vc0101 inhibited tumor growth more potently than control huNeg8.8-vc0101 and carboplatin standard of care chemotherapy.

Furthermore, the data demonstrates that hu28-vc0101 dosed at 3 mg/kg Q4dx4 had similar potency to paclitaxel dosed at 60 mg/kg,Q7dx4 which is the maximum tolerated dose of paclitaxel in SHO mice.

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Table 28. Efficacy of anti-Notch3 ADC hu28-vc0101 in OVCAR3 ovarian xenografts.

		OVCAR3 Ovarian Xenografts, tumor volume (mm3 ± S,E.M.)						
	PBS	hu28-vc0101			huNeg8.8-vc0101		Carboplatin	Paclitaxel
Dose mg/kg	0	0.3	1	3	1	3	20	60
DAY 0	170 ±14	168 ±18	169 ±20	169 ±18	171 ±21	168 ±14	167 ±13	169 ±13
DAY 3	255 ±18	291 ±29	255 ±31	253 ±35	261 ±36	286 ±18	242 ±20	225 ±29
DAY 6	277 ±25	302 ±35	226 ±33	180 ±19	267 ±38	287 ±30	246 ±17	139 ±13
DAY 11	378 ±18	394 ±40	216 ±35	113 ±9	369 ±68	329 ±27	311 ±25	85 ±8
DAY 14	501 ±33	510 ±50	242 ±39	104 ±9	467 ±75	437 ±23	426 ±27	84 ±7
DAY 18	578 ±43	601 ±57	212 ±38	72 ±9	516 ±101	431 ±26	473 ±29	61 ±5
DAY 21	693 ±48	685 ±83	190 ±38	57 ±6	587 ±123	407 ±26	475 ±26	50 ±4
DAY 25	806 ±59	767 ±84	216 ±52	49 ±5	638 ±131	447 ±27	552 ±25	41 ±2
DAY 28	912 ±71	841 ±112	282 ±69	47 ±6	772 ±171	518 ±35	640 ±26	44 ±4
DAY 31	970 ±68	926 ±118	292 ±74	48 ±4	854 ±176	564 ±48	692 ±28	43 ±3
DAY 34	1105 ±83	1038 ±148	350 ±95	48 ±6	1002 ±216	665 ±41	811 ±44	50 ±3
DAY 38	1166 ±83	1191 ±156	497 ±124	49 ±4	903 ±88	751 ±56	869 ±44	55 ±6
DAY 41	1285 ±89	1254 ±188	559 ±137	40 ±6	979 ±95	786 ±56	906 ±44	38 ±3
DAY 45	1616 ±126	1486 ±203	750 ±160	39 ±3	1109 ±115	904 ±67	1009 ±62	39 ±3
DAY 49	1836 ±142	1479 ±236	781 ±178	33 ±4	1289 ±129	994 ±95	1149 ±52	40 ±4
DAY 53			934 ±200	34 ±3	1486 ±138	1127 ±129	1290 ±65	40 ±5
DAY 56			991 ±201	30 ±2	1481 ±131	1224 ±141	1370 ±70	39 ±4
DAY 59			1149 ±228	33 ±4	1739 ±182	1325 ±163	1514 ±81	37 ±3
DAY 63			1421 ±328	35 ±3		1510 ±258	1783 ±128	42 ±4
DAY 67				32 ±3			1844 ±112	33 ±2
DAY 70				33 ±5				28 ±2
DAY 74				41 ±8				32 ±2

DAY 77				50 ±10				31 ±3
DAY 80				56 ±15				31 ±2
DAY 83				63 ±16				38 ±4
DAY 87				87 ±26				28 ±1
DAY 90				84 ±26				32 ±2
DAY 94				137 ±49				30 ±2
DAY 97				129 ±47				31 ±3
DAY 102				153 ±55				26 ±2
DAY 105				176 ±66				31 ±2
DAY 108				232 ±93				28 ±3
DAY 111				271 ±102				26 ±2
DAY 115				323 ±134				31 ±3
DAY 118				318 ±137				26 ±4
DAY 122				399 ±176				31 ±4

Example 17

Cysteine Mutant Generation for Site-Specific Conjugation

Anti-Notch3 ADCs described in the previous **Examples** were generated through conventional, non-specific conjugation of linker-payloads to cysteine amino acid residues of the target antibody. Site-specific conjugation of linker-payloads to antibodies was conducted to facilitate homogeneous drug loading and avoid ADC subpopulations with potentially altered antigen-binding or pharmacokinetics properties, which may be observed in some ADCs generated by conventional conjugation methods. One such site-specific conjugation strategy has been designed to introduce cysteine residues at specific sites in the amino acid sequence of the target antibody. A number of amino acid positions in the constant heavy chain and constant light chain were identified, as described in International Publication No. WO/2013/093809, and substituted with a cysteine residue at the specific amino acid position in the hu28 antibody.

Site-specific cysteine substitutions were engineered into the Fc polypeptide or human IgG1 heavy chain constant domain (Cγ) at positions L443 and K392 (herein L443C and K392C). Site-specific cysteine substitutions were engineered into the human kappa (κ) light chain constant domain (Cκ) polypeptide at position K183 (herein κK183C). CDRs of cysteine mutants remain the same as parental wildtype hu28. **FIGS 17A** and **17B** provide the amino acid and nucleotide sequences of the hu28 antibody with the site-specific cysteine substitutions described. Cysteine substitutions in the hu28 antibody resulted in the generation of the following antibodies: hu28-(L443C), hu28-(L443C/K392C) and hu28-(L443C/κK183C). All cysteine mutations were constructed by site-directed mutagenesis, as described in International Publication No. WO/2013/093809. Expression constructs for these mutant antibodies were generated in the site-specific integration (SSI) expression vector (Lonza) for stable expression in CHO cells. Stable pools of transfected CHO cells expressing the cysteine mutant antibodies were established and conditioned media generated. Standard Protein A affinity purification followed by size exclusion chromatography were employed to purify the anti-Notch3 cysteine mutant antibodies from conditioned media, as described in previous Examples.

Example 18
Characterization of Cysteine Mutants

Unconjugated cysteine mutant humanized anti-Notch3 antibodies were screened for cell surface binding activity to Notch3 expressing cell lines in a cell-based ELISA, as described in previous Examples. **Table 29** shows EC_{50} (nM) values calculated from cell surface Notch3 binding ELISAs for unconjugated cysteine mutants, hu28-(L443C), hu28-(L443/K382) and hu28-(L443/κK183C). The data demonstrates that the cysteine mutant antibodies have EC_{50} values that are similar to the parental wildtype hu28 antibody in binding to full-length human Notch3 expressed on the cell surface of U2OS/hNotch3, HCC2429 and OVCAR3 cells. Further, the data demonstrates that substitution of cysteine amino acids into the constant regions of the heavy chain or both

the heavy and light chains of the humanized hu28 antibody did not affect or alter their binding characteristics. The negative control huNeg8.8 antibody did not bind any of the cell lines tested. For the control antibody that lacked binding (LB), EC_{50} values were not generated as indicated.

Table 29. EC_{50} (nM) values for unconjugated cysteine mutant anti-Notch3 antibodies

Antibody	EC_{50} (nM Ab/ml)			
	U2OS/hNotch3		HCC2429	OVCAR3
hu28 VH1.0/VL1.0	0.3	0.26	0.10	0.46
hu28-(L443C)	0.28	0.30	0.08	0.20
hu28-(L443C/κK183C)	0.38	0.27	0.11	0.20
hu28-(L443C/K392C)	0.31	0.29	0.11	0.31
huNeg8.8	LB	-	LB	LB

Example 19

In vitro Cytotoxicity Assay of Cysteine Mutant ADCs

The conjugation of maleimide functionalized linker-payloads to the anti-Notch3 hu28 cysteine mutant antibodies was achieved via complete reduction of the antibodies with 100-fold molar excess of tris(2-carboxyethyl)phosphine (TCEP) in 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer), pH 7.0 and 1 mM diethylenetriaminepentaacetic acid (DTPA) for 2 hours at 37 °C followed by desalting to remove excess TCEP. The reduced antibodies were incubated in 2mM dehydro ascorbic acid (DHA), 100 mM HEPES, pH 7.0 and 1mM DTPA for 16 hours at 4 °C to reform the inter-chain disulfide bonds. After desalting, the vc0101 linker-payload was added to the reaction mixture at a linker-payload/antibody molar ratio of about 7 and reacted for an additional 1 hour at 25°C in the presence of 15% v/v of dimethylacetamide (DMA). After the 1 hour incubation period, 6-fold excess L-Cys was added to quench any unreacted linker-payload. The reaction mixture was dialyzed overnight at 4°C in phosphate buffered saline (PBS), pH 7.4, and purified via SEC (AKTA explorer, Superdex 200). The ADC was further characterized via SEC for purity, hydrophobic interaction chromatography (HIC), and liquid chromatography electrospray

ionization tandem mass spectrometry (LC-ESI MS) to calculate drug-antibody ratio (loading). The protein concentration was determined via UV spectrophotometer. Additional conjugation techniques are described in International Publication No. WO/2013/093809.

This process generated the anti-Notch3 cysteine mutant ADCs: hu28-(L443C)-vc0101 and hu28-(L443C/κK183C)-vc0101. The activity of the anti-Notch3 cysteine mutant ADCs were assessed on cell lines endogenously expressing Notch3 protein: HCC2429 (lung cancer) and OVCAR3 (ovarian cancer) using an MTS cellular viability indicator as described in previous Examples.

Table 30 shows IC_{50} (ng Ab/mL) values of the anti-Notch3 cysteine mutant ADC treatments. The data demonstrate that hu28-(L443C /κK183C)-vc0101 with a DAR=3.9 was active and had similar potency to the wildtype hu28-vc0101 ADC with DAR=3.9, and both induced cell death in the Notch3 expressing cancer cell lines HCC2429 and OVCAR3. The data further demonstrates that the single cysteine mutant hu28-(L443)-vc0101 with a DAR=2 and the non-targeted control huNeg8.8 ADC with DAR=4 were minimally active at the highest doses tested.

Table 30. IC_{50} (ng Ab/mL) values of cysteine mutant humanized anti-Notch3 ADCs.

ADC	DAR	IC_{50} (ng Ab/ml)					
		HCC2429			OVCAR3		
hu28-vc0101	3.9	124	463	145	404	1450	1173
hu28-(L443C)-vc0101	2	20308	16879	14324	13129	15840	12189
hu28-(L443C /κK183C)-vc0101	3.9	219	352	10	3020	3673	1250
huNeg8.8-vc0101	4.0	18850	13944	18302	9944	9826	7564

Example 20

In vivo Efficacy of Cysteine Mutant ADCs

Similar in vivo experiments were performed with the anti-Notch3 cysteine mutant ADCs using the OVCAR3 ovarian cell line as described in previous Examples. The OVCAR3 model was dosed intravenously Q4dx4 with PBS vehicle, hu28-vc0101, anti-

hu28-(L443C)-vc0101, hu28-(L443C/κK183C)-vc0101, and control huNeg-8.8 ADC at the doses provided in **Table 31**. The data demonstrates that the double cysteine mutant hu28-(L443C/κK183C)-vc0101 inhibited growth of OVCAR3 ovarian xenografts with activity similar to wildtype hu28-vc0101 at the 3 mg/kg dose, but less at the 1 mg/kg dose. The data further demonstrates that the single cysteine mutant hu28-(L443C)-vc0101 with DAR=2 was efficacious in vivo, but was less active than the wildtype hu28-vc0101 and hu28-(L443C/κK183C)-vc0101 at both the 3 1 mg/kg and 3 mg/kg doses. The data indicates that anti-Notch3 cysteine mutant ADCs generated by site-specific conjugation are efficacious in vivo and inhibit tumor growth.

Table 31. In vivo efficacy of anti-Notch3 cysteine mutant ADCs in OVCAR3 ovarian xenograft

	OVCAR3 Ovarian Xenografts, tumor volume (mm3 ± S,E.M.)								
	PBS	hu28-vc0101		hu28-(L443C)-vc0101		hu28-(L443C/κK183C)-vc0101		huNeg8.8-vc0101	
	0	1	3	1	3	1	3	1	3
DAY -2	161 ±10	160 ±13	160 ±10	160 ±10	160 ±7	160 ±11	159 ±12	160 ±11	160 ±10
DAY 2	248 ±27	249 ±28	246 ±21	235 ±17	244 ±16	247 ±31	247 ±24	245 ±34	265 ±18
DAY 6	312 ±32	257 ±30	219 ±19	295 ±23	259 ±17	281 ±39	223 ±24	323 ±43	325 ±31
DAY 9	404 ±42	282 ±29	192 ±13	346 ±26	240 ±20	322 ±35	169 ±25	394 ±51	386 ±26
DAY 12	477 ±61	257 ±28	133 ±10	359 ±35	198 ±23	325 ±41	133 ±21	454 ±60	400 ±36
DAY 15	590 ±71	263 ±24	99 ±9	349 ±49	142 ±15	331 ±51	98 ±11	524 ±72	433 ±43
DAY 19	655 ±95	236 ±34	73 ±5	383 ±63	115 ±13	319 ±62	76 ±5	585 ±89	466 ±42
DAY 22	742 ±99	251 ±40	63 ±5	427 ±77	94 ±9	363 ±89	68 ±5	666 ±101	452 ±45
DAY 26	831 ±135	303 ±49	63 ±5	519 ±101	85 ±12	473 ±123	63 ±4	780 ±108	497 ±70
DAY 29	1008 ±171	396 ±69	56 ±4	624 ±118	97 ±14	583 ±167	54 ±3	834 ±124	555 ±80
DAY 33	1168 ±201	555 ±96	58 ±4	789 ±138	114 ±28	691 ±194	58 ±4	1007 ±155	695 ±88
DAY 36	1207 ±81	683 ±125	56 ±6	942 ±164	146 ±39	872 ±246	48 ±2	1152 ±155	782 ±93
DAY 40	1330 ±91	811 ±144	58 ±8	1046 ±191	205 ±56	1128 ±311	46 ±3	1328 ±220	872 ±131
DAY 42	1363 ±111	850 ±157	55 ±10	1130 ±190	246 ±71	955 ±247	51 ±5	1307 ±200	910 ±130
DAY 47	1656 ±98	1121 ±197	76 ±23	1493 ±243	378 ±103	1140 ±255	44 ±2	1470 ±223	1032 ±116
DAY 50	1661 ±95	1107 ±204	74 ±25		452 ±131	1152 ±248	40 ±2		1101 ±110

DAY 54	2005 ±140	1322 ±228	102 ±40		604 ±184	1463 ±317	48 ±4		1272 ±161
DAY 57		1286 ±235	117 ±51		826 ±264		43 ±4		1270 ±120
DAY 61			152 ±69		820 ±248		63 ±10		1610 ±160
DAY 64			182 ±88				55 ±9		
DAY 68			217 ±102				67 ±15		
DAY 71			242 ±120				73 ±20		
DAY 75			286 ±146				84 ±24		
DAY 78							107 ±32		
DAY 82							99 ±36		
DAY 85							119 ±50		
DAY 89							173 ±63		
DAY 99							328 ±130		
DAY 111							525 ±203		

WHAT IS CLAIMED:

1. An isolated antibody, or antigen-binding fragment thereof, that binds to Notch3 comprising:

a VH CDR1, a VH CDR2, and a VH CDR3 of a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 13; and

a VL CDR1, a VL CDR2, and a VL CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 25.

2. The isolated antibody, or antigen-binding fragment thereof, of claim 1, wherein the antibody or antigen-binding fragment:

(a) internalizes into a cell,

(b) does not inhibit Notch3 signaling, or

(c) does not activate Notch3 signaling.

3. The isolated antibody, or antigen-binding fragment thereof, of claim 1, wherein the antibody or antigen-binding fragment:

(a) binds to the Lin 12/Notch repeat (LNR)-C and heterodimerization (HD)-1 domains of the Notch3 Negative Regulatory Region (NRR),

(b) does not maintain the Notch3 NRR in an auto-inhibitory conformation, or

(c) does not inhibit S2-cleavage.

4. The isolated antibody, or antigen-binding fragment thereof, of claim 1, comprising:

a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 15 or 16;

a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 19 or 20;

a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 23;

a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 27;

a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 29; and

a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 31.

5. The isolated antibody, or antigen-binding fragment thereof, of claim 1, comprising a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 13 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 25.

6. The isolated antibody, or antigen-binding fragment thereof, of claim 5, comprising a heavy chain variable region amino acid sequence of SEQ ID NO: 13.

7. The isolated antibody, or antigen-binding fragment thereof, of claim 6, comprising a heavy chain amino acid sequence of SEQ ID NO: 33.

8. The isolated antibody, or antigen-binding fragment thereof, of claim 5, comprising a light chain variable region amino acid sequence of SEQ ID NO: 25.

9. The isolated antibody, or antigen-binding fragment thereof, of claim 8, comprising a light chain amino acid sequence of SEQ ID NO: 35.

10. An antibody-drug conjugate comprising a cytotoxic agent conjugated to the antibody, or antigen-binding fragment thereof, of claim 1.

11. A pharmaceutical composition comprising the antibody-drug conjugate of claim 10 and a pharmaceutically acceptable carrier.

12. A composition comprising the antibody of claim 1.

13. An isolated antibody, or antigen-binding fragment thereof, that binds to Notch3 comprising:

a VH CDR1, a VH CDR2, and a VH CDR3 of a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 37; and

a VL CDR1, a VL CDR2, and a VL CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 49.

14. The isolated antibody, or antigen-binding fragment thereof, of claim 13, comprising:

a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 39 or 40;

a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 43 or 44;

a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 47;

a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 51;

a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 53; and

a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 55.

15. The isolated antibody, or antigen-binding fragment thereof, of claim 13, comprising a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 37 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 49.

16. The isolated antibody, or antigen-binding fragment thereof, of claim 15, comprising a heavy chain variable region amino acid sequence of SEQ ID NO: 37.

17. The isolated antibody, or antigen-binding fragment thereof, of claim 16, comprising a heavy chain amino acid sequence of SEQ ID NO: 57.

18. The isolated antibody, or antigen-binding fragment thereof, of claim 15, comprising a light chain variable region amino acid sequence of SEQ ID NO: 49.

19. The isolated antibody, or antigen-binding fragment thereof, of claim 18, comprising a light chain amino acid sequence of SEQ ID NO: 59.

20. An antibody-drug conjugate comprising a cytotoxic agent conjugated to the antibody, or antigen-binding fragment thereof, of claim 13.

21. A pharmaceutical composition comprising the antibody-drug conjugate of claim 20 and a pharmaceutically acceptable carrier.

22. A composition comprising the antibody of claim 13.

23. An antibody-drug conjugate of the formula:

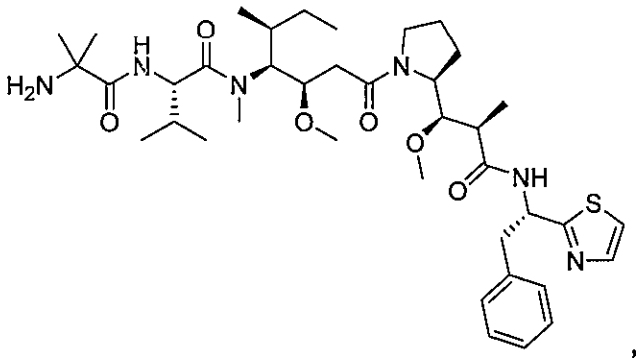
Ab-(L-D)*p*,

or a pharmaceutically acceptable salt thereof wherein:

(a) Ab is an antibody, or antigen-binding fragment thereof, that binds to Notch3;

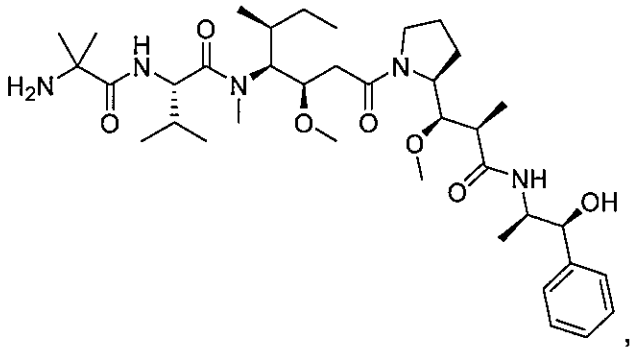
(b) L-D is a linker-drug moiety, wherein L is a linker selected from the group consisting of maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl (vc), maleimidocaproyl (mc), maleimido-heptanoyl (me) and maleimido-Peg6C2 (MalPeg6C2), and D is a drug selected from the group consisting of:

(i) 0101 having the formula:



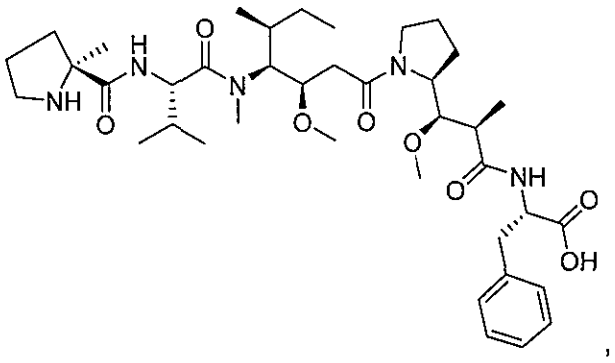
,

(ii) 6780 having the formula:

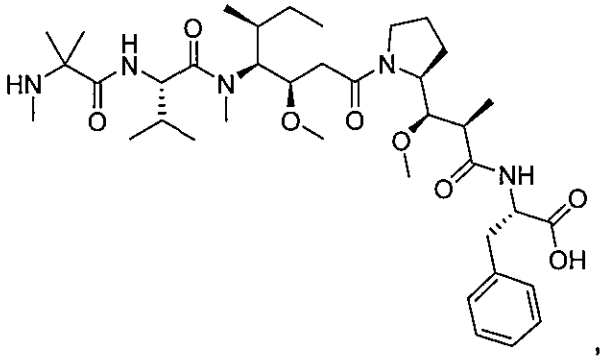


,

(iii) 0131 having the formula:

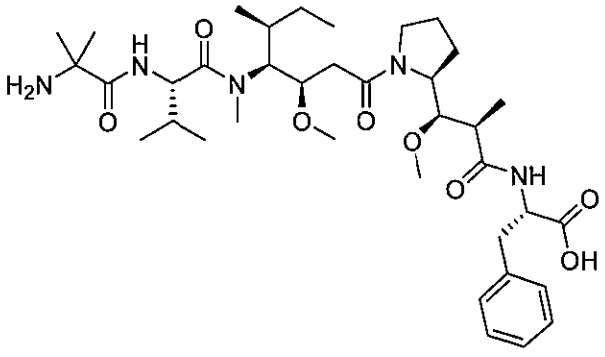


(iv) 3377 having the formula:



, and

(v) 8261 having the formula:



; and

(c) *p* is an integer from 1 to 12.

24. A pharmaceutical composition comprising the antibody-drug conjugate of claim 23 and a pharmaceutically acceptable carrier.

25. The antibody-drug conjugate of claim 23, wherein Ab comprises: a VH CDR1, a VH CDR2, and a VH CDR3 of a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 13; and a VL CDR1, a VL CDR2, and a VL

CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 25; or a VH CDR1, a VH CDR2, and a VH CDR3 of a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 37; and a VL CDR1, a VL CDR2, and a VL CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 49.

26. The antibody-drug conjugate of claim 23, wherein Ab comprises: a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 15 or 16; a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 19 or 20; a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 23; a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 27; a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 29; and a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 31; or a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 39 or 40; a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 43 or 44; a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 47; a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 51; a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 53; and a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 55.

27. The antibody-drug conjugate of claim 26, wherein Ab comprises: a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 13 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 25; or a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 37 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 49.

28. The antibody-drug conjugate of claim 27, wherein Ab comprises a heavy chain variable region amino acid sequence of SEQ ID NO: 13; or a heavy chain variable region amino acid sequence of SEQ ID NO: 37.

29. The antibody-drug conjugate of claim 28, wherein Ab comprises a heavy chain amino acid sequence of SEQ ID NO: 33; or a heavy chain amino acid sequence of SEQ

ID NO: 57.

30. The antibody-drug conjugate of claim 27, wherein Ab comprises a light chain variable region amino acid sequence of SEQ ID NO: 25 or a light chain variable region amino acid sequence of SEQ ID NO: 49.

31. The antibody-drug conjugate of claim 30, wherein Ab comprises a light chain amino acid sequence of SEQ ID NO: 35; or a light chain amino acid sequence of SEQ ID NO: 59.

32. An antibody-drug conjugate of the formula:

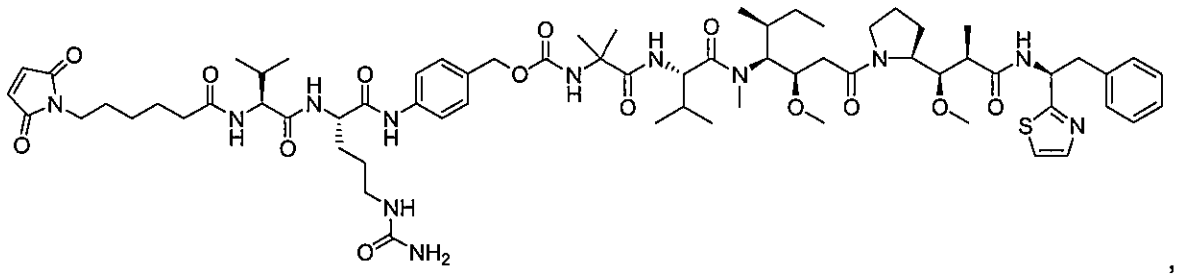
Ab-(L-D)*p*,

or a pharmaceutically acceptable salt thereof wherein:

(a) Ab is an antibody, or antigen-binding fragment thereof, that binds to Notch3;

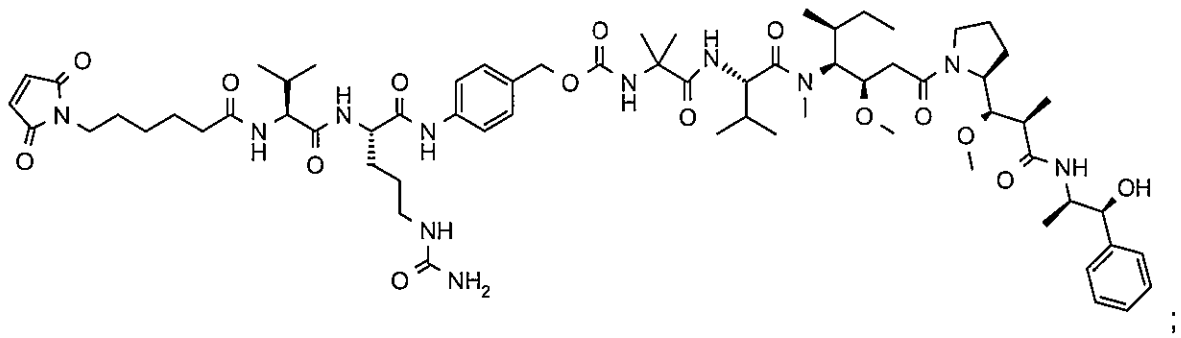
(b) L-D is a linker-drug moiety, wherein L is a linker, and D is a drug, wherein L-D is selected from the group consisting of:

vc0101 having the formula:



,

and vc6780 having the formula:



;

and

(c) *p* is an integer from 1 to 12.

33. A pharmaceutical composition comprising the antibody-drug conjugate of claim 32 and a pharmaceutically acceptable carrier.

34. The antibody-drug conjugate of claim 32, wherein Ab comprises: a VH CDR1, a VH CDR2, and a VH CDR3 of a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 13; and a VL CDR1, a VL CDR2, and a VL CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 25; or a VH CDR1, a VH CDR2, and a VH CDR3 of a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 37; and a VL CDR1, a VL CDR2, and a VL CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 49.

35. The antibody-drug conjugate of claim 32, wherein Ab comprises: a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 15 or 16; a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 19 or 20; a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 23; a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 27; a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 29; and a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 31; or a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 39 or 40; a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 43 or 44; a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 47; a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 51; a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 53;

and a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 55.

36. The antibody-drug conjugate of claim 35, wherein Ab comprises: a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 13 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 25; or a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 37 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 49.

37. The antibody-drug conjugate of claim 36, wherein Ab comprises a heavy chain variable region amino acid sequence of SEQ ID NO: 13; or a heavy chain variable region amino acid sequence of SEQ ID NO: 37.

38. The antibody-drug conjugate of claim 37, wherein Ab comprises a heavy chain amino acid sequence of SEQ ID NO: 33; or a heavy chain amino acid sequence of SEQ ID NO: 57.

39. The antibody-drug conjugate of claim 36, wherein Ab comprises a light chain variable region amino acid sequence of SEQ ID NO: 25 or a light chain variable region amino acid sequence of SEQ ID NO: 49.

40. The antibody-drug conjugate of claim 39, wherein Ab comprises a light chain amino acid sequence of SEQ ID NO: 35; or a light chain amino acid sequence of SEQ ID NO: 59.

41. An antibody-drug conjugate of the formula:

Ab-(L-D)*p*,

or a pharmaceutically acceptable salt thereof wherein:

(a) Ab is an antibody, or antigen-binding fragment thereof, that binds to Notch3 comprising a VH CDR1, a VH CDR2 and a VH CDR3 of a heavy chain variable region

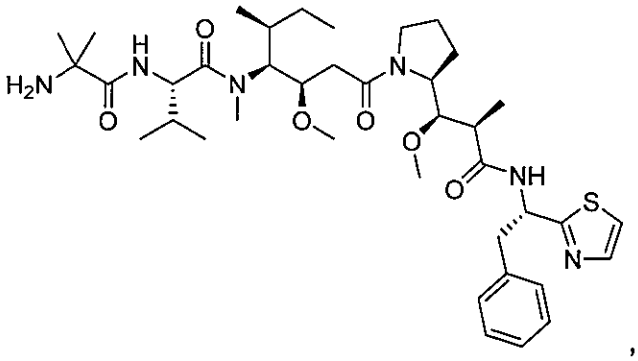
(VH) comprising the amino acid sequence set forth in SEQ ID NO: 13 and a VL CDR1, a VL CDR2 and VL CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 25;

(b) L-D is a linker-drug moiety, wherein L is a linker, and D is a drug; and

(c) p is an integer from 1 to 12.

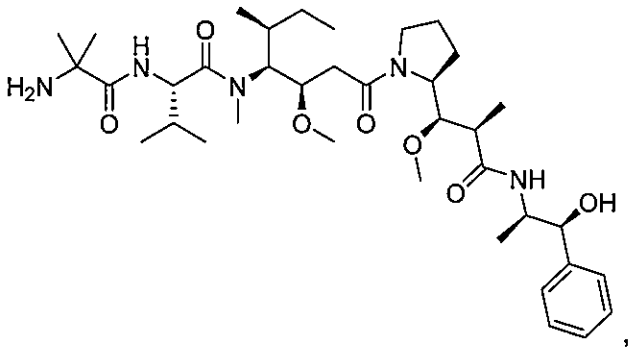
42. The antibody-drug conjugate of claim 41, wherein L is selected from the group consisting of maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl (vc), maleimidocaproyl (mc), maleimido-heptanoyl (me) and maleimido-Peg6C2 (MalPeg6C2).

43. The antibody-drug conjugate of claim 42, wherein D is selected from the group consisting of:

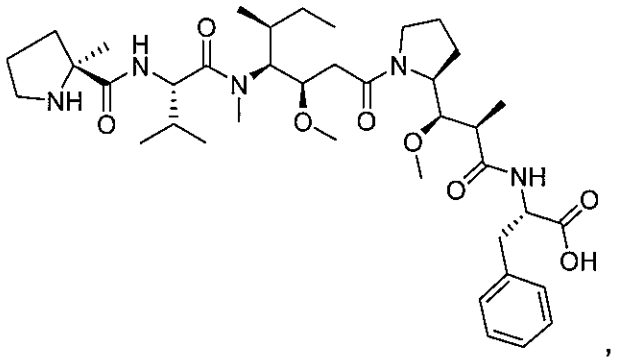


(a) 0101 having the formula:

(b) 6780 having the formula:

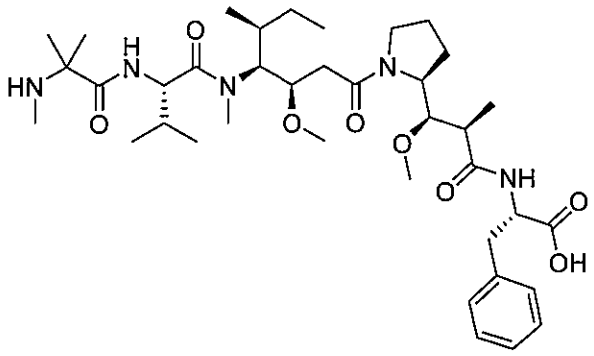


(c) 0131 having the formula:



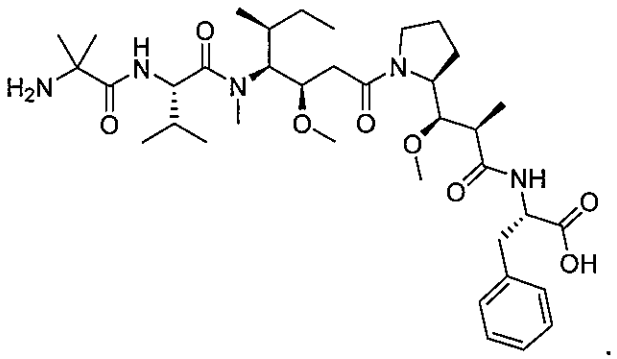
,

(d) 3377 having the formula:



, and

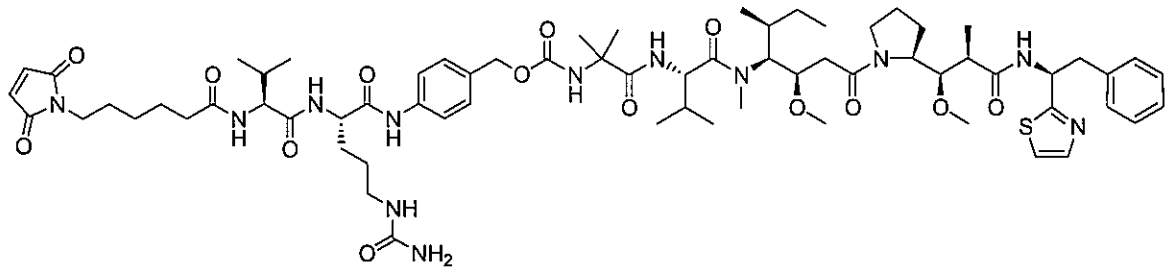
(e) 8261 having the formula:



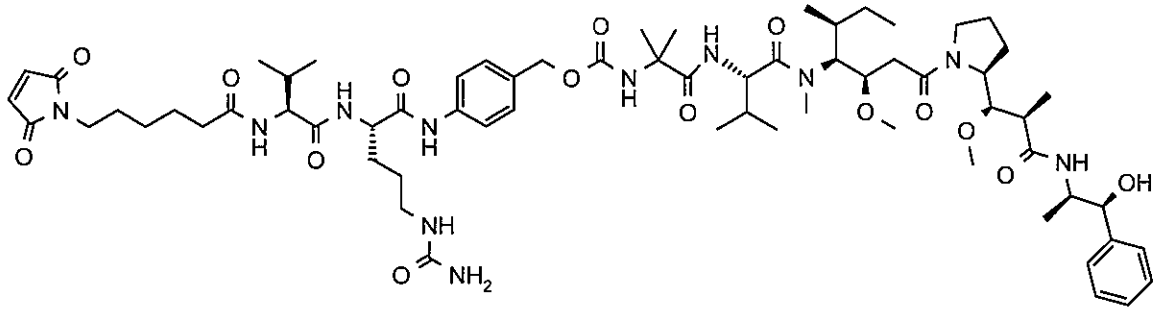
.

44. The antibody-drug conjugate of claim 41, wherein L-D is selected from the group consisting of:

vc0101 having the formula:



and vc6780 having the formula:



45. A pharmaceutical composition comprising the antibody drug conjugate of claim 41 and a pharmaceutically acceptable carrier.

46. The antibody-drug conjugate of claim 41, wherein Ab comprises: a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 15 or 16; a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 19 or 20; a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 23; a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 27; a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 29; and a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 31.

47. The antibody-drug conjugate of claim 41, wherein Ab comprises a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID

NO: 13 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 25.

48. The antibody-drug conjugate of claim 47, wherein Ab comprises a heavy chain variable region amino acid sequence of SEQ ID NO: 13.

49. The antibody-drug conjugate of claim 48, wherein Ab comprises a heavy chain amino acid sequence of SEQ ID NO: 33.

50. The antibody-drug conjugate of claim 47, wherein Ab comprises a light chain variable region amino acid sequence of SEQ ID NO: 25.

51. The antibody-drug conjugate of claim 50, wherein Ab comprises a light chain amino acid sequence of SEQ ID NO: 35.

52. An antibody-drug conjugate of the formula:

Ab-(L-D)*p*,

or a pharmaceutically acceptable salt thereof wherein:

(a) Ab is an antibody, or antigen-binding fragment thereof, that binds to Notch3 comprising a VH CDR1, a VH CDR2 and a VH CDR3 of a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 37 and a VL CDR1, a VL CDR2 and a VL CDR3 of a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 49;

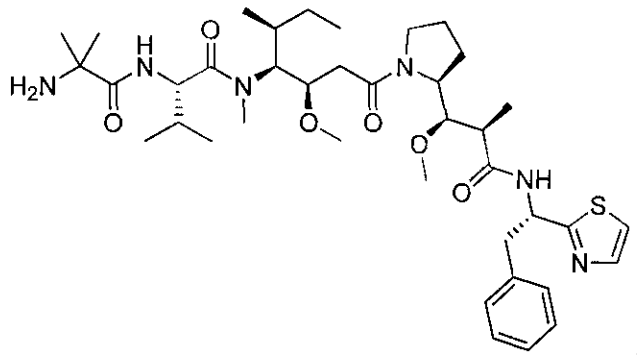
(b) L-D is a linker-drug moiety, wherein L is a linker, and D is a drug; and

(c) p is an integer from 1 to 12.

53. The antibody-drug conjugate of claim 52, wherein L is selected from the group consisting of maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl (vc), maleimidocaproyl (mc), maleimido-heptanoyl (me) and maleimido-Peg6C2 (MalPeg6C2).

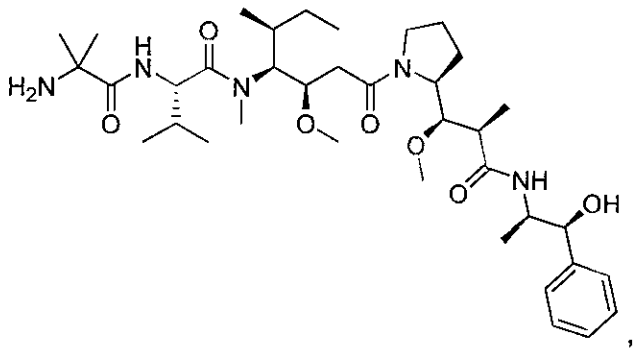
54. The antibody-drug conjugate of claim 53, wherein D is selected from the group consisting of:

(a) 0101 having the formula:



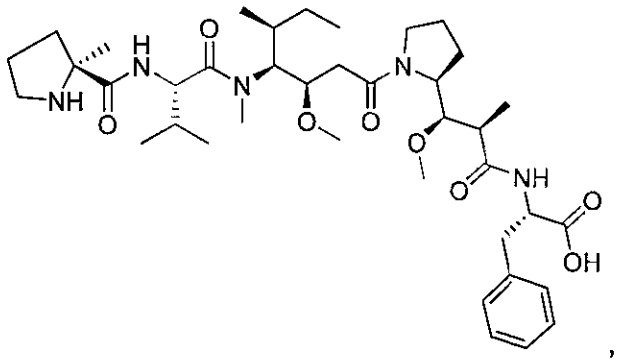
,

(b) 6780 having the formula:



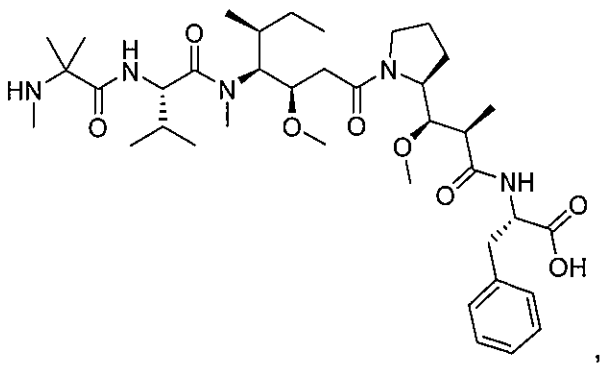
,

(c) 0131 having the formula:



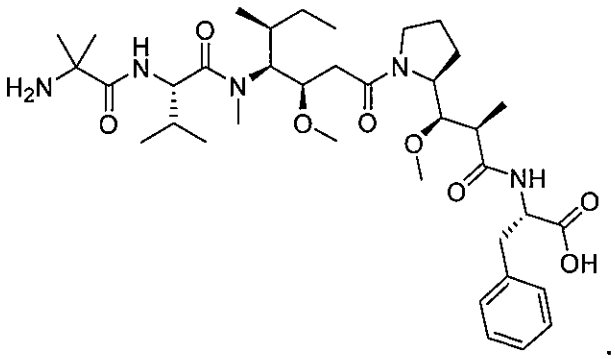
,

(d) 3377 having the formula:



, and

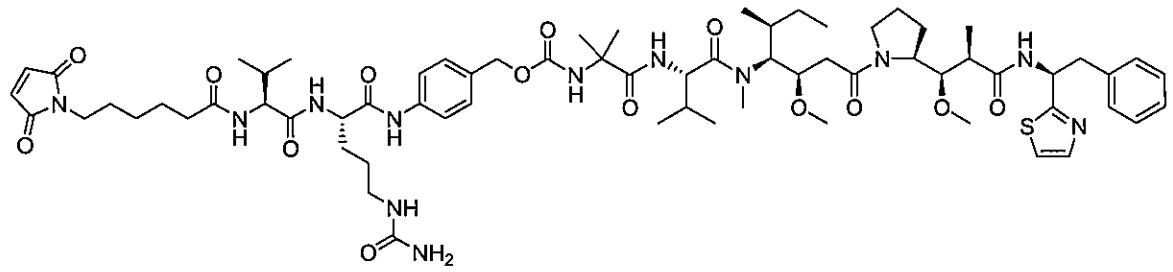
(e) 8261 having the formula:



.

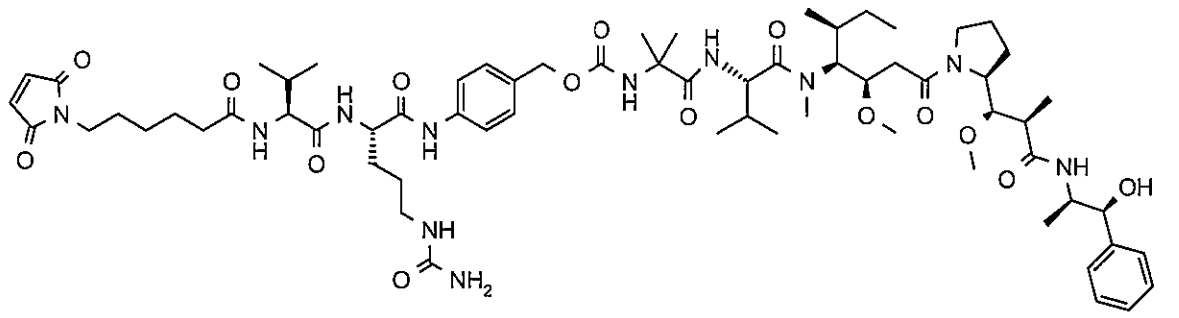
55. The antibody-drug conjugate of claim 52, wherein L-D is selected from the group consisting of:

vc0101 having the formula:



,

and vc6780 having the formula:



56. The antibody-drug conjugate of claim52, wherein Ab comprises: a VH CDR1 comprising the amino acid sequence of SEQ ID NO: 39 or 40; a VH CDR2 comprising the amino acid sequence of SEQ ID NO: 43 or 44; a VH CDR3 comprising the amino acid sequence of SEQ ID NO: 47; a VL CDR1 comprising the amino acid sequence of SEQ ID NO: 51; a VL CDR2 comprising the amino acid sequence of SEQ ID NO: 53; and a VL CDR3 comprising the amino acid sequence of SEQ ID NO: 55.

57. The antibody-drug conjugate of claim 52, wherein Ab comprises a heavy chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 37 or a light chain variable region having an amino acid sequence that is at least 90% identical to SEQ ID NO: 49.

58. The antibody-drug conjugate of claim 57, wherein Ab comprises a heavy chain variable region amino acid sequence of SEQ ID NO: 37.

59. The antibody-drug conjugate of claim 58, wherein Ab comprises a heavy chain amino acid sequence of SEQ ID NO: 57.

60. The antibody-drug conjugate of claim 57, wherein Ab comprises a light chain variable region amino acid sequence of SEQ ID NO: 49.

61. The antibody-drug conjugate of claim 60, wherein Ab comprises a light chain amino acid sequence of SEQ ID NO: 59.

62. A pharmaceutical composition comprising the antibody-drug conjugate of claim52 and a pharmaceutically acceptable carrier.

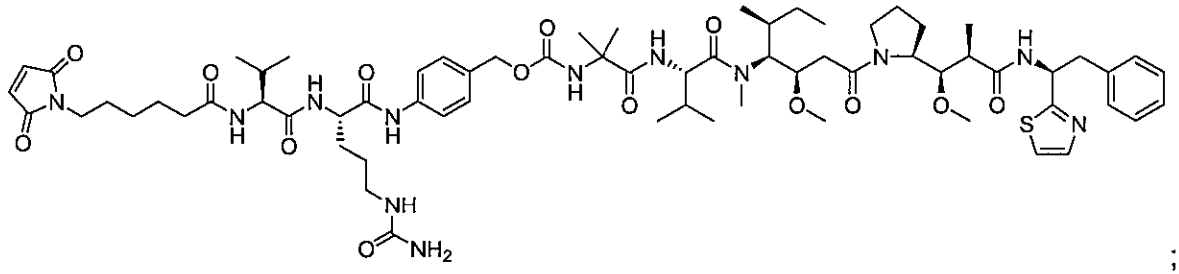
63. An antibody-drug conjugate of the formula:

Ab-(L-D)*p*,

or a pharmaceutically acceptable salt thereof wherein:

(a) Ab is an antibody, or antigen-binding fragment thereof, comprising a heavy chain variable region amino acid sequence of SEQ ID NO: 13 and a light chain variable region amino acid sequence of SEQ ID NO 25;

(b) L-D is a linker-drug moiety, wherein L is a linker, and D is a drug, wherein the L-D is vc0101 having the formula



;

and

(c) *p* is an integer from 1 to 12.

64. A pharmaceutical composition comprising the antibody-drug conjugate of claim 63 and a pharmaceutically acceptable carrier.